Université de Montréal

Brain tumour and brain endothelial cells' response to ionizing radiation and phytochemical treatments

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Cette thèse intitulée :

Brain tumour and brain endothelial cells' response

to ionizing radiation and phytochemical treatments

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RÉSUMÉ EN FRANÇAIS

Le glioblastome multiforme (GBM) représente la tumeur cérébrale primaire la plus agressive et la plus vascularisée chez l'adulte. La survie médiane après le diagnostic est de moins d'un an en l'absence de traitement. Malheureusement, 90% des patients traités avec de la radiothérapie après la résection chirurgicale d'un GBM développent une récidive tumorale. Récemment, le traitement des GBM avec radiothérapie et témozolomide, un agent reconnu pour ses propriétés antiangiogéniques, a permis de prolonger la survie médiane à 14,6 mois. Des efforts sont déployés pour identifier des substances naturelles capables d'inhiber, de retarder ou de renverser le processus de carcinogenèse. Epigallocatechin-3-gallate (EGCG), un polyphénol retrouvé dans le thé vert, est reconnu pour ses propriétés anticancéreuses et antiangiogéniques. L'EGCG pourrait sensibiliser les cellules tumorales cérébrales et les cellules endothéliales dérivées des tumeurs aux traitements conventionnels.

Le chapitre II décrit la première partie de ce projet de doctorat. Nous avons tenté de déterminer si l'EGCG pourrait sensibiliser la réponse des GBM à l'irradiation (IR) et si des marqueurs moléculaires spécifiques sont impliqués. Nous avons documenté que les cellules U-87 étaient relativement radiorésistantes et que Survivin, une protéine inhibitrice de l'apoptose, pourrait être impliquée dans la radiorésistance des GBM. Aussi, nous avons démontré que le pré-traitement des cellules U-87 avec de l'EGCG pourrait annuler l'effet cytoprotecteur d'une surexpression de Survivin et potentialiser l'effet cytoréducteur de l'IR.

Au chapitre III, nous avons caractérisé l'impact de l'IR sur la survie de cellules endothéliales microvasculaires cérébrales humaines (HBMEC) et nous avons déterminé si l'EGCG pouvait optimiser cet effet. Bien que les traitements individuels avec l'EGCG et l'IR diminuaient la survie des HBMEC, le traitement combiné diminuait de façon synergique la survie cellulaire. Nous avons documenté que le traitement combiné augmentait la mort cellulaire, plus spécifiquement la nécrose.

Au chapitre IV, nous avons investigué l'impact de l'IR sur les fonctions angiogéniques des HBMEC résistantes à l'IR, notamment la prolifération cellulaire, la migration cellulaire en présence de facteurs de croissance dérivés des tumeurs cérébrales, et la capacité de tubulogenèse. La voie de signalisation des Rho a aussi été étudiée en relation avec les propriétés angiogéniques des HBMEC radiorésistantes. Nos données suggèrent que l'IR altère significativement les propriétés angiogéniques des HBMEC. La réponse aux facteurs importants pour la croissance tumorale et l'angiogenèse ainsi que la tubulogenèse sont atténuées dans ces cellules.

En conclusion, ce projet de doctorat confirme les propriétés cytoréductrices de l'IR sur les gliomes malins et propose un nouveau mécanisme pour expliquer la radiorésistance des GBM. Ce projet documente pour la première fois l'effet cytotoxique de l'IR sur les HBMEC. Aussi, ce projet reconnaît l'existence de HBMEC radiorésistantes et caractérise leurs fonctions angiogéniques altérées. La combinaison de molécules naturelles anticancéreuses et antiangiogéniques telles que l'EGCG avec de la radiothérapie pourrait améliorer l'effet de l'IR sur les cellules tumorales et sur les cellules endothéliales associées, possiblement en augmentant la mort cellulaire. Cette thèse supporte l'intégration de nutriments avec propriétés

anticancéreuses et antiangiogéniques dans le traitement des gliomes malins pour sensibiliser les cellules tumorales et endothéliales aux traitements conventionnels.

MOTS CLÉS

Astrocytome

Angiogenèse

Epigallocatechin-3-gallate

Glioblastome multiforme

Irradiation

Radiothérapie

Rho, voie de signalisation

Survivin

SUMMARY

Glioblastoma multiform (GBM) represents the most aggressive and vascularised primary cerebral neoplasm in adults. Median length of survival without further therapy is usually less than one year from the time of diagnosis. Unfortunately, 90% of patients receiving radiotherapy following GBM resection develop a tumor recurrence. More recently, treatment of GBM with combined radiotherapy and temozolomide, an agent recognized for its antiangiogenic activity, increased the median survival to 14,6 months. Efforts have been oriented towards identifying naturally occurring substances capable of inhibiting, delaying or reversing the multi-stage carcinogenesis process. Epigallocatechin-3-gallate (EGCG), a green tea polyphenol, has been recognized for its anticancerous and antiangiogenic property. EGCG may represent a potential agent capable of sensitizing brain tumor cells and their derived endothelial cells (ECs) to conventional treatments.

In chapter II, the first part of this doctorate project aimed at determining if EGCG, in synergy with radiotherapy, can sensitize GBM's response to radiation and whether specific molecular markers are involved. We documented that U-87 cells were relatively radioresistant and that Survivin, an inhibitor of apoptosis protein, may be involved in GBM's radioresistance. We also found that pre-treatment of U-87 cells with EGCG could overcome the cytoprotective effect of Survivin overexpression and potentiate the cytoreductive effect of irradiation (IR).

In chapter III, we characterized the impact of IR on human brain microvascular endothelial cell (HBMEC) survival and determined whether EGCG, could optimize this effect. We found that although EGCG treatment and IR individually decreased

HBMEC survival, the combined treatment synergistically reduced survival. We documented that the combined treatment increased cell death, more specifically necrosis.

In chapter IV, we investigated the impact of IR exposure on the angiogenic functions i.e. cell proliferation, cell migration in response to brain tumor-derived growth factors, and capacity for tubulogenesis of surviving human brain tumor-derived ECs. The Rho signalling pathway was also investigated in relation to the functional properties of radioresistant HBMEC. Our data suggests that IR significantly alters radioresistant HBMEC migration response to tumor-secreted growth factors and tubulogenesis. Response to growth factors important for tumor expansion and angiogenesis is significantly attenuated in these cells.

In conclusion, this doctorate project confirmed IR's cytoreductive properties on malignant gliomas. We proposed a novel mechanism to explain GBMs' radioresistance. This project documented for the first time IR's cytotoxic effect in HBMEC. It also described the existence of radioresistant HBMEC and characterized their altered angiogenic functions. The combination of natural anticancerous and antiangiogenic molecules such as EGCG with radiotherapy could improve IR's effect on human malignant glioma cells and microvascular ECs, especially through increased necrosis of HBMEC. The thesis supports integrating nutrients bearing anticancerous and antiangiogenic properties, such as EGCG, in the management of gliomas to sensitize tumor and tumor-associated ECs to conventional therapies.

KEY WORDS

Astrocytoma

Angiogenesis

Epigallocatechin-3-gallate

Glioblastoma multiform

Irradiation

Radiotherapy

Rho, signalisation pathway

Survivin

TABLE DES MATIÈRES

Résumé i

Mots clés en français iii

Summary iv

Key words vi

Table des matières vii

Liste des tableaux xiii

Liste des figures xiv

Liste des sigles et abbréviations xvii

Dédicace xxii

Remerciements xxiii

CHAPTER I INTRODUCTION 1

1.1 The brain 1

1.1.1 General anatomy 1

1.1.2 Brain cellular components and their function 2

1.1.2.1 Nerve cells 2

1.1.2.2 Glial cells 4

1.1.2.3 Endothelial cells 5

1.2 Primary brain tumors 6

1.2.1 Generalities 6

1.2.1.1 Epidemiology of primary brain tumors 6

1.2.1.2 Classification of primary brain tumors 9

1.2.2 Classification and grading of astrocytomas 10

1.2.3. Cellular origins of gliomas 12

1.3 Glioblastoma multiforme 16

1.3.1 Epidemiology of glioblastoma 16

1.3.2 Risk factors 17

1.3.2.1 Hereditary syndromes and familial aggregation 17

1.3.2.2 Polymorphisms in genes relevant to cancer causation or prevention 18

1.3.2.3 Ionizing radiation 19

1.3.3 Clinical presentation of glioblastomas 19

1.3.4 Radiological characteristics 21

1.3.5 Histological characteristics 23

1.3.6 Primary and secondary glioblastomas 25

1.3.7 Overview of altered molecular pathways in glioblastoma 27

1.3.7.1 Signal transduction 27

1.3.7.2 Cell cycle 27

1.3.7.3 Programmed cell death 28

1.4 Angiogenesis 30

1.4.1 Distinction between vasculogenesis and angiogenesis 30

1.4.2 Steps in angiogenesis and implicated molecular players 31

1.4.2.1 The angiogenic switch 31

1.4.2.2 Mechanism of sprouting angiogenesis 34

1.4.3 Tumor vasculature 35

1.4.3.1 Types of tumor angiogenesis 35

1.4.3.2 Macroscopic and microscopic characteristics of tumor blood vessels 37

1.4.3.3 Cellular characteristics of brain tumor endothelial cells 38

1.4.4 Angiogenesis as a therapeutic target 40

1.5 Therapeutic management of glioblastomas 41

1.5.1 Surgery 41

1.5.2 Radiotherapy 42

1.5.2.1 Overview of radiobiology 42

1.5.2.1.1 Types of rays 42

1.5.2.1.2 Effects of radiation : physical, cellular, tissular 43

1.5.2.1.3 Types of radiotherapy 46

1.5.2.2 Radiosensitivity and radioresistance 47

1.5.3 Chemotherapy 49

1.5.3.1 Principles of CNS pharmacology and obstacles to chemotherapy 49

1.5.3.2 Description of frequently used agents 50

1.6 Nutratherapy 53

1.6.1 Diet and cancer prevention 53

1.6.2 Nutritional sciences 54

1.6.3 Dietary polyphenolic phytochemicals 57

1.6.3.1 Polyphenol catechins 57

1.6.3.2 Epigallocatechin-3-gallate anticancerous properties 59

1.6.3.3 Mechanisms of action of EGCG 62

1.7 Hypothesis, goals, and cellular models 63

1.7.1 Hypothesis 63

1.7.2 Goals 67

1.7.3 Cellular models used 67

1.7.3.1 Brain tumor-derived cell lines 67

1.7.3.2 Brain-derived endothelial cell line 68

CHAPTER II The Survivin-mediated radioresistant phenotype of glioblastomas is regulated by RhoA and inhibited by the green tea polyphenol (-)-epigallocatechin-3-gallate. (Brain Res. 2006;1071(1):1-9) 69

Abstract 70

Introduction 72

Material and Methods 75

Results 79

Discussion 84

Acknowledgements 88

References 89

Figures 98

CHAPTER III Combined low dose ionizing radiation and green tea-derived epigallocatechin-3-gallate treatment induces human brain endothelial cells death. (J Neurooncol. 2006 Nov;80(2):111-21) 106

Abstract 107

Introduction 109

Material and Methods 112

Results 117

Discussion 121

Acknowledgements 127

References 128

Figures 137

CHAPTER IV The response to brain tumor-derived growth factors is altered in radioresistant human brain endothelial cells.
(Cancer Biol Ther. 2006 Nov;5(11):1539-45) 147

Abstract 148

Introduction 150

Material and Methods 153

Results 159

Discussion 163

Acknowledgements 168

References 169

Figures 177

CHAPTER V DISCUSSION 184

5.1 Summary 184

5.2 Discussion 185

5.2.1 Radioresistance of cells 185

5.2.2 Tumor-associated endothelial cells as a therapeutic target 191

5.2.3 Necrosis, an important goal of adjuvant therapies 198

5.2.4 Nutritherapy as a complementary therapeutic modality 203

5.3 Conclusion 208

CHAPITRE VI BIBLIOGRAPHIE 210

LISTE DES TABLEAUX

CHAPTER I

Table 1.	Distribution and incidence rate of primary brain and central nervous system tumors by histology	8
Table 2.	Classification of malignant gliomas and prognostic significance	11
Table 3.	Principal tumor suppressor genes altered in brain tumors and involved in hereditary tumoral syndromes	18
Table 4.	Karnofsky performance scale for brain tumor patients	20
Table 5.	Cell death pathway characteristics	29
Table 6.	Total dose of irradiation tolerated by normal organs	45
Table 7.	Partial list of bioactive food components that may influence cancer risk and tumor behavior	55
Table 8.	Mechanistic findings of EGCG against tumor development and progression	61

LISTES DES FIGURES

CHAPTER I

Figure 1. The central nervous system 1

Figure 2. Neurons, the functional cellular unit of the nervous system 3

Figure 3. Relationships between astrocytes and other central nervous system cells. 5

Figure 4. Distribution of all primary brain and central nervous system tumors by location 7

Figure 5. Current concept of the stem cell niche 13

Figure 6. Comparison of the niches under normal and cancerous conditions 14

Figure 7. Incidence rates of primary brain tumors by major neuro-epithelial tissue and meningeal histologic types and age group 17

Figure 8. Characteristic radiological features of glioblastomas 22

Figure 9. Characteristic histopathological features of glioblastomas 24

Figure 10. Formation of primary and secondary glioblastoma multiform 26

Figure 11. The angiogenic balance 33

Figure 12. Mechanisms of tumor angiogenesis 35

Figure 13. Glomeruloid vascular proliferation in glioblastoma multiform 37

Figure 14. Mechanisms of action of ionizing radiations 44

Figure 15. Diet may influence genetic & epigenetic events associated with several cancer processes 56

Figure 16. Structures of the major polyphenolic catechins present in green tea 58

CHAPTER II

Figure 1.	Effects of ionizing radiation on the proliferation rates of malignant glioma and medulloblastoma cell lines	98
Figure 2.	Ionizing radiation effects on caspase-3 activity in malignant glioma and medulloblastoma cell lines.	99
Figure 3.	Expression of prosurvival proteins in malignant glioma cells exposed to ionizing radiation.	100
Figure 4.	Effect of ionizing radiation on the proliferation rates of malignant glioma cells transfected with proteins involved in radioresistance.	101
Figure 5.	Survivin overexpression does not antagonize EGCg's anti-proliferative effect.	103
Figure 6.	Effect of combined EGCg and low dose IR on U-87 glioma cell proliferation.	105

CHAPTER III

Figure 1.	Effects of EGCg treatment or ionizing radiation exposure on HBMECs' survival.	137
Figure 2.	Ionizing radioation's influence on HBMEC cell cycle phase distribution in not modulated by EGCg pre-treatment	139
Figure 3.	Induction of cyclin kinase inhibitors p21 and p27 by EGCg treatment and IR	140

Figure 4. Combined EGCg and ionizing radiation treatments increased sub-G1 population 142

Figure 5. IR-induced caspase-dependant mechanisms are not increased by EGCg pre-treatment. 143

Figure 6. Cell necrosis significantly increases following EGCg/IR combined treatments 145

CHAPTER IV

Figure 1. Ionizing radiation decreases HBMEC survival 177

Figure 2. Ionizing radiation inhibits HBMECs' migratory response to brain tumor-derived growth factors 178

Figure 3. Ionizing radiation induces RhoA, ROK and Caveolin-1 expression in radioresistant HBMEC 179

Figure 4. Inhibition of RhoA/ROK signalling pathway produces similar diminution in migration responses of RhoA-transfected and of irradiated HBMEC. 180

Figure 5. Ionizing radiation and RhoA overexpression decrease HBMEC capacity to form tubes *in vitro* 182

CHAPTER V

Figure 1. Differences between apoptosis and necrosis 199

Figure 2. Direct synergy of EGCG and curcumin 207

LISTE DES SIGLES ET DES ABRÉVIATIONS

67LR	67-kDa laminin receptor
Akt/PKB	Thymoma viral proto-oncogene / protein kinase B
Ang1	Angiopoietin-1
Ang-2	Angiopoietin-2
α-SMA	Alpha-smooth muscle actin
ATP	Adenosine triphosphate
BAEC	Bovin aortic endothelial cells
BBB	Blood brain barrier
Bcl-2	B-cell leukemia/lymphoma 2
Bcl-x(L)	B-cell lymphoma-extra large
BFGF	Basic fibroblast growth factor
bFGFR	Basic Fibroblast growth factor receptor
BTSC	Brain tumor stem cells
CD31	Cluster designation 31, PECAM-1 (Platelet Endothelial Cell Adhesion Molecule-1)
CD34	Cluster designation 34
CD105	Cluster designation 105/ endoglin
CD133	Cluster designation 133/prominin-1
CD144	Cluster designation 144/ vascular endothelial (VE)-cadherin
cdk4	Cyclin-dependent kinase 4
cdk6	Cyclin-dependent kinase 6
Chk1	Checkpoint kinase -1

Chk2	Checkpoint kinase - 2
c-kit	Receptor of stem cell factor (SCF)
CNS	Central nervous system
COX-2	Cyclooxygenase-2
Crm1	Chromosome region maintenance
CT	Computed tomography
DNA	Deoxyribonucleic acid
DXM	Dexamethasone
EC	(-)Epicatechin
ECs	Endothelial cells
EGC	(-)Epicatechin-3-gallate
EGCG	(-)Epigallocatechin-3-gallate
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor/ErbB-1/HER1
EPC	Endothelial progenitor cell
ErbBR	Tyrosine kinase receptors
ERK	Extracellular-related kinase
ET-1	Endothelin-1
GBM	Glioblastoma multiform
GC	Gallocatechin
Gy	Gray
HBMEC	Human brain microvascular endothelial cells
HER2	Human Epidermal growth factor Receptor 2/ ErbB-2

HGF	Hepatocyte growth factor
HIF-1	Hypoxia inducible factor-1
HUVEC	Human umbilical vein endothelial cells
IAP	Inhibitor of apoptosis protein
IGFR-1	Insulin-like growth factor-1 receptor
iNOS	Inducible nitric oxide synthase
Interleukin-8	IL-8
IR	Irradiation
JNK	Jun N-terminal kinase
KPS	Karnofsky performance scale
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
MDM2	Murine double minute 2
MDR1	Multidrug resistance gene
MGMT	O6-methylguanine-DNA methyltransferase
MMP-2	Collagenase type IV/gelatinase A
MMP-9	Collagenase type IV/gelatinase B
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
mRNA	Messenger Ribonucleic acid
mTOR	Mammalian target of rapamycin
NAD	b-nicotinamide adenine dinucleotide
NFkB	Nuclear factor kappa B

NSC	Neural Stem cells
p14/ARF	Cyclin-dependent kinase inhibitor, produced by an alternative reading frame (ARF) of the human INK4 locus
p16(INK4a)	Cyclin-dependent kinase inhibitor
p21/WAF1/Cip1	Cyclin-dependent kinase inhibitor
p27/Kip1	Cyclin-dependent kinase inhibitor
PARP-1	Poly(ADP-ribose) polymerase member 1
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
Pgp	P-glycoprotein
PI3K	Phosphatidylinositol-3'-OH kinase
PTEN	Phosphatase and tensin homolog
PVC	Multiagent regimen including Procarbazine – Lomustine – Vincristine
Rb	Retinoblastoma gene
Rho	Small GTP-binding proteins (GTPases)
RhoA	Small GTP-binding proteins (GTPases), type A
RhoB	Small GTP-binding proteins (GTPases), type B
RhoBN19	Inducible dominant negative form of RhoB
RNA	Ribonucleic acide
ROK	Rho Kinase
ROS	Reactive oxygen species
S1P	Sphingosine-1 phosphate

S1P(3) receptor	Receptor of sphingosine-1-phosphate, subtype 3
SAPK/JNK	Stress activated protein kinase or c-Jun N-terminal kinase
SCID	Severe combined immunodeficiency
SCF	Stem cell factor
siRNA	Small interfering RNA
SRS	Stereotactic radiosurgery
SV40-LT	Simian virus 40, large T antigens
TMZ	Temozolomide
U-87	Human glioblastoma cell line
U-87 GF	Brain tumor-derived growth factors
uPA	Urokinase-plasminogen activator
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor,
vWF	Von Willebrand factor
WHO	World health organisation

To my mother and father, for their unconditional love

To my husband, for every sunrise and sunset shared, for his eternal love

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CHAPTER I INTRODUCTION

1.1 The brain

1.1.1 General anatomy

The human central nervous system (CNS) is divided in the spinal cord, the cerebellum, and the brain. The brain is further subdivided in two cerebral hemispheres, a diencephalon, a brain stem, and a cerebellum (Figure 1). Each specific region has general and more specific functions, working in unison to realize the most complex movements and translate perceptions, emotions, and thoughts.

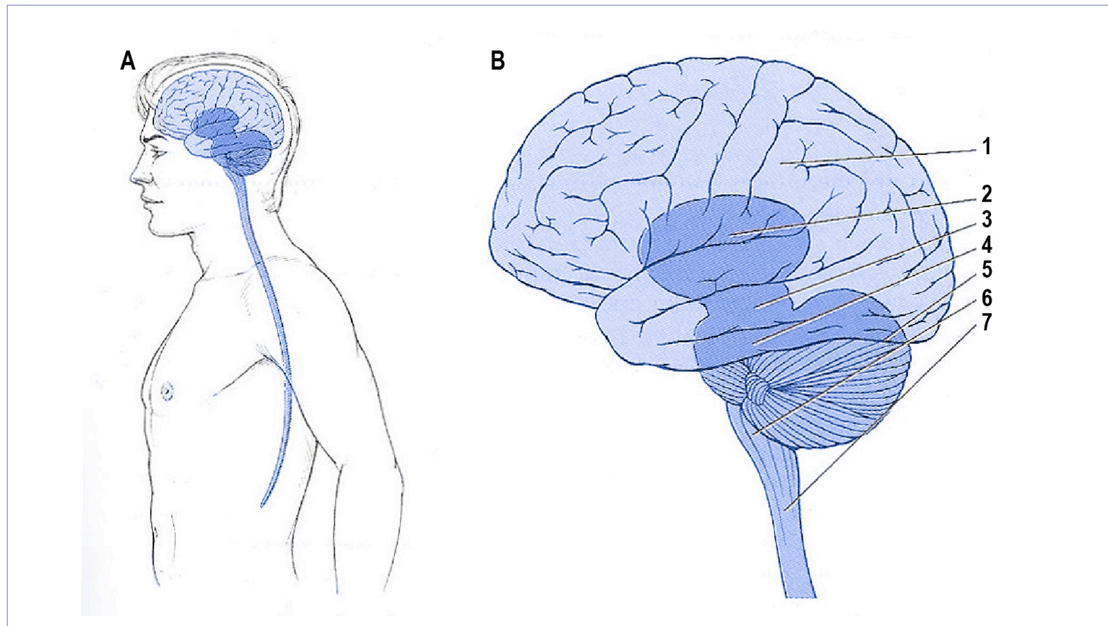

Figure 1. The central nervous system. A) Location of the central nervous system in the body. B) The major divisions of the central nervous system are: (1) cerebral hemispheres, (2) diencephalon, the brain stem composed of the(3) midbrain, (4) pons, and (6) medulla, (5) the cerebellum and (7) spinal cord [1].

1.1.2 Brain cellular components and their function

1.1.2.1 Nerve cells

Three types of cells compose the nervous tissue: nerve cells, glial cells, and endothelial cells. These cells interact together within the extracellular matrix. The nerve cell, or neuron, is the functional cellular unit of the nervous system. There is a great diversity of structural and functional characteristics possessed by the various cells that are named neuron. However, they have properties related to their function in common. Neurons are polarized cells derived from epithelial origins. They are composed of dendrites, cell body and axon terminals (Figure 2). Dendrites and the cell body receive information from other neurons at specialized contact sites called synapses. The nucleus and cellular organelles reside in the cell body. The axon conducts electrical information encoded in action potentials to axon terminals.

Dendrites and neuronal cell bodies in the CNS are located in cortical areas and in nuclei located beneath the cortical surface, giving the appearance of grey matter. Regions rich in axons surrounded by cells rich in myelin form the white matter.

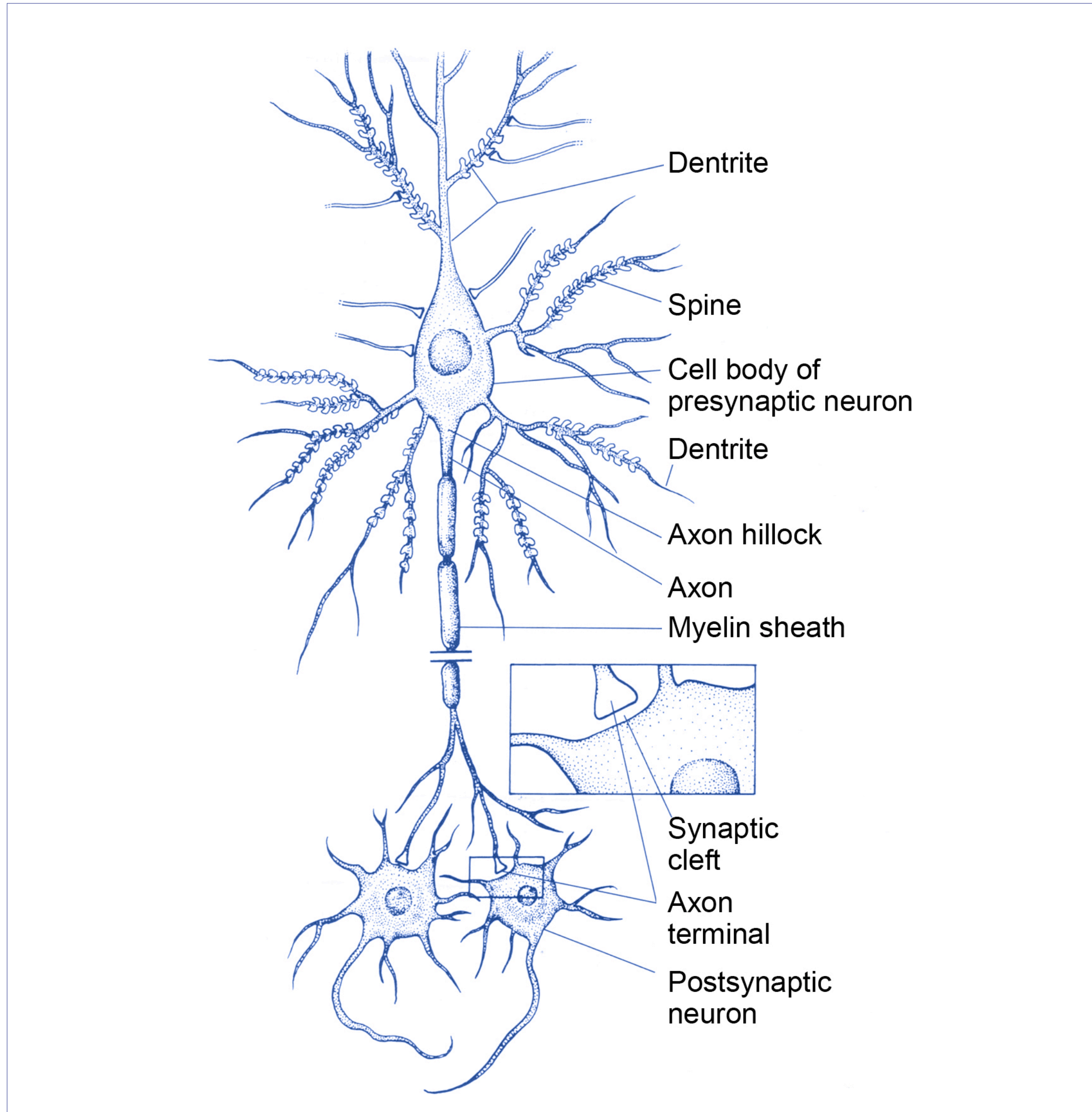

Figure 2. Neurons, the functional cellular unit of the nervous system. A schematic nerve cell is shown, illustrating the dendrites, cell body, and axon. The presynaptic terminals of the neuron are shown synapsing on the cell body of the postsynaptic neurons. The inset showns the spatial relation of three components of the synapse, the axon terminal, the synaptic cleft, and the cell body of the postsynaptic neuron. Adapted from [1]

1.1.2.2 Glial cells

Glial cells outnumber neurons in a 10:1 ratio. They are divided in two major classes: microglia and macroglia. Most microglia originate from bone marrow-derived monocytes which enter the brain during early development. They function as resident immune cells and phagocytes in the CNS, exerting a scavenger role in response to nervous system infection, trauma, and inflammation. Macroglia, including oligodendrocytes, Schwann cells, ependymal cells, and astrocytes, subserve support and nutritive functions. Oligodendrocytes form the myelin shealth around axons in the CNS. In addition to myelinization and axonal support in the peripheral nervous system, Schwann cells can promote axonal regeneration. Ependymal cells line the ventricles and the central canal in the spinal cord. Astrocytes are the most abundant cell within the CNS. Although there are many subtypes of astrocytes, the presence of a unique population of intermediate filaments enriched in glial fibrillary acidic protein is common to all astrocytes. These cells are important for structural support, homeostatic regulation of the CNS microenvironment and regulation of energy metabolism. Astrocytes processes surround many other CNS (Figure 3) such as blood vessels, implicating them in the blood-brain barrier (BBB) [2]. In the presence of CNS pathology, reactive astrocytes' may secrete a variety of substances that can inhibit or promote axonal regeneration, brain repair, and neuronal function [2].

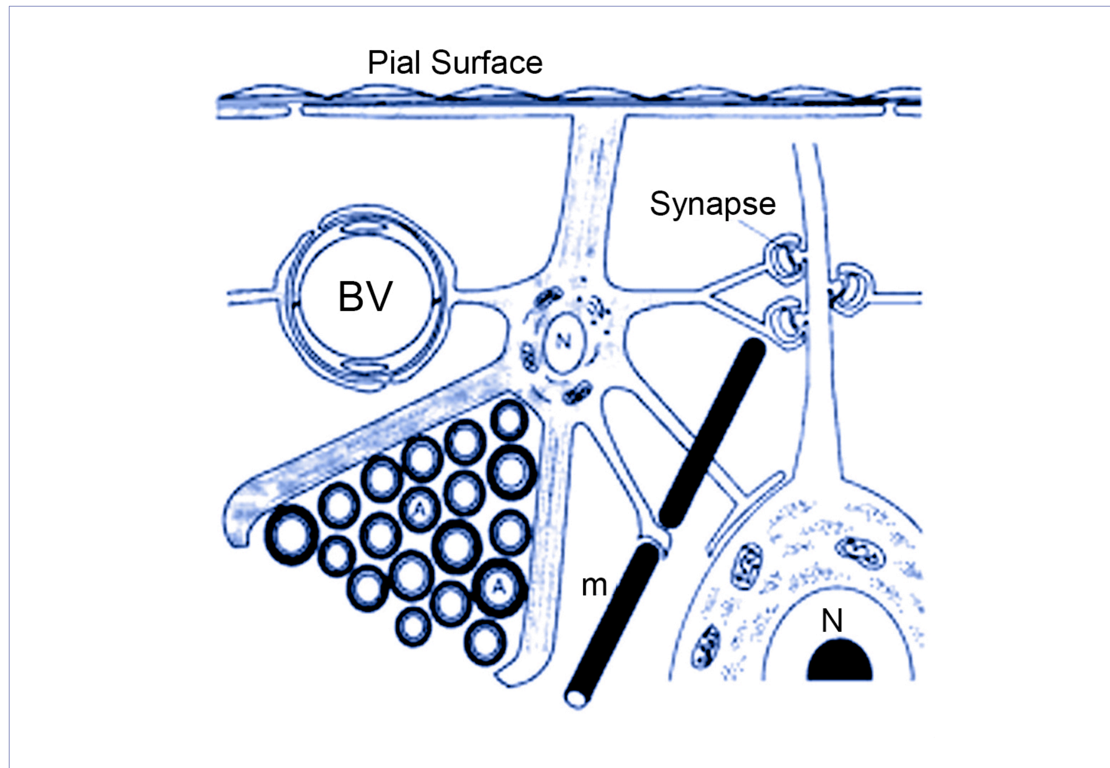

Figure 3. Relationships between astrocytes and other central nervous system cells. Astrocyte processes surround blood vessels (BV), synapses, nodes of Ranvier, neuronal cell bodies (N), and groups of myelinated axons (A). Myelin (m) Adapted from [2]

1.1.2.3 Endothelial cells

In the adult brain, the total surface area of microvasculature is 12m^2. A BBB is present in more than 99% of the brain capillaries [2]. Brain capillary endothelial cells (ECs) are surrounded by astrocytes, pericytes, neurons, microglia and extracellular matrix. In addition to composing the BBB, they have highly specialized properties and functions. Brain capillary ECs constitute a continuous lining given the lack of fenestrations and the presence of tight junctions between each ECs [3], hindering the passage of small and large molecules between blood and brain. Also, brain capillary ECs are characterized by low pinocytic activity. Only highly lipophylic molecules can

relatively easily pass the BBB, other molecules depend on selective specialized transport mechanisms.

1.2 Primary brain tumors

1.2.1 Generalities

1.2.1.1 Epidemiology of primary brain tumors

Primary brain tumors arise from intrinsic cellular elements found in the CNS. In contrast, secondary tumors originate outside the nervous system and reach neural tissues either by contiguity or hematogenous spread. In the most recent compilation of primary brain and CNS tumors in the United States, the overall annual incidence rate of primary brain and CNS tumors was 11.5/100 000 persons/year. The calculated annual incidence rates for benign tumors varied from 2.1-8.9/100 000 persons/year throughout the various regions analyzed. The calculated annual incidence rates for malignant tumors varied from 5.9-7.8/100 000 persons/year [4].

Tumors most frequently occur within the brain parenchyma with 39% located in the supratentorial region [4] (Figure 4).

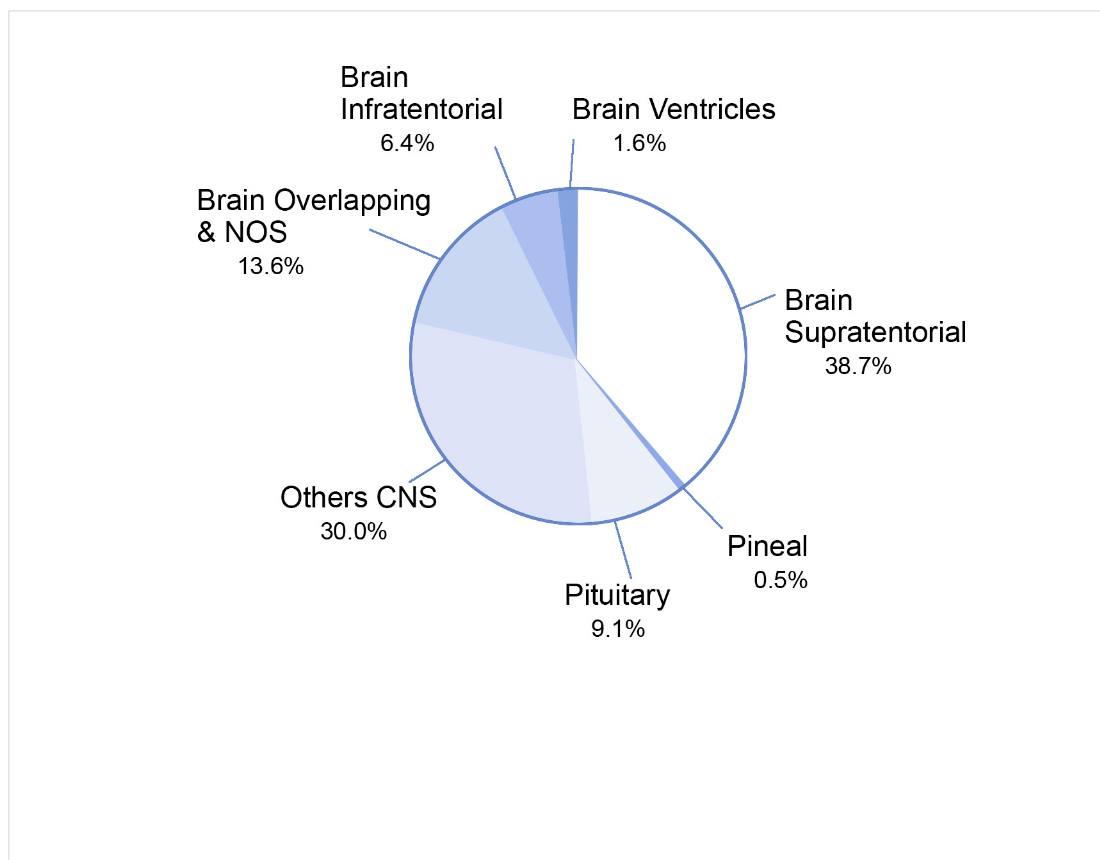

Figure 4. Distribution of all primary brain and central nervous system tumors by location, CBTRUS 1990-1994. CBTRUS, Central Brain Tumor Registry of the United States; not otherwise specified (NOS) [4].

The most frequently reported histopathologies are meningiomas (24.0%), glioblastomas (22.6%), and astrocytomas (13,7%, including diffuse, anaplastic, not otherwise specified) [4] (Table 1). The highest mean age at diagnosis is found in meningiomas (62 years) and glioblastomas (62 years). The lowest mean age at diagnosis is found in pilocytic astrocytomas (17 years) and medulloblastomas (14 years) [4].

Table 1. Distribution and incidence rate of primary brain and central nervous system tumors by histology

Histology	Total (n)	Reported brain tumors (%)
Tumors of the neuroepithelial tissue		
-Diffuse astrocytoma	273	1.3
-Anaplastic astrocytoma	885	4.3
-Glioblastoma	4695	22.6
-Pilocytic astrocytoma	371	1.8
-Oligodendroglioma	540	2.6
-Anaplastic oligodendroglioma	117	0.6
-Ependymoma/ anaplastic ependymoma	420	2.0
Tumors of the cranial and spinal nerves		
-Nerve sheath, benign and malignant	1356	6.5
Tumors of the meninges		
-Meningioma	4989	24.0
-Other mesenchymal, benign and malignant	73	0.4
-Hemangioblastoma	195	0.9
Lymphoma and hematopoietic neoplasms		
-Lymphoma	846	4.1
Germ cell tumors and cysts		
-Germ cell tumors, cysts, heterotopias	127	0.6
Tumors of the sellar region		
-Pituitary	1670	8.0
-Craniopharyngioma	183	0.9
Extension from regional tumors		
-Chordoma/chondrosarcoma	45	0.2

Adapted from [4]

1.2.1.2 Classification of primary brain tumors

Many classification systems have been proposed to organize tumors of the CNS. In the nineteenth century, Cohnheim proposed that neoplasms develop from nests of embryonic cells [5]. The suffix –oma was added to the name of the cell from which the tumor was believed to originate. This cytogenetic concept was used in the first tumor classification scheme written by Bailey and Cushing [5]. They attributed a histopronostic value to each tumor depending on its cellular differentiation.

Later on, Kernohan proposed that tumor cells originate from differentiated cells that undergo anaplasia. He introduced in his classification grades of malignancy depending on their differentiation and degree of anaplasia.

The most recent classification, the 2007 World Health Organization (WHO) classification of tumours of the CNS, builds on the previous classification schemes [6]. It is based on the consensus of an international working group of pathologists and geneticists and represents the standard for the definition of brain tumors [6]. Two basic concepts are used in the WHO system to classify tumors. First, recognition of the cellular component of the tumor either by histology or immunocytochemical methods [7]. Primary CNS tumors may origin from neuroepithelial tissue, cranial and paraspinal nerves, meninges, germ cells, lymphomas and haematopoetic neoplasm, sellar region [7].

Second, each tumor is graded according to a scheme that is a malignancy scale, predicting the biological behavior of the neoplasm [5, 6]. Grade I is reserved for lesions with low proliferative potential and the possibility of cure following surgery. Grade II designates infiltrative lesions with low proliferative activity. Grade III is attributed to lesions with anaplastic characteristics including nuclear atypia and brisk mitotic activity. Grade IV is reserved for cytologically malignant lesions with high mitotic activity, endothelial proliferation and necrosis [6]. Adequate sampling of the tumor is important in order to determine its type and judge its malignant potential

1.2.2 Classification and grading of astrocytomas

Tumors that originate from glial cells are grouped together as gliomas. Gliomas include tumors that originate from astrocytes (astrocytomas), oligodendrocytes (oligodendrogliomas), ependymal cells (ependymomas) and choroids plexus. Furthermore, some tumors of glial origin contain more than one type of neoplastic cells and are referred to as mixed tumors (oligoastrocytomas) [6].

Numerous grading systems have been systematically evaluated and successfully applied to astrocytomas. Currently, the two most commonly used classifications are the St. Anne/Mayo, also named the Daumas-Duport system [8] and the WHO [7] grading systems. The St. Anne/Mayo classification system is restricted to fibrillary, protoplasmic, gemistocytic, anaplastic astrocytomas and glioblastomas. It assesses the presence or absence of four morphologic criteria: nuclear atypia, mitoses, endothelial proliferation, and necrosis. The summary score is translated into a grade

as follows: 0 criterion = grade 1, 1 criterion = grade 2, 2 criteria = grade 3, 3 or 4 criteria = grade 4 [8]. Grade I is attributed to diffuse astrocytoma without atypia.

The WHO classification includes in grade I pilocytic astrocytoma and subependymal giant cell astrocytoma. The diffusely infiltrative astrocytic tumors are graded II through IV. Grade II astrocytic tumors (diffuse astrocytoma) present little cellularity and minimal pleomorphic changes. Grade III (anaplastic astrocytoma) is reserved for tumors showing moderate cellularity and pleomorphism but no necrosis. Grade IV (glioblastoma) is attributed to highly cellular tumors with nuclear and cellular pleomorphism, numerous mitotic figures, endothelial proliferation or glomeruloid microvascular proliferations and necrosis [6, 9]. Tumor grade has an important prognostic significance, influencing the choice of adjuvant therapies such as radiation and chemotherapy (Table 2) [10].

Table 2. Classification of malignant gliomas and prognostic significance

WHO Grade	Designation	Frequency (% of Gliomas)	5-Year Survival (%)	Median Survival
II	Diffuse astrocytoma	1.7	46.9	3-8 y
	Oligodendroglioma	9.2	70.5	3-8 y
III	Anaplasic Astrocytoma	7.9	29.4	7-10 y
	Anaplasic oligodendroglioma	5.1	40.1	3-5 y
IV	Glioblastoma	50.7	3.3	9-15 mo
	Others	20.5		

Adapted from [10]

1.2.3. Cellular origins of gliomas

Although the WHO classification scheme implies a cell of origin for most brain tumors, that cell of origin has not been unequivocally identified for any of them. As such, astrocytomas and oligodengrogliomas are believed to arise from astrocytic or oligodendroglial precursors. Stem cells, progenitor cells and differentiated cells may acquire genetic alterations leading to the development of neoplastic cells.

Analysis of the genetic profile of both cellular components of mixed tumors oligoastrocytomas has revealed that both cell types present loss of heterozygosity of 1p and 19q, suggesting that oligodendroglial and astrocytic cells derived from a single precursor cell [11]. Similarly, genetic analysis of the glial and sarcomatous elements revealed that both components carried identical p53 mutations, suggesting a common origin of the two cellular components from a multipotent neural stem cell or an early glial progenitor cell [12].

Neural stem cells have been identified in the adult CNS within its specialized microenvironment named the niche which can regulate stem cell quiescence, asymmetrical self-renewal, and differentiation into mature astrocytes and neurons (Figure 5) [13].

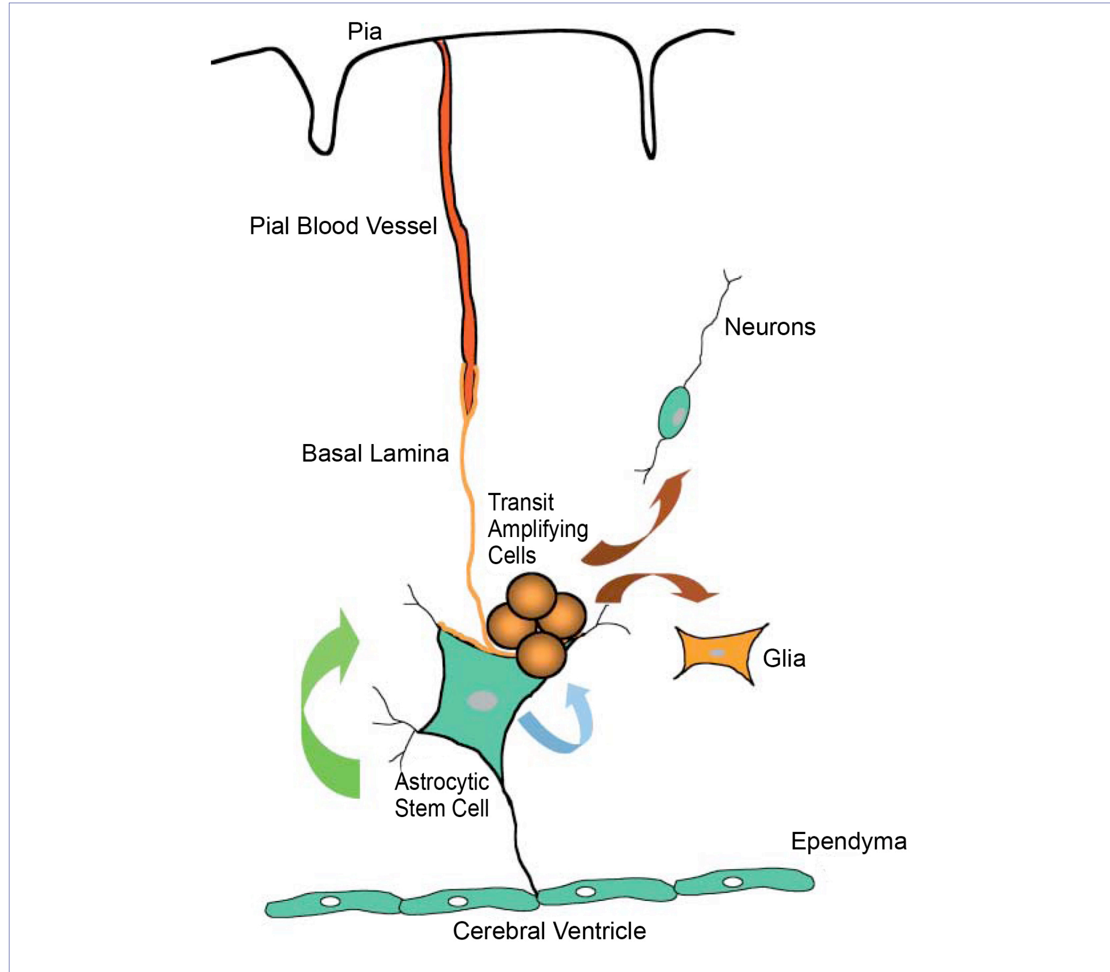

Figure 5. Current concept of the stem cell niche. The multipotent stem-cell like astrocytes are closely opposed to the ventricular lining and basal lamina associated with the pial microvasculature. Asymmetric division gives rise to self-renewal (green arrow) and a transient amplifying population (blue arrow). These cells can migrate out of the germinal niche and differentiate into neurons and glia (brown arrow) [13].

The occurrence of a genetic mutation in neural stem cells (NSC) or early glial progenitors could lead the cells to become independent of growth signals or to resist antigrowth signal. These cells could undergo uncontrolled proliferation and possible tumorigenesis (Figure 6) [14].

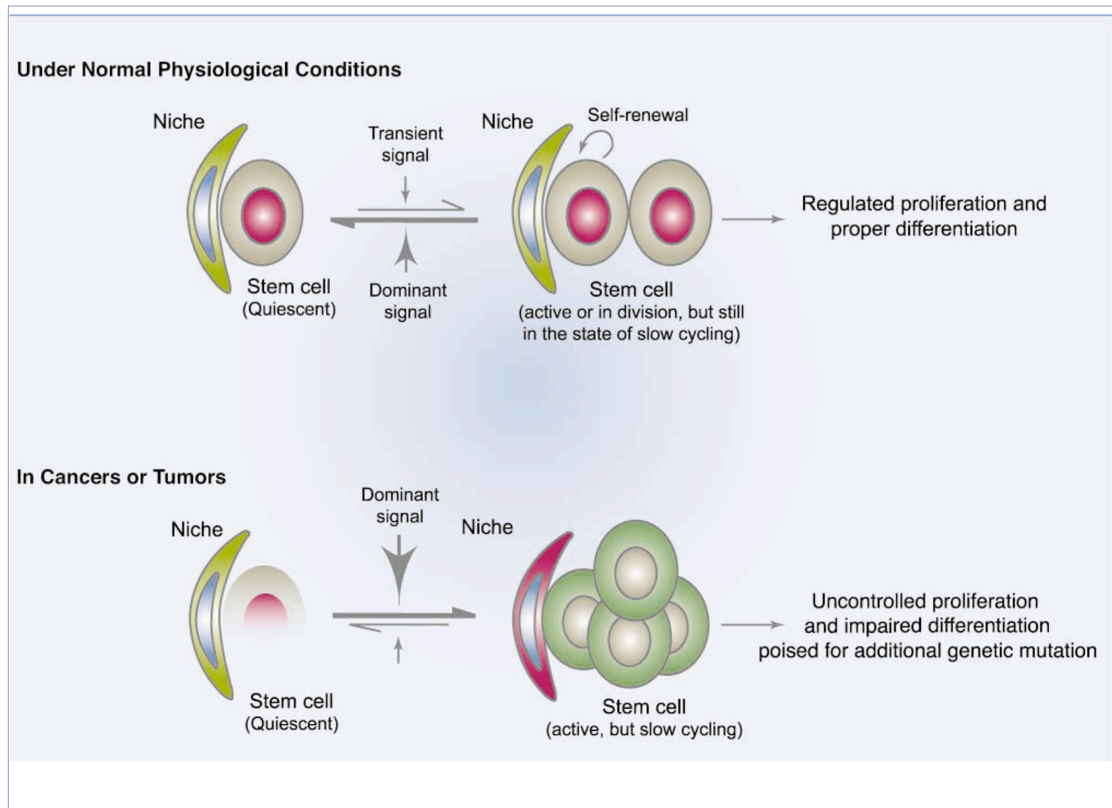

Figure 6. Comparison of the niches under normal and cancerous conditions. The stem cell niche under normal physiological conditions provides an environment that predominantly inhibits proliferation and differentiation. A transient signal is required to support ongoing tissue regeneration. In cancer, internal mutations or change in the niche's signals results in abnormal cell proliferation and growth [14].

Brain tumor stem cells (BTSC) have been identified in human brain tumors of different phenotypes from both children and adults [15]. Studies have shown that BTSC isolated from astrocytomas, when explanted into naive mouse brains, result in the development of tumors identical to the parent tumor [16]. Isolated tumor stem cells form neurospheres, possess the capacity for self-renewal, express genes associated with NSC such as the neural precursor cell surface marker CD133, generate daughter cells of different phenotypes from one mother cell, and differentiate into the phenotypically diverse populations of cells similar to those present in the initial GBM [16]. Also it has been found that CD133(+) and CD133(-) primary glioblastoma-derived cancer stem cells show molecular differences and different biological growth pattern *in vitro* an *in vivo* [17]. This might suggest that CD133(+) and CD133(-) cancer stem cell lines might reflect two biologically different glioblastoma subtypes in primary glioblastomas [17].

Another possible source of transformed glial cell with properties resembling those of stem cells is the mature astrocyte or oligodendrocyte that may be brought to dedifferentiate in response to extrinsic signals or genetic mutations [18]. Recent studies have shown that explanted mature astrocytes isolated from embryonic cortical tissue are able to dedifferentiate in the recipient brain into an earlier glia-like phenotype and acquire proliferative and migratory capabilities that these progenitor cells possess early in CNS development [18].

1.3 Glioblastoma multiforme

1.3.1 Epidemiology of glioblastoma

Every year, approximately 18,000 patients are diagnosed with malignant primary brain tumors in the United States [19]. More than half of these patients have glioblastoma multiforme (GBM), making this the most common malignant brain tumor in adults [4, 19]. The incidence of GBM is highest in elderly patients, peaking at age 65 to 74 years (Figure 7) [20]. GBM seem to occur more frequently in men and Caucasians [4, 20].

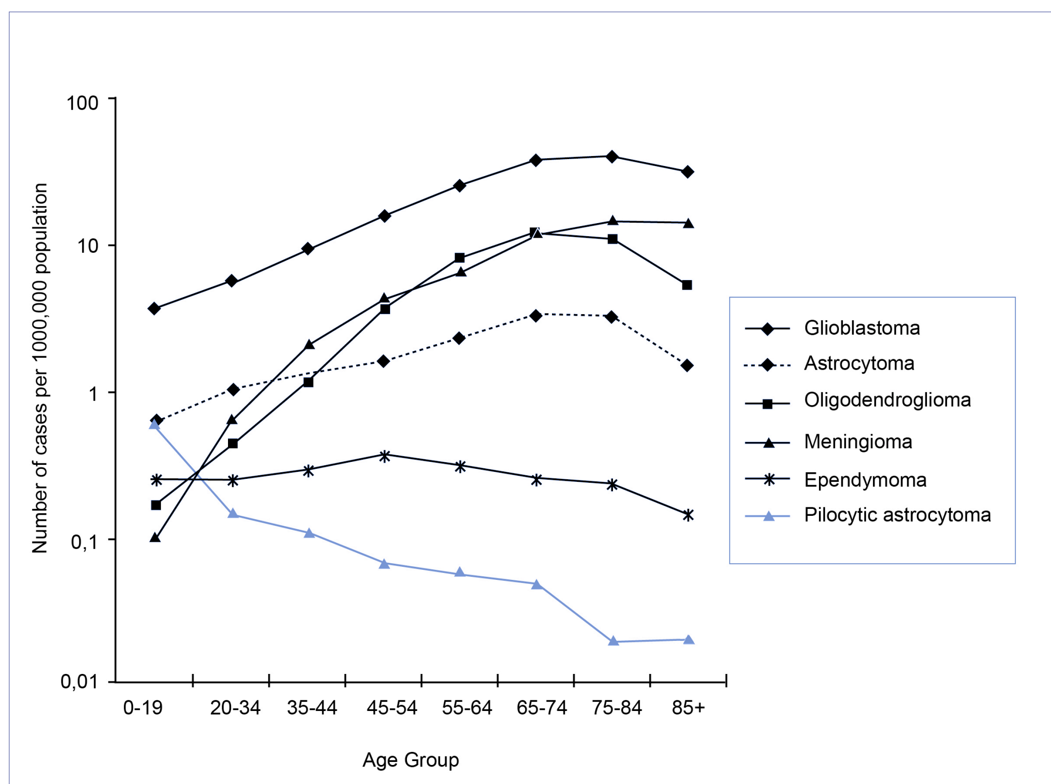

Figure 7. Incidence rates of primary brain tumors by major neuroepithelial tissue and meningeal histologic types and age group. CBTRUS, 1992-1997. The astrocytoma category includes diffuse astrocytomas, anaplastic astrocytomas, unique astrocytomas and astrocytomas not otherwise specified. Adapted from [20].

1.3.2 Risk factors

1.3.2.1 Hereditary syndromes and familial aggregation

In most cases, malignant gliomas occur sporadically. However, the inheritance of certain genes may influence the risk of developing primary brain tumors. Patients with some hereditary syndromes – such as tuberous sclerosis, neurofibromatosis type 1 and 2, nevoid basal cell carcinoma syndrome, Li-Fraumeni syndrome, and syndromes involving adenomatous polyps – seem to be predisposed to malignant gliomas [20] (Table 3). Interestingly, primary brain tumors can also occur in families

without a known predisposing hereditary disease [21]. Environmental exposures may be important in the etiology of this familial aggregation [21].

Table 3. Principal tumor suppressor genes altered in brain tumors and involved in hereditary tumoral syndromes.

Suppresor gene	Germinal mutation	Somatic mutation in tumors of the nervous system
P53	Li-Fraumeni syndrome	Low grade astrocytoma
P16/CDKN2A	Hereditary melanoma	Anaplastic astrocytoma Anaplastic oligodendroglioma
PTEN/MMAC1	Cowden syndrome (multiple hamartomas)	Glioblastoma
RB1	Hereditary retinoblastoma	Anaplastic astrocytoma Glioblastoma
NF1	Neurofibromatosis type 1	Pilocytic astrocytoma ?
NF2	Neurofibromatosis type 2	Neurinoma Meningioma Ependymoma
Patched	Gorlin syndrome (also known as naevoid basal cell carcinoma syndrome)	Medulloblastoma
APC	Rectocolic polyposis	Medulloblastoma

Adapted from [22]

1.3.2.2 Polymorphisms in genes relevant to cancer causation or prevention

It has been proposed that polymorphisms in genes might influence the susceptibility to brain tumors in concert with other external factors [20]. Alteration in genes involved in oxidative metabolism, detoxification of carcinogens, DNA stability and repair might confer genetic susceptibility to brain tumors [20].

1.3.2.3 Ionizing radiation

Therapeutic ionizing radiation (IR) has been recognized as a risk factor for brain tumors [20, 23, 24]. Authors observed a high prevalence (17%) of previous radiation therapy with an average dose of 48.5 Gray (Gy) and an average latency period of 15 years between initial therapy and GBM diagnosis [23]. Studies have found that radiation-induced gliomas are nearly all astrocytic in their differentiation, present as high grade lesions, and occur in a younger patient population than would be expected [24, 25]. Unfortunately, little is known regarding the susceptibility of individuals within the general population to radiation-induced tumorigenesis [26] Diagnostic radiation techniques have not been associated with an increased risk of glioma [27].

1.3.3 Clinical presentation of glioblastomas

Glioblastomas become clinically eloquent due to infiltration, compression and destruction of normal brain structures by tumor, edema, and sometimes hemorrhage. Cerebral spinal fluid flow may be compromised by the tumor, leading to further increased intracranial pressure. Most often, symptoms install progressively, over weeks to months. However, acute apoplectic clinical presentations do occur, for example following intratumoral hemorrhage or *de novo* seizures due to cortical stimulation. The signs and symptoms depend on the tumor's mass effect and its location. General manifestations may occur such as mental changes, headaches, nausea, vomiting and generalized seizures. Focal manifestations include focal seizures, weakness, sensory abnormalities, speech disturbances, and visual deficits [2].

The clinical features at presentation may be used as a measure of the outcome of treatment. The karnofsky performance scale (KPS) is a standardized tool describing the patient's capabilities (Table 4) [2]. Although a KPS of 70 or more at diagnosis has favourable impact on survival, clinical parameters do not fully account for the variation of survival rates [28].

Table 4. Karnofsky performance scale for brain tumor patients

SCORE	PATIENT STATUS
100	Normal: no complaints; no evidence of disease
90	Able to carry on normal activity: minor symptoms
80	Normal activity with effort: some symptoms
70	Cares for self: unable to carry on normal activity
60	Requires occasional assistance: cares for most needs
50	Requires considerable assistance and frequent care
40	Disabled: requires special care and assistance
30	Severely disabled: hospitalized; death not imminent
20	Very sick: active supportive treatment needed
10	Moribund: fatal processes are rapidly progressing

Adapted from [2]

1.3.4 Radiological characteristics

Magnetic resonance imaging (MRI) is more sensitive and accurate than computed tomography (CT) in studying gliomas [29, 30]. Glioblastomas typically present as irregular supratentorial white matter masses with infiltrated margins, poor demarcation and extensive edema. Necrosis, cysts and thick irregular margin are common findings [31]. Spontaneous hemorrhage can be seen and is believed to correlate with histologic grade [32]. Following contrast injection, a thick ring of enhancement surrounding central necrosis is commonly found, although a solid, nodular or patchy appearance may be visualized. The enhancement seen on MRI and CT following contrast injection correlates with the area of greatest vascularity [2, 31] (Figure 8). The aggressiveness of GBM may be suggested by its infiltration through the adjacent brain parenchyma, best approximated by the hyperintensity on T2-weighted images [33] (Figure 8). However, tumor cells are found infiltrating the brain beyond the hyperintensity signal changes in T2-weighted images when results of stereotactic biopsies are compared with MRI [33, 34]. Newer techniques are being developed to improve diagnosis accuracy, to determine the most appropriate biopsy site, and to evaluate tumor recurrence [31] such as MRI perfusion, magnetic resonance spectroscopy, positron emission tomography, diffusion tensor imaging.

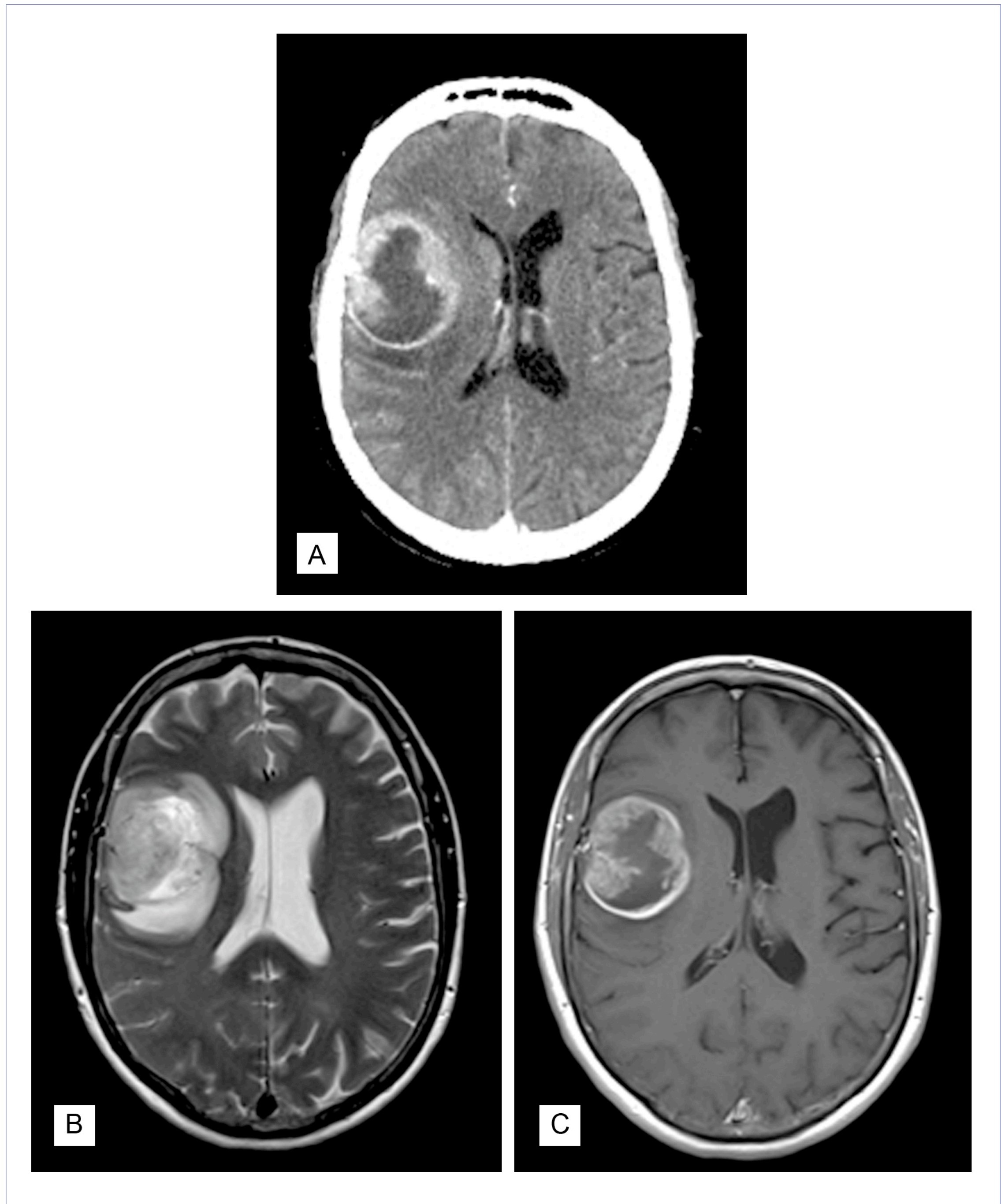

Figure 8. Characteristic radiological features of glioblastoma.
A) Axial contrast enhanced CT shows strong, heterogeneous, irregular rim enhancement surrounding a hypodense necrotic core. B) Axial T2 weighted image MR shows a heterogeneous mass with surrounding hyperintense signal compatible with tumor infiltration and vasogenic edema. C) Axial T1 weighted image MR with gadolineum demonstrating a hazy, heterogeneous peripheral enhancement.

The most common differential diagnosis include brain metastasis, enhancing anaplastic astrocytoma, primary CNS lymphoma, and non-neoplastic disorders such as an abscess, multiple sclerosis, cerebral infarction, vascular malformation [31]. The final diagnosis can be confirmed only by histopathological examination.

1.3.5 Histological characteristics

Intraneoplasm and interneoplasm heterogeneity is characteristic of GBM. Although some areas have the characteristics of a low grade astrocytoma or anaplastic astrocytoma, the significant amount of microvascular proliferation and the presence of necrosis are key features distinguishing GBM from grade 2 and 3 astrocytomas [9].

The cellular morphology of GBM is highly variable. A spectrum ranging from small tightly packed, round or elongated cells to giant elements with significant nuclear atypia can be encountered within the same tumor. GBM are highly cellular tumors with cellular pleomorphism and numerous mitotic figures with corresponding elevated proliferation marker indexes. Endothelial proliferation or glomeruloid microvascular proliferations are highly suggestive of GBM. Necrosis on the other hand is characteristic of grade IV astrocytomas as it results of a pathological tumor microvasculature [9] (Figure 9).

GBM cells tend to infiltrate adjacent brain by spreading along white matter tracts, through the adjacent extracellular matrix or along the basement membranes [2]. Therefore islets of GBM cells can be found at distance from the primary tumor site.

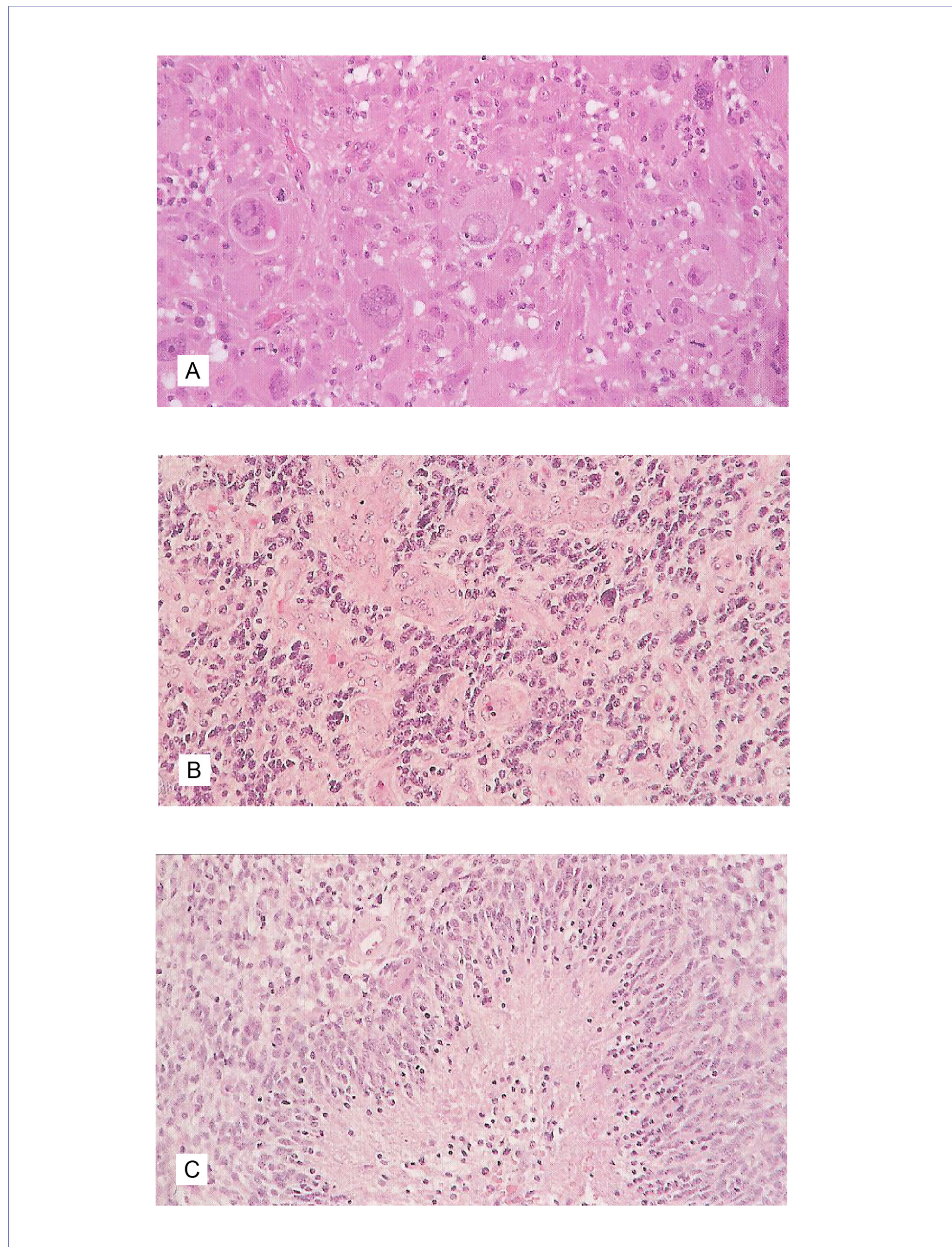

Figure 9. Characteristic histopathological features of glioblastomas.
A) Cellular pleomorphism seen as variable nuclear:cytoplasmic ratio. B) Neovascularisation seen as buds of capillary endothelial proliferation among neoplastic cells. C) Pseudopalisading of cells around necrotic areas. Adapted from [9].

1.3.6 Primary and secondary glioblastomas

GBM seem to develop from one of two ways. The majority of cases (>90%) are primary glioblastomas that develop rapidly *de novo* from glial cells without clinical or histological evidence of a less malignant precursor lesion. Typically, they become clinically manifest within less than 6 months of diagnosis. Approximately 50% of patients with primary GBM have a clinical history of less than 3 months [35]. Primary GBM is most common in older patients [36]. Secondary GBM develops over months to years from pre-existing low-grade astrocytomas [37]. Neuroimaging or histologic evidence of evolution from a less malignant precursor lesion is mandatory to diagnose secondary GBM. This subtype of GBM predominantly affects younger patients, average age at diagnosis is 39 years [36].

Primary and secondary GBM represent distinct entities that arise through different genetic pathways. The development of primary GBM is associated with over-expression or amplification of the epidermal growth factor (EGF) gene. Additional genetic alterations leading to this subtype of GBM include amplification or over-expression of murine double minute 2 (MDM2), loss of heterozygosity (LOH) 10q, deletion or mutation of the phosphatase and tensin homolog deleted from chromosome 10 (PTEN), p16(INK4a) deletion [37]. In the pathway to secondary glioblastoma, p53 mutations are the most frequent and earliest detectable genetic alteration, already present more than 60% of precursor low-grade astrocytomas. It is also associated with overexpression of platelet-derived growth factor (PDGF) ligands and receptors and LOH 10q [35, 37, 38] (Figure 10). Although histological characteristics of primary and secondary glioblastomas are reported identical [39],

differences in their pattern of promoter methylation and in expression profiles at RNA and protein levels seem to have implications for therapeutic options [37].

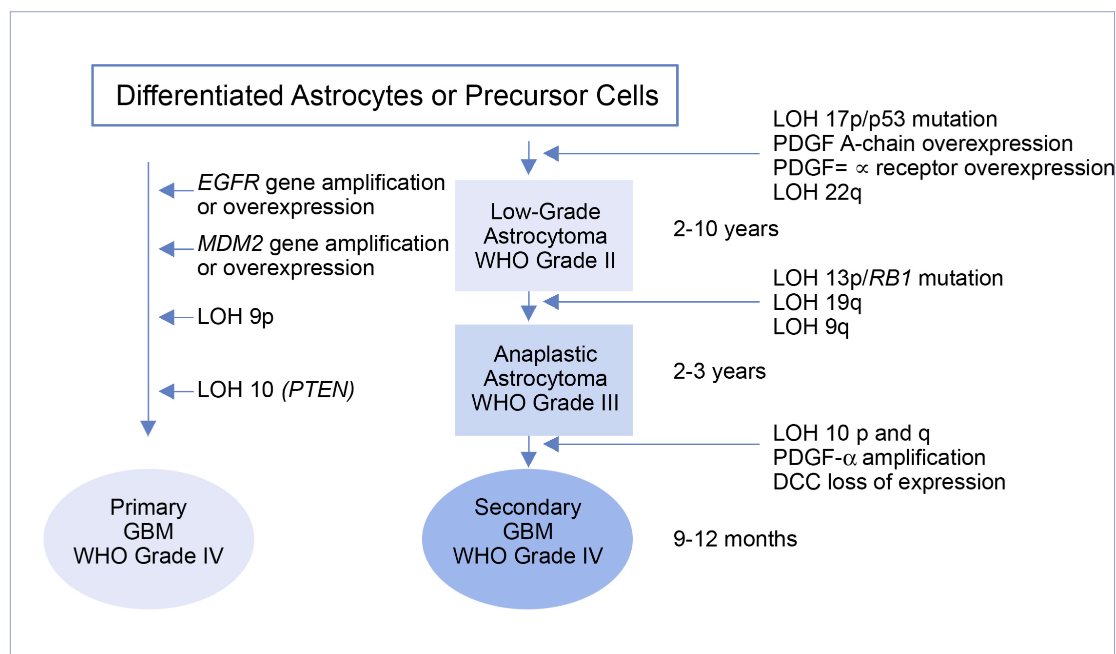

Figure 10. Formation of primary and secondary glioblastoma multiform (GBM). Multiple genetic changes are involved in the development of primary and secondary glioblastomas.Abbreviation: DCC, deleted in colorectal cancer; EGFR, epidermal growth factor receptor; LOH, loss of heterozygosity; MDM2, murine double minute 2; PDGF, platelet-derived growth factor; PTEN, phosphate and tensin homolog deleted on chromosome 10; RB1, retinoblastoma 1 gene; WHO, World Health Organization. Adapted from [38]

1.3.7 Overview of altered molecular pathways in glioblastoma

1.3.7.1 Signal transduction

Altered signal transduction may contribute to gliomatogenesis. Critical signals are initiated in GBM by members of the tyrosine kinase receptor family involving, for instance, EGF receptor (EGFR), PDGF receptor (PDGFR), basic fibroblast growth factor receptor (bFGFR). Increased production of the growth factor or increased production or activity of the growth factor receptor may occur through various mutations. As a result, downstream pathways exhibit increased activity such as increased PI3K, Akt, or Ras which may contribute to tumor formation and tumor progression [40]. Also, mutations of tumor suppressor genes may result in an oncogenic signal. For example loss of PTEN function in glioma cells may participate in astrocytic tumor pathogenesis and progression through activation of the prosurvival PI3K/Akt pathway [39, 40].

1.3.7.2 Cell cycle

In glioblastoma, regulatory controls of the cell cycle are often disrupted, leading to uncontrolled proliferation of transformed cells. The Rb and p53 pathways normally maintain cells in G_1 arrest. Other cell cycle regulators such as p21/WAF1/Cip1 and p27/Kip1 also regulate the progression from the G_1 to the S phase. Loss of function of such regulators promotes accelerated growth and malignant transformation in astrocytes [2, 41].

1.3.7.3 Programmed cell death

Apoptosis, a specialized mechanism of cell death, is an important mechanism for maintaining genetic stability. If damage is beyond repair, p53 activates a series of pro-apoptotic members. In glioblastoma cells, altered signal transduction pathways modify the balance between pro-apoptotic and anti-apoptotic activities. An example of such a protein is Survivin, an inhibitor of apoptosis protein (IAP), that has been reported to increase survival in GBM by suppressing caspase-mediated apoptosis [42-44]. Survivin can bind specifically to the terminal effector cell death proteases, caspase-3 and caspase-7 [45]. Studies with a transgenic mouse model of transgenic expression of Survivin in the skin have shown that this IAP can also be antiapoptotic by inhibiting the intrinsic caspase-9-dependent pathway [46]. Recently Survivin has been recognized to also inhibit cell death in various cell lines through a caspase-independent pathway although the mechanisms have not yet been elucidated [47, 48]. In addition to being involved in the modulation of apoptosis[42-44, 49]. Survivin is implicated in the regulation of cell growth [42], in the regulation of mitotic events such as chromosomal segregation and cytokinesis [50, 51], and in the process of angiogenesis [52, 53].

Other types of cell death less well studied in glioblastoma possibly contribute to the survival versus death balance such as autophagy, necrosis, and mitotic catastrophe [54]. In the context of cancer therapy, senescence consisting in permanent growth arrest, is also considered a type of cell death. The biological and morphological characteristics of each cell death pathway are presented in table 5.

Table 5. Cell death pathway characteristics

	Apoptosis	Autophagy	Necrosis	Senescence	Mitotic catastrophe
Morphologic changes					
Cell membrane	Blebbing,membrane integrity maintained	Blebbing	Loss of membrane integrity	Flattening; Increase in cell size	?
Nucleus	Chromatin conden-sation, DNA laddering, nuclear fragmentation	Partial chromatin condensation, no DNA laddering	Random DNA degradation	Accumulation of heterochromatin foci	Mis-segregation of chromosomes during cytokinesis; micronudei
Cytoplasm	Condensed membrane-bound cellular fragments; depolymerization of cytoskeleton	Increased number of autophagic vesicles,degradation of Golgi, polyribosomes, and the ER	Swelling of cellular organelles	Granularity	?
Detection methods	Annexin V staining, DNA fragmentation assays, caspase activation	LC3 localization	Early permeability to vital dyes, release of intra-cellular contents	Senescence-associated β-galactosidase activity	Visualization of multinucleated cells
Release of cellular contents	Lysophosphatidyl-chlorine	?	HMGB1, S100 molecules, purine metabolites, heat-shock proteins, uric acid, HDGF	?	?
Immunologic response	Suppressive, engulfment of cell carcass	?	Stimulatory, initiation of cell growth and tissue repair	?	?

Adapted from [54]. Abbreviations: ER, endoplasmic reticulum; HDGF, hepatoma-derived growth factor; HMGB1, high-mobility group box 1 protein; LC3, microtubule-associated protein 1, light chain 3.

1.4 Angiogenesis

In addition to their agressive character, malignant gliomas are also recognized as the most intensively vascularized solid tumors [2]. The process of angiogenesis is essential for tumor progression. Indeed, malignant gliomas become more angiogenic with increasing tumor grade, suggesting that the vascular component plays an important role in their malignant progression [55].

1.4.1 Distinction between vasculogenesis and angiogenesis

New vessel formation occurs via two distinct processes: vasculogenesis and angiogenesis [56]. Vasculogenesis is defined as the formation of blood vessels from endothelial-cell precursors, the angioblasts. During embryologic and fetal development, angioblasts arise in the mesoderm and differentiate into endothelial cells that proliferate to form a primitive vascular network in an avascular tissue [56, 57]. In the postnatal period, circulating bone marrow-derived endothelial progenitor cells (EPCs) may home to sites of physiological and pathological neovascularisation [58, 59]. Postnatal vasculogenesis is subject to regulation by many factors, including cytokines such as vascular endothelial growth factor (VEGF) and growth factors such as PDGF [59].

The primitive vascular network is modified by the process of angiogenesis leading to the formation of new vascular segments. Normal angiogenesis occurs in physiological conditions such as wound healing and female reproduction system [56]. Aberrant angiogenesis is present in pathological non-malignant conditions such as ischemia and inflammatory reactions as well as in malignancies [56].

1.4.2 Steps in angiogenesis and implicated molecular players

1.4.2.1 The angiogenic switch

Initially, it was proposed that tumors exist in two phases: avascular and vascular. In the avascular phase, solid tumors of 2-3 mm^3 or less obtain the necessary oxygen and nutrient supplies required for growth and survival and eliminate metabolic waste products by simple passive diffusion. However, tumors larger than 2mm^3 require blood supply for further growth. This concept has been challenged by the discovery of a subset of tumors that initiate growth by co-opting existing host vessels. Co-opted vessels overexpress angiopoietin-2 (Ang-2) which leads to vessel regression [60]. Vessel collapse and increased tumor growth occurs when anaplastic astrocytomas progress to glioblastoma. Hypoxic conditions induce the activation of a hypoxia-inducible factor (HIF-1). This transcription factor binds to hypoxia-responsive elements and induces the transcription of many angiogenic factors such as VEGF, PDGF, Angiopoietin (Ang)-1 and -2 [61], stem cell factor (SCF) [62]. In addition, hypoxia increases VEGF messenger RNA (mRNA) stability through binding of several RNA-binding proteins [56]. Hypoxia and subsequent necrosis is pathognomonic of glioblastoma and is the principal stimulus for new vessels formation. Interestingly, high SCF expression has been documented in glioma cells but also in normal host neurons, possibly in response to the glioma-induced damage in normal brain parenchyma [62]. The areas of neuronal and glioma SCF overexpression correspond to areas of sprouting angiogenesis. Therefore, normal brain cells may also participate to induce pathological angiogenesis and support tumor growth and infiltration [62]. Activation of SCF/c-Kit signalling pathway in ECs has been found to

enhance ECs' proliferation, survival and migration, even in the absence of growth factors believed to be obligate such as VEGF [62]. Most probably, various pro-angiogenic factors have complementary roles in tumors with significant angiogenesis such as gliomas.

In all circumstances, tumor progression requires the induction of a tumor vasculature, termed the angiogenic switch [63]. The angiogenic switch can occur at different stages of the tumor-progression pathway, depending on the tumor type and its environment [57]. Although angiogenesis is necessary, it is not in itself sufficient for tumor growth [63]. Induction of the angiogenic switch depends on the balance between angiogenic stimulators and inhibitors, termed the angiogenic balance (Figure 11) [63].

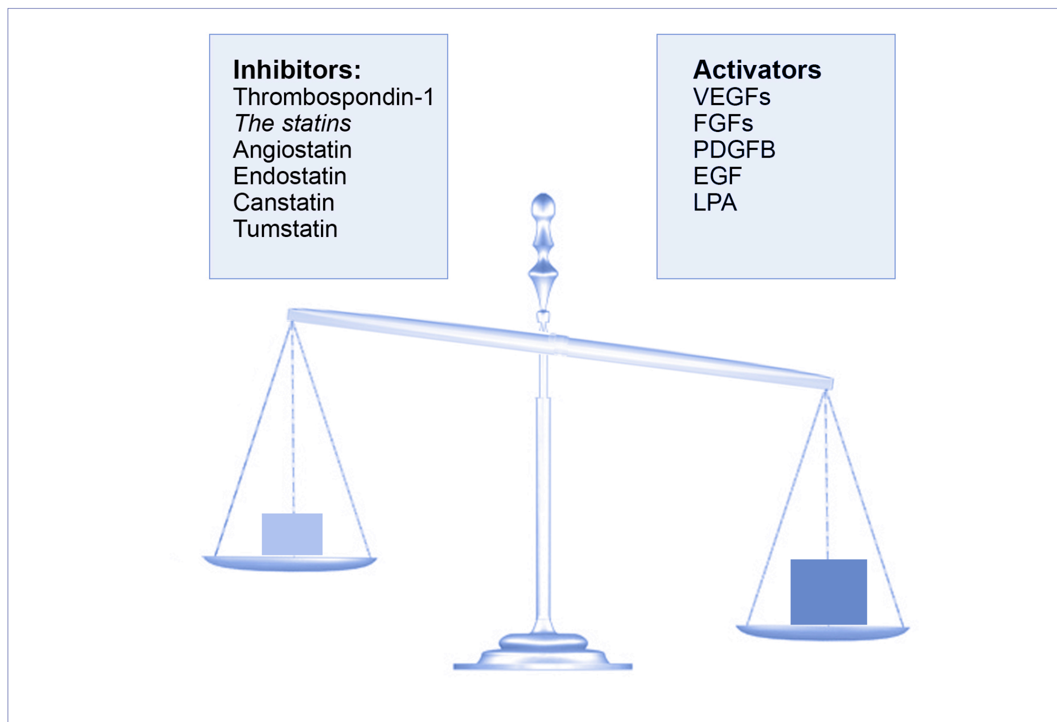

Figure 11. The angiogenic balance. Angiogenesis is orchestrated by a variety of activators and inhibitors – only a few of which are listed above. Activators are mainly receptor tyrosine kynase ligands such as vascular endothelial growth factor (VEGF), fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), but can also be of various origin such as lysophosphatic acid (LPA). Inhibitors include thrombospondin-1 and the statins. Adapted from [57].

Expression of such regulators may be in response to physiological stimuli, such as hypoxia, resulting from increased tissue mass, and also to oncogene activation or tumor suppressor mutation in tumor cells [57]. Tumor cells may upregulate expression of angiogenic activators or downregulate expression of angiogenic inhibitors, mobilize angiogenic proteins from the extracellular matter, recruit host cells such as macrophages which produce their own angiogenic proteins [63].

1.4.2.2 Mechanism of sprouting angiogenesis

Angiogenesis may occur via sprouting and non-sprouting mechanisms. Sprouting angiogenesis is a multi-step process involving interplay between cells, soluble factors, and extracellular matrix components. Angiogenic activators such as VEGF, placental growth factor and Ang-1 may initiate angiogenesis [57]. Angiogenesis begins with vasodilation and increased vascular permeability in response to VEGF and loosening of pericytes covering host vessels. The vascular basement membrane and extracellular matrix are locally degraded by proteolytic enzymes such as cathepsin B and matrix metalloproteinases (MMPs) [57]. Endothelial cells may migrate into the interstitial space towards chemotactic angiogenic stimuli. Endothelial cells proliferate at the migrating tip, forming a solid vessel sprout. The sphingolipid sphingosine-1-phosphate (S1P) and S1P(3) receptors enhance ECs proliferation and migration, playing a key role in angiogenesis [64]. Endothelial cells change shape and adhere to each other to form a lumen. A new basement membrane is produced around the newly formed blood vessel with recruitment of pericytes. Finally vascular sprouts fuse with other sprouts to form loops and blood may flow in the newly vascularized area [57].

1.4.3 Tumor vasculature

1.4.3.1 Types of tumor angiogenesis

Tumor vasculature is not necessarily derived from sprouting angiogenesis: it can also occur through non-sprouting processes [65]. Cancer tissue can acquire its vasculature by intussusceptive angiogenesis, recruitement of circulating endothelial precursor cells, co-option of pre-existing vessels, mosaic vessel formation, and vasculogenic mimicry [65] (Figure 12).

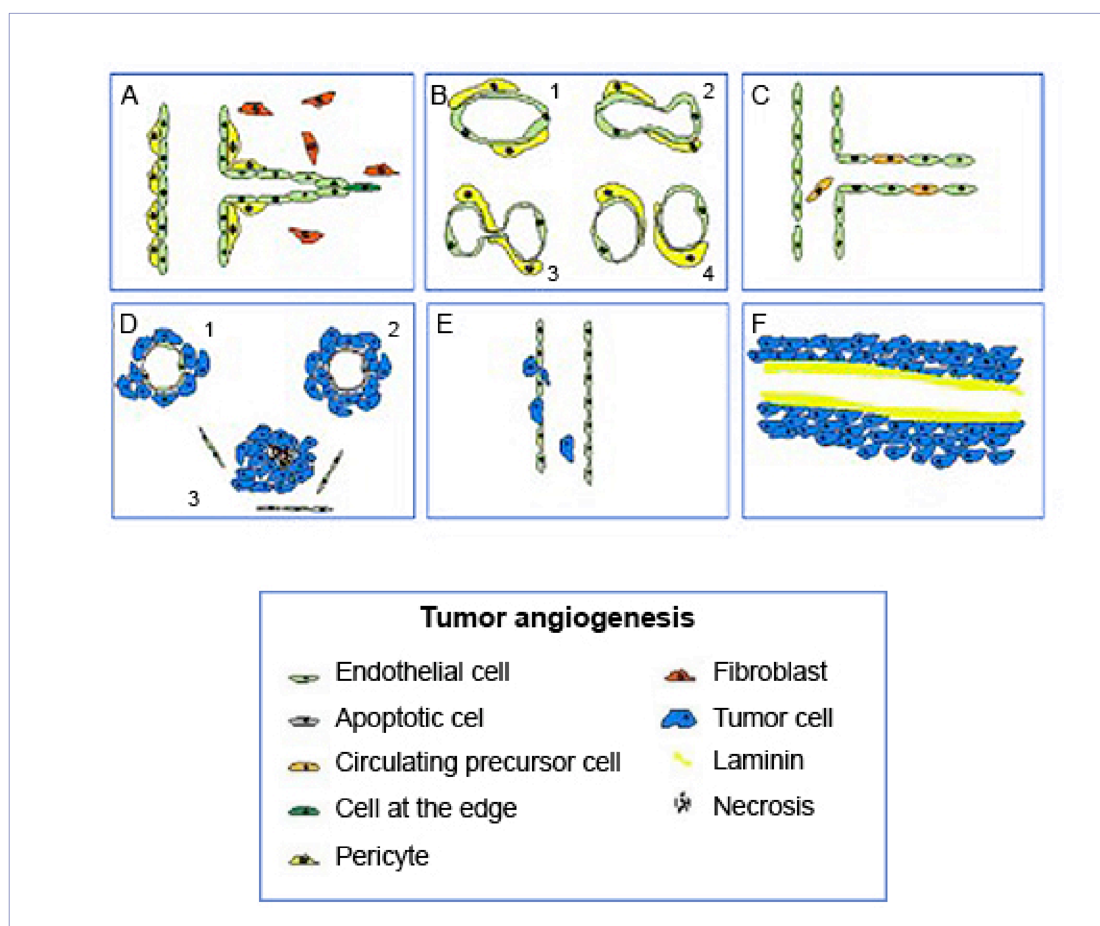

Figure 12. Mechanisms of tumor angiogenesis. The mechanisms of tumor angiogenesis are: A) Sprouting; B) Intussuceptive angiogenesis; C) Recruitment of precursor cells; D) Vessel co-option; E) Mosaic vessel formation; F) Vasculogenic mimicry. Adapted from [65].

Intussusceptive angiogenesis refers to vessel network formation by insertion of connective tissue columns, called tissue pillars into the vessel lumen and to subsequent growth of these pillars, resulting in partitioning of the vessel lumen [66]. Vessel co-option may occur as cancer cells proliferate along pre-existing microvessels, without a tumor capsule, eliciting an invasive character [57, 60, 66]. Mosaic vessels refers to the presence of tumor cells within the walls of tumor vasculature. Vasculogenic mimicry may contribute to tumor angiogenesis in various malignancies such as melanomas, breast, prostate and lung cancers. These cancers have the ability to express an endothelial cell phenotype and to form three dimentional vessel-like networks, mimicking the pattern of embryonic vascular networks [67, 68].

Astrocytomas also feature another mechanism of tumor angiogenesis named glomeruloid angiogenesis. In fact, glomeruloid bodies are best known in high grade astrocytomas where they are one of the diagnostic histopathological features of GBM [69]. These bodies are tufted collections of newly formed microvessels surrounded by variably thickened basement membrane with an incomplete layer of pericytes [66, 69]. It has been proposed that hypoxic astrocytoma cells in areas surrounding central necrosis up-regulate the expression and secretion of VEGF, which induces vascular hyperplasia of nearby vessels that develops in glomeruloid bodies (Figure 13) [70].

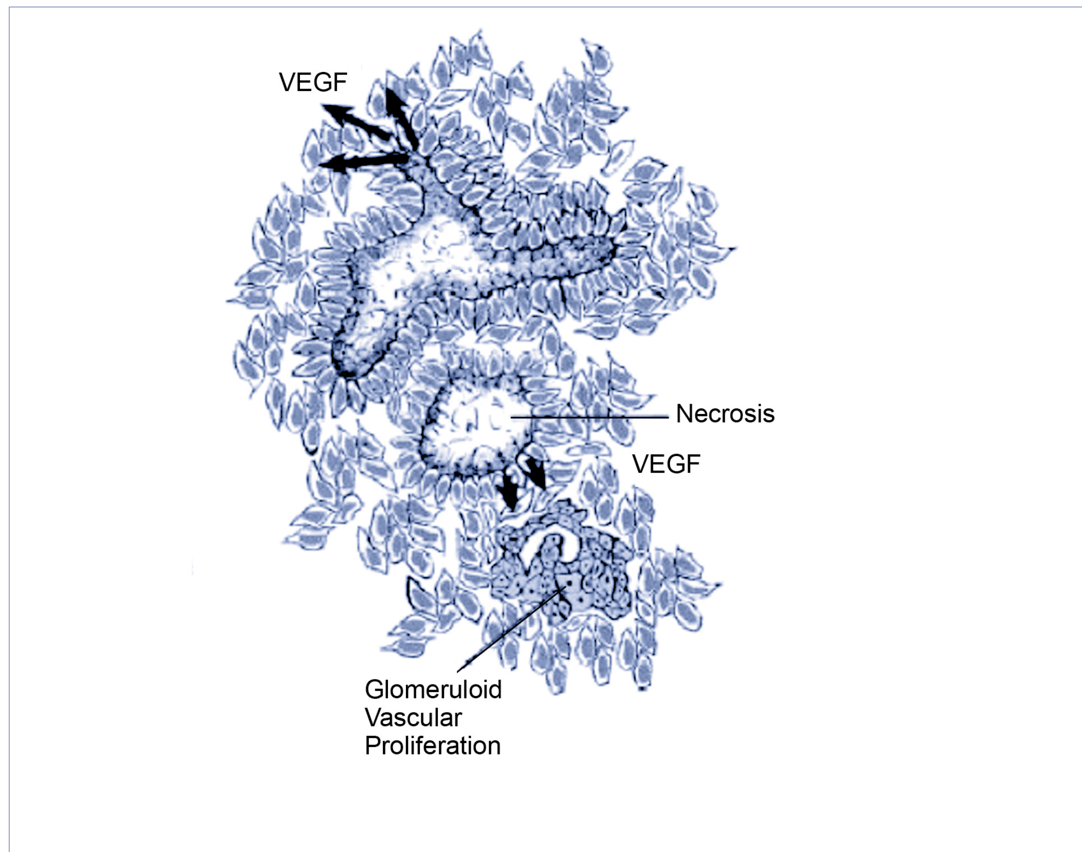

Figure 13. Glomeruloid vascular proliferation in glioblastoma multiform. Hypoxic astrocytoma cells in zones surrounding central necrosis up-regulate the expression and secretion of VEGF, which acts on nearby vessels to cause vascular hyperplasia, inducing glomeruloid vascular proliferation. Adapted from [70].

1.4.3.2 Macroscopic and microscopic characteristics of tumor blood vessels

The appropriate balance between activators and inhibitors is lost in tumor angiogenesis, resulting in extensive blood vessel growth and failure to mature. Histological examination of glioblastoma tissue reveals an architecturally specific vasculature, different from normal brain counterparts. Macroscopically, two main types of vascular patterns have been described in glioblastoma [71, 72]. The glomeruloid/garland like type, which refers to glomeruloid angiogenesis, is characterized by unevenly distributed vascular formations. The 'classic' capillary like

vascular pattern shows irregularly shaped, dilated and tortuous vessels, with occasional dead ends [57]. Tumor blood vessels share characteristics of venules, arterioles and capillaries. Blood flow is irregular in tumor vessels, moving slowly, sometimes oscillating and even flowing in reverse sense [73]. The vascular network is often leaky and hemorrhagic [74]. The perivascular cells that are usually in contact with endothelial cells are more loosely apposed and less abundant than in normal vasculature [57]. Endothelial cells derived from GBM have a flat appearance, with large nuclei, abundant cytoplasm and multiple nucleoli [75]. Normal brain endothelial cells are smaller, with limited cytoplasm [76].

It has been proposed that vascular patterns influence clinical outcome of patients with astroglial brain tumors [77]. However, other authors observed that poor observer agreement on vascular patterns in patients with glioblastoma limits the clinical utility of these factors [78]. Improved methodologies for morphologic assessment of glioblastoma vascularization need to be identified. In addition, regional tumor heterogeneity may limit the clinical relevance of these histopathological assessments [79].

1.4.3.3 Cellular characteristics of brain tumor endothelial cells

GBM-associated ECs express typical endothelial markers such as vWF, CD105, CD31, similarly to normal ECs. For some markers, the level of expression and the distribution of expression varies between studies given the heterogeneity of ECs sampled [75]. For instance, the expression of CD34, a marker for EPCs, has been reported elevated, similar, or decreased in comparison to control ECs [75]. Expression

of CD144 (VE-cadherin), a tight junction protein, is reduced in GBM-associated ECs [76]. This may contribute to the leakiness of GBM's vessels [80]. The reduced expression of other tight junction proteins in tumor microvessels such as claudin-1, claudin-5, and occludin may also contribute to the tumor's leakiness [81]. Interestingly, 50% of GBM-associated ECs express α-SMA, a cytosqueletal protein implicated in initiation of cell contraction that is mostly expressed by mural cells and absent from control ECs [75]. Its expression may be related to tumor-associated ECs' enhanced migratory potential.

Although some authors have found that GBM endothelial cells proliferate faster than normal ECs [80], others have documented a slower rate of replication [75, 76]. This discrepancy might be explained by tumor vasculature heterogeneity: endothelial cells isolated from the periphery of a tumor may proliferate more than those obtained close to hypoxic or necrotic regions [75]. Furthermore, glioblastoma-derived ECs were found to migrate more than those in normal brain, based on results of modified Boyden chamber migration assays. [76]. Glioblastoma-derived ECs also have enhanced survival properties. Authors have documented that these cells undergo less apoptosis after serum starvation [76]. Migration activity and proliferative and apoptotic activities might be mutually exclusive behaviours in tumor ECs, as was proposed for astrocytoma cells [82].

Tumor endothelial cells also produce angiogenic growth factors [83]. Studies have documented an increased expression of VEGF, endothelin-1 (ET-1) and interleukin-8 (IL-8) in comparison to normal counterparts [76, 83]. Also, the expression of VEGF receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), as well

as that of IL-8 receptors CXCR1 and CXCR2, was documented on GBM-derived endothelial cells [83]. Endothelial cells derived from GBM maintain angiogenic properties, they have the capacity to form tubules [84].

1.4.4 Angiogenesis as a therapeutic target

Given the requirement of angiogenesis for growth and progression of tumors, the tumor vasculature is an attractive target for tumor therapy. Therapeutic vascular targeting is of two types: anti-antiangiogenic approaches which aims to prevent neovascularization in tumors and vascular disrupting approaches which aims to disrupt established tumor vasculature [85], both approaches culminate in tumor cell death.

Many targets have been explored and the VEGF/VEGFR-2 signalling has been strongly suggested as the primary target [86]. However, studies have shown that targeting only one signalling pathway may result in the activation of alternative pro-angiogenic pathways [86]. Therefore, targeting multiple angiogenic signalling pathways by polyvalent inhibitors or combinations of agents with distinct antiangiogenic properties has been attempted to optimize antiangiogenic agents [86].

1.5 Therapeutic management of glioblastomas

1.5.1 Surgery

Depending on the clinical state of the patient and the radiological characteristics, surgical goals should be adjusted to each patient. Surgery should enable to obtain a tissue diagnosis. Although the diagnosis of a malignant glioma is highly suspected with the presence of contrast enhancement on CT and MRI, up to approximately 40% of malignant gliomas do not enhance following contrast injection [87]. Given that a radiological diagnosis is not reliable, a tissue sample is required in essentially all cases [88]. For patients in whom resection is not possible because of advanced age, multiple or severe comorbidities, tumor location and/or extent, a biopsy should be performed to determine the histology of the tumor [88]. Biopsies may be performed either openly, through a mini-craniotomy or through a burr hole with stereotaxy or neuronavigation guidance.

When surgical resection is possible, total tumor resection should be the goal. Decreasing local tumor mass can improve neurological function, reduce steroid dependence, and prevent early death [2]. Decrease in tumor burden has been reported to be a significant prognostic indicator for survival. In deed, gross-total tumor resection is associated with longer survival in patients with GBM. Functional mapping has been used to maximize the extent of tumor removal and avoid injury to cortex essential for language, motor and sensory functions. Intraoperative magnetic resonance guidance, fluorescence-guided surgery, and neuronavigation systems have been used to achieve a more complete removal of deep-seated tumor than with

conventional techniques [89]. However, the benefit of such technologies have yet to be clearly demonstrated.

The amount of tumor remaining after surgery has been shown to be a significant prognostic indicator for survival [90]. In addition, the post-operative residual tumor area is an important baseline variable that affects the efficacy of adjuvant therapies [91].

1.5.2 Radiotherapy

1.5.2.1 Overview of radiobiology

1.5.2.1.1 Types of rays

Radiations used for radiotherapy are of two types either particulate or electromagnetic. Particulate radiation includes electrons but also neutrons, protons, alpha particles, heavy ions, helium ions [92]. These charged particles do not penetrate deeply the tissue as they interact with tissue cells within the first centimetres penetrated [93]. Photon radiation such as x-rays and gamma rays have no electric charge and are called electromagnetic radiation. They are generated electrically by machines such as the linear accelerator, or produced through spontaneous desintegration of natural or artificial radioactive material such as cobalt 60, iodine 125 [93, 94]. The unit of dose delivery in radiation therapy is the Gray (Gy), which is defined by the amount of energy delivered to a mass of tissue.

1.5.2.1.2 Effects of radiation: physical, cellular, tissular

Immediately after exposure, particular and electromagnetic radiations exert their effect at the physical and cellular level. The physical reaction is almost instantaneous. Energy deposition to a cell occurs very quickly, within 10^{-18} s, in a random fashion [95]. Radiation causes ionization, the process by which a neutral atom acquires a positive or negative charge. The passage of an ionizing ray induces a series of ionization in the atoms encountered along its trajectory. Photon radiation is indirectly ionizing since it is not charged and its activity depends on particles encountered. Electrons, protons, and alpha particles are directly ionizing: these charged particles produce ionization by collision or repulsion within the matter [93, 94].

Following radiation indirect or direct interaction in a cell may occur [95]. Indirect interaction occurs when radiations interact with cellular water, resulting in a hydrogen molecule and a hydroxyl free radical molecule. If two hydroxyl molecules recombine, they form a hydrogen peroxide. As this highly unstable molecule diffuses throughout the cell, it may lose a hydrogen ion resulting in a peroxide hydroxyl molecule which can combine with organic molecules, an enzyme for example, to form a stable organic hydrogen peroxide molecule, impairing the molecule's function [95]. These indirect interactions begin within seconds following irradiation. On the other hand, radiations may interact directly with cellular macromolecules such as DNA or other proteins. Many different types of DNA alterations may be induced within minutes after radiation exposure such as single strand breaks, double strand breaks, base damage of various types and DNA-protein cross-links [96]. Direct hits are

mostly repaired with high fidelity since the undamaged strand of DNA with its complementary base sequence can be used as a template for repair. However, the probability of perfect repair reduces with increasing complexity or clustered damage to DNA. Cellular damage might result in division delay and reproductive failure, allowing time for repair of DNA [95]. Cell death may occur promptly or many generations after IR exposure (Figure 14) [93].

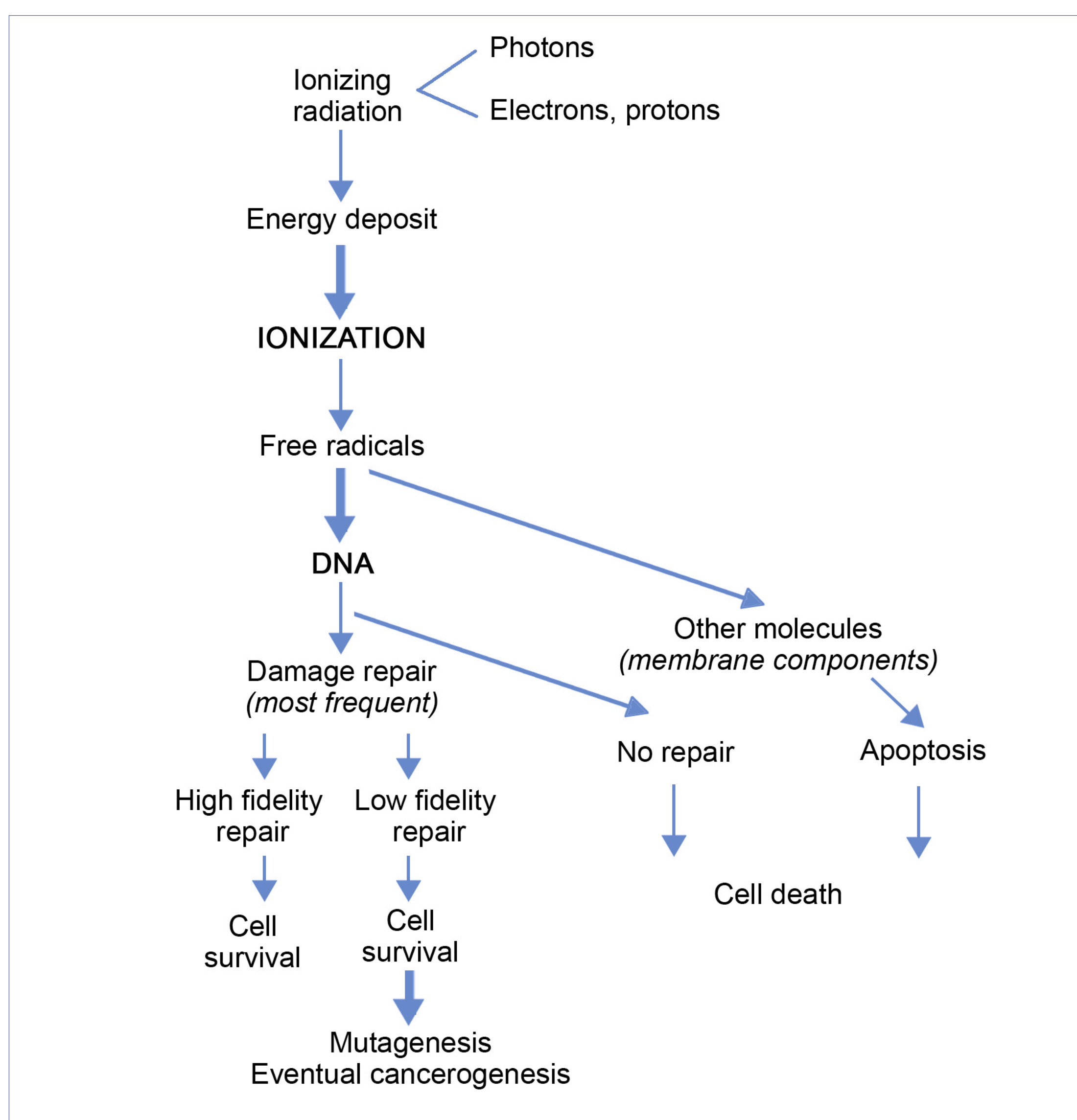

Figure 14. Mechanisms of action of ionizing radiations. Adapted from [93].

Tissular alterations which result of cellular damage occur within a few days to a few years after radiation exposure. In addition to the intrinsic radiosensibility of the tissue exposed to radiations, the length of the treatment and if it is fractionated influence the occurrence of adverse effects. Increasing the duration of the treatment gives time to tissues with rapid renewal rates such as the skin or mucosa to repopulate normal tissues and prevent acute reactions. However, since tumors often behave like rapidly renewing tissues they also have the opportunity to multiply. Multiple fractions of IR with at least 6hres between each treatment enables tissues with low renewal rates, for example the brain, to repair the radio-induced lesions between treatments. Most secondary effects occur in adjacent normal tissues after a certain dose of radiation is administered (Table 6). After this dose, the risk of developing secondary effects due to IR increases with the total dose delivered [93].

Table 6. Total dose of irradiation tolerated by normal organs

Normal organ	Tolerated total dose when administered in classic fractions (in Gy)
Bone marrow	1-5
Gonads	5-6
Cristallin	10
Kidney	20
Lung	20
Heart	30
Spinal cord	45-50
Brain (as a whole)	40
Peripheral nerves	50-60
Skin (variable depending of surface)	50-70
Bone	50-70

Adapted from [93]

1.5.2.1.3 Types of radiotherapy

Conventional, or fractionated, radiotherapy delivers a fraction of the complete radiation dose to an involved field over many sessions. A standard algorithm is 60 Gy of external-beam radiotherapy delivered in 1.8 to 2.0 Gy fractions 5 times per week. Conventional radiotherapy has been shown to prolong median survival for 6-10 months [10, 97]. Many studies have assessed the effect of dose intensification and modified radiation profile such as hypofractionation and accelerated fractionation to improve outcomes of patients with GBM, however results were equivalent to conventional radiotherapy schemes [98].

Stereotactic radiosurgery (SRS) is a technique of external beam radiation that uses convergent beams to deliver a high single dose of radiation to a small tumor volume, limiting radiation exposure to surrounding brain. SRS can be performed by X-rays (linear accelerator), gamma radiation (gamma knife), and proton particles (proton beam). SRS followed by conventional radiotherapy and chemotherapy (carmustine) did not improve survival of patients with GBM or quality-of life measures [99].

Several experimental techniques are being evaluated. Interstitial brachytherapy, in which the radioactive source is implanted in the tumor bed, delivers continuous low-dose radiation. Boron Neutron capture therapy is based on the selective uptake of boron-10, a non-radioactive isotope, by tumor cells, and the subsequent irradiation of the area with an appropriate neutron beam. The boron-10 desintegrates into densely ionizing particles that kill cells in proximity. Radioimmunotherapy delivers tumor-associated radiolabelled monoclonal antibodies

directly to the surgical cavity. A recent study conducted in newly diagnosed patients with malignant brain tumors treated with direct injections of (131)I-labeled anti-tenascin monoclonal antibody into surgical cavity followed by conventional radiotherapy and chemotherapy have shown an increase in the mean survival of GBM patients [100]. More research is needed regarding these experimental radiotherapy techniques.

1.5.2.2 Radiosensitivity and radioresistance

Tumors, as well as normal tissue, vary in their sensitivity to radiation. In addition to the intrinsic radiosensitivity of tumor cells, there are other factors that influence a tumor's response to radiation. Cellular oxygenation is an important factor. Well oxygenated tumors show a greater response to radiotherapy than poorly oxygenated tumors. Theoretically, the oxygen can combine with free radicals formed during ionization, producing new and toxic combinations [94]. The cell cycle also influences response to radiation. Quiescent cells are the most radioresistant and proliferating cells, the most radiosensitive [93]. Increased fractionation of the total dose protects tissues with slow renewal rate, enabling cell damage repair between radiation sessions. Spacing the treatments protects tissues with rapid renewal rate, enabling cellular repopulation between sessions [93, 94].

An alternative approach involves identifying and targeting the mechanisms responsible for tumour resistance. Indeed, glioblastoma cells that escape the cytotoxic effects of radiation may develop a radiation-induced resistant phenotype. Recent studies have proposed that GBMs' recurrence following IR is partially mediated by an

enhanced invasive character of radioresistant tumor cells, which makes them more difficult to treat [101, 102]. Through radiation-induced proto-oncogene or a tumor suppressor gene alterations, tumor cells may acquire a more malignant character. Although DNA repair and DNA damage cell cycle checkpoint mechanisms constitute the main defense systems for maintaining genetic integrity in the event of DNA damage, failure of these processes can lead to destabilization of the genome with a resultant enhancement of the rate with which deleterious modifications arise [103].

Until recently, the mechanisms underlying tumor radioresistance have remained elusive. Survivin, belonging to the family of inhibitor of apoptosis proteins, has been proposed as a major factor for radioresistance in glioblastoma [104]. Its expression has been associated with enhanced malignant potential of gliomas and increased cell viability after IR exposure [105, 106]. Rho proteins, more specifically, farnesylated RhoB pathway, have been suggested as a key factor in glioblastoma resistance to IR. Overexpression of RhoB in radiosensitive cells increased cell survival after IR [107]. Conversely, inhibition of RhoB led to decreased glioma cell survival [108]. Although studies have shown that the expression of RhoA and RhoB were similar in brain tumors of grades II to IV [109], the specific role of RhoA, in conjunction with Caveolin-1, a protein associating RhoA to endothelial caveolae-enriched membrane domains [110, 111], had not been studied.

Recent data suggests that these high grade malignancies harbour a population of glioma stem cells that are both radioresistant and capable of initiating tumour regrowth. Glioma stem cells contribute to radioresistance through preferential

activation of the DNA damage checkpoint response and an increase in DNA repair capacity [112].

1.5.3 Chemotherapy

1.5.3.1 Principles of CNS pharmacology and obstacles to chemotherapy

A first obstacle in chemotherapy for brain tumors is getting the drug to the involved target, either tumor or tumor-associated endothelial cells. Factors such as route of administration, drug's bioavailability, capability to cross the BBB and blood-tumor barrier all limit the delivery of the cytotoxic drug to the brain tumor. High-dose chemotherapy has been tried to increase the delivery of chemotherapeutic agents but resulted in increased systemic cytotoxicity. Infusion of chemotherapeutic agents directly in the arterial supply of a tumor have shown encouraging responses [113]. BBB disruption achieved either with osmotic agents or modulation of specific receptors of the BBB are experimental strategies used to circumvent the BBB [113].

A second problematic step is getting the agent to stay in the brain tumor cell and exert its effect. However, multiple mechanisms exist in malignant astrocytomas to resist the effect of cytotoxic therapy. The MDR1 (multidrug resistance) gene encodes a protein, p-glycoprotein (Pgp), which acts as a transmembrane efflux pump on the cytosolic membrane of neoplastic cells as well as endothelial cells of tumor capillaries [114, 115]. Pgp in this special localization can exclude chemotherapeutic agents from tumor cells that are located around the capillaries. This pump can actively exclude a huge variety of lipophilic drugs from tumor cells located adjacent to capillaries [114].

A third obstacle is maintaining the induced damage. The O^6-methylguanine-DNA methyltransferase (MGMT) gene codes for an excision repair enzyme removing alkyl-groups from the O^6-position of guanine, one of the targets of alkylating agents. MGMT gene promoter is inactivated through methylation and the results in suppression of MGMT activity. Studies have shown that epigenetic silencing of the MGMT gene by promoter methylation is an independent prognostic factor associated with a significant response rate to temozolomide chemotherapy [116-118].

1.5.3.2 Description of frequently used agents

Chemotherapy is currently used either as an adjuvant, concurrent, or pre-irradiation treatment for malignant gliomas. The addition of standard cytotoxic chemotherapy offers a minimal survival advantage [97, 119, 120]. Commonly used agents can be grouped in three categories: alkalating agents, alkaloids, topoisomerase inhibitors.

Of the alkalating agents, the nitroureas are the most frequently used and studied agents in the treatment of malignant astrocytomas. Carmustine and lomustine are the two main drugs in this class. They exert their cytotoxic effect by methylation of DNA, generating different types of DNA adducts especially O6methylguanine, but also N3-methylguanine and N7-methylguanine [121]. Interestingly, measuring the level of DNA adducts following alkylation of the N7 guanine has been proposed as a molecular dosimeter to evaluate the delivery of alkylating agents to brain tumors [122]. Temozolomide (TMZ) is one of the newer alkylating agents that is recognized for its complete bioavalability after oral intake and good BBB penetration [123].

Temozolomide also generates different types of methyl adducts (70% N7-methylguanine, 10% N3-methyladenine and 9% O6-methylguanine) [121]. Its cytotoxic activity has been mainly attributed to the formation of O6-methylguanine adducts. Tumor cells' chemosensitivity to TMZ is influenced by the functional status of DNA repair systems, implicated either in the removal of methyl adducts from O6G or in the apoptotic signaling triggered by O6-methylG:T mispairs [121]. Although initially studied at GBM recurrence, a major phase III study was designed in which newly diagnosed GBM patients were randomized to either radiotherapy alone or radiotherapy and TMZ [124]. Median survival time was 14,6 months against 12,1 months in favor of patients receiving TMZ. Two-year survival increased from 10,4 to 26,5% when on TMZ [124]. In view of these results, concurrent TMZ and radiation therapy is now considered by many investigators as standard primary treatment for newly diagnosed glioblastomas.

Of the alkaloid agents, vincristine is a cell-cycle specific agent binding to tubulin during the S phase and inducing a metaphase arrest [2]. This agent has been commonly administered to treat primary brain tumors, especially as part of multiagent regimens such as PCV (Procarbazine – Lomustine – Vincristine). However, a randomized trial of radiotherapy alone or radiotherapy with PVC in newly diagnosed anaplastic astrocytomas or glioblastomas failed to show significant survival benefit in patients treated with radiotherapy and PVC [125]. Experimentally, it has been demonstrated that vincristine has negligible penetration in normal rat brain or brain tumor, despite intra-arterial delivery [126]. In the light of such data, some authors

have proposed that vincristine should be withdrawn from the chemotherapeutic options for high grade gliomas [126].

Of the topoisomerase inhibitor agents, etoposide is the most commonly used agent. It prevents the topoisomerase enzymes to detach from the DNA strand, which leads to replication of defective DNA, arrest in the G_2 phase and eventually apoptosis [123].

When administered in cyclic high-dose, conventional cytotoxic chemotherapeutic agents exert a negligible antiangionegic effect since endothelial cells are provided enough time to repair induced damage [86]. A strategy combining low-dose regimens of conventional chemotherapeutic agents and antiangiogenic treatment recently showed in animal studies a marked decrease in tumor volume, vascularity, and proliferative index, and an increased apoptosis [127]. Results of clinical studies administering a chemotherapeutic drug, most often TMZ, and antiangiogenic compounds such as thalidomide or interferon, seem promising [86].

1.6 Nutratherapy

1.6.1 Diet and cancer prevention

There are geographic and ethnic differences in the incidence of various cancers around the world as well as trends for each population over time [128, 129]. Diet, a modifiable factor, can play an important role either in precipitating or preventing diseases such as cancer. Indeed, diet-related factors are thought to account for about 30% of cancers in developed countries [129]. The association of different cancers with diet is illustrated by the fact that the incidence patterns of cancers observed among immigrants changes according to where they live [128]. Other environmental and lifestyle factors such as cigarette smoking, alcohol, sun exposure, environmental pollutants, infections, stress, obesity, and physical inactivity may also modify patterns of cancers among immigrants [130].

Over the past 20 years, a large number of epidemiological studies, particularly case-control and cohort studies, have been conducted to investigate the role of diet and the risk of developing different types of cancer. The concept of chemoprevention is to control the occurrence of cancer by slowing, blocking, attacking, or reversing the development of the disease by the administration of naturally occurring or synthetic compounds [131, 132]. A review of over 200 studies found a significant protective effect of fruit and vegetable intake against lung, colon, breast, cervix, oesophagus, oral cavity, stomach, bladder, pancreas and ovary cancer [133]. For most cancer sites, persons with low fruit and vegetable intake (at least the lower one-fourth of the population) experience about twice the risk of cancer compared with those with high intake [133]. Such observations are supported by convincing evidence of

chemopreventive efficacy of specific dietary constituents in cellular and rodent models of carcinogenesis. The ultimate proof of efficacy of chemopreventive dietary constituents will be provided through long-term phase III clinical studies.

1.6.2 Nutritional sciences

Nutratherapy, nutritional chemotherapy, is a type of chemotherapy that uses the arsenal of anticancerous molecules naturally found in dietary constituents to combat cancer cells. Nutratherapy is a complementary approach that should be integrated to the existing standard cancer treatments such as surgery, radiotherapy and chemotherapy.

An incomplete list of some of the numerous food components that may be important in modifying cancer risk and tumor behavior is provided in table 7 [132].

Table 7. Partial list of bioactive food components that may influence cancer risk and tumor behavior

Class	Bioactive component	Dietary source
Vitamins	Vitamin D Folic acid Vitamin A Vitamin E (-tocophenol) Ascorbic acid	Dairy products Vegetables Vegetables Vegetable oils Vegetables, Fruits
Minerals	Calcium Selenium Zinc	Dairy products, vegetables Cereal grains, meat, fish Meat, vegetables
Carotenoids	Lycopene Lutein β-Carotene	Tomatoes Dark green vegetables Orange-yellow vegetables
Flavonoids	Genistein Resveratrol Quercetin Tangeretin Catechins (-)-Epigallocatechin-3-gallate	Soybeans, soy products Grapes, red wine Vegetables, fruits Citrus fruits Grapes Green tea
Organosulfur	Allyl sulfur Diallyl sulfide	Allium vegetables Garlic
Isothiocyanates	Allyl isothiocyanate Bebzyl isothiocyanate Sulforaphane	Cabbage Garden cress Broccoli
Indoles Monoterpenes	Indole-3-carbinol D-Limonene D-Carvone	Cruciferous vegetables Citrus fruit oils Caraway seed oil
Phanolic acids	Curcumin Caffeic acid Ferulic acid Chlorogenic acid	Tumeric, curry, mustard Fruits, coffee beans, soybeans Fruits, soybeans Fruits, coffee beans, soybeans
Chlorophyll	Chloropyll Chlorophyllin	Green vegetables Green vegetables

Adapted from [132].

It is probable that most bioactive food components simultaneously modify multiple steps in the cancer process (Figure 15) [132]. Multiple variables influence how cancer cells respond to bioactive food constituents: the fluctuations in the amount of bioactive components in the diet, the variations in the ability of bioactive constituents to reach or affect critical molecular targets, the presence of genetic polymorphisms that can influence the absorption, metabolism, or sites of action of bioactive components [132].

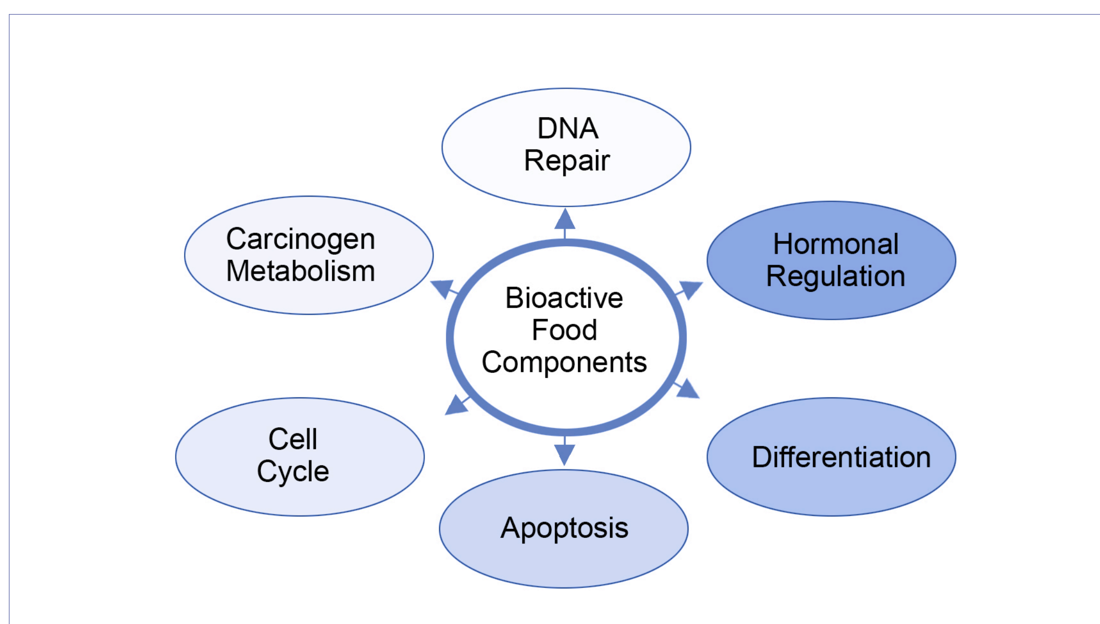

Figure 15. Diet may influence genetic & epigenetic events associated with several cancer processes. Bioactive food components are known to influence multiple biological processes. Determining which is most instrumental in bringing about a phenotypic change is critical to the future of nutrition and health. Adapted from [132].

The advent of nutriproteomics which studies the interaction of bioactive components with proteins [134] and nutrigenomics which studies the ability of bioactive food constituents to modulate gene expression [135] will help better understand how the bioactive components influence cancer.

1.6.3 Dietary polyphenolic phytochemicals

Plants interact with their environment by producing phenolic compounds as secondary metabolites. There are more than ten types of polyphenols, depending on their chemical structure [136]. Flavonoids and phenolic acids account for 60% and 30% respectively of total dietary polyphenols [136].

1.6.3.1 Polyphenol catechins

Polyphenolic catechins are characterized by di- or trihydroxyl group substitution of the B ring and meta5,7-dihydroxy substitution of the A ring of flavanols [136]. Catechins contribute to the bitterness and astringency of food. There are many catechins in green tea: (-)epicatechin (EC), (-)epicatechin-3-gallate (ECG), (-)epigallocatechin-3-gallate (EGCG), (+)catechin, and (-gallocatechin-) (GC) (Figure 16) [137].

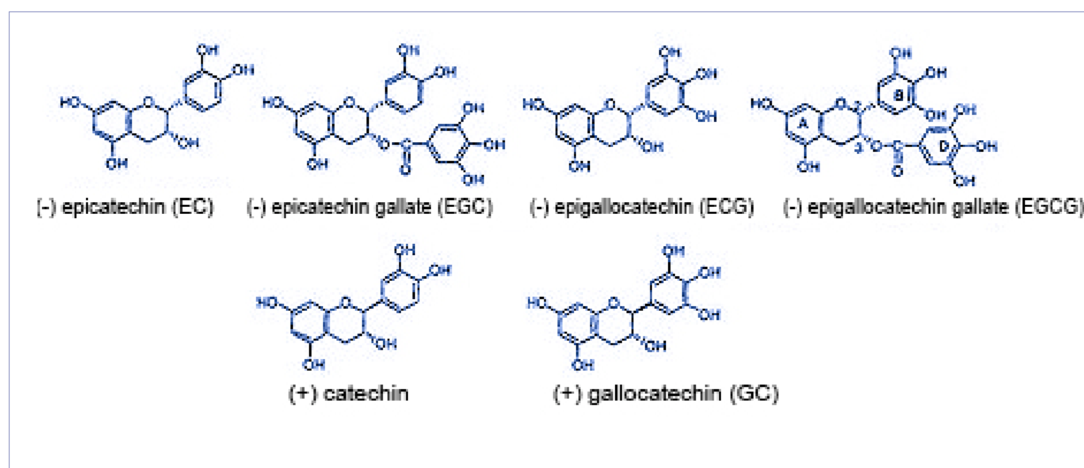

Figure 16. Structures of the major polyphenolic catechins present in green tea. Reproduced from [137].

Green tea has been shown to have many beneficial health properties. Green tea consumption seems to afford significant protection against Parkinson's disease, Alzheimer's disease, ischemic insults [138] and cardiovascular diseases [139]. Green tea has also shown anti-diabetic, anti-bacterial, anti-HIV, anti-aging and anti-inflammatory activities [137]. Results of epidemiological studies assessing the effect of green tea on chemoprevention have been inconsistent; some finding a reduced cancer incidence and recurrence associated with green tea consumption and others failing to show such an effect [140]. Results from *in vitro* and animal models have been more convincing regarding the properties of green tea and its catechins [136].

1.6.3.2 Epigallocatechin-3-gallate anticancerous properties

EGCG is the most abundant catechin in green tea, it accounts for 50-80% of the total catechin content, representing 200-300 mg in a brewed cup of green tea [141]. EGCG has been recognized as having many anticancerous properties verified in several cell types including malignant primary brain tumors. EGCG has exhibited antiproliferative properties on brain tumor cells [142]. EGCG has been shown to inhibit antiapoptotic Bcl-2-family proteins, Bcl-x(L) and Bcl-2 [143], induce apoptosis through caspase-3 activation and poly(ADP)ribose polymerase-1 (PARP-1) cleavage [144, 145] and induce cell cycle arrest through regulation of cyclin D1, cdk4, cdk6, p21/WAF1/CIP1 and p27/KIP1 [145, 146].

EGCG inhibits cancer invasion and metastasis [147]. EGCG has been shown to inhibit matrix metalloproteinases MMP-2 and MMP-9 in tumor and endothelial cells, these enzymes play an important role in degrading the basement membrane and facilitating cell invasion, metastasis and angiogenesis [145, 148]. Also, EGCG has been found to inhibit the activity of urokinase-plasminogen activator (uPA), a protease that can stimulate cell migration and angiogenesis and modulate cell adhesion [148, 149].

EGCG bears recognized antiangiogenic properties. It can inhibit VEGF's promoter activity and cellular production of VEGF [150] as well as interfere with VEGF receptor expression [151] and activity [152] in ECs. EGCG can inhibit ECs proliferation and induce their apoptosis, possibly by blocking VEGF induction [153]. Also, EGCG has been shown to inhibit ECs migration [154] and *in vitro* tubulogenesis [152].

EGCG has been recognized for its antioxydant properties. Many mechanisms have been proposed for this, including EGCG capacity to neutralize free radicals, act as a metal chelator by binding transition metal ions such as copper and iron that would otherwise catalyse free radical formation, and inhibit pro-oxidant enzymes with peroxidase activity [155]. However, EGCG may in itself be pro-oxydant and induce DNA damage [156]. EGCG exposure may induce differential oxidative environments depending on the type of cell studied and the treatment dose [157]. It is proposed that pathways activated by EGCG in normal epithelial versus tumor cells create different oxidative environments, favoring either normal cell survival or tumor cell destruction [158]. Table 8 summarizes the mechanistic findings of EGCG against tumor development and progression [153].

Table 8. Mechanistic findings of EGCG against tumor development and progression

Mechanistic findings of EGCG against tumour development and progression
Introduction of apoptosis and cell cycle arrest
Inhibition of carcinogenesis
Regulation of transcription factor
AP-1
NF-κB
Inhibition of gene expression
TNF-α
VEGF
NOS
Modulation of enzyme activities
Ornithine decarboxylase
Matrix metalloproteinase
Urokinase plasminogen activator
Protein kinase C and protein phosphatase 2A
Cyclooxygenase and lipooxygenase
Protein tyrosine kinase
Mitogen-activated protein kinase
EGF-R tyrosine kinase
Inhibition of tumour progression
Adhesion and invasion
Angiogenesis
Metastasis
Antioxidation
Radioprotection

Adapted from [153].

1.6.3.3 Mechanisms of action of EGCG

Until now, it is unknown through which cell signalling pathways EGCG exerts its inhibitory effect on carcinogenesis. The antitumor effects of EGCG may be due either to binding to multiple cellular targets and subsequent inhibition of the activation of downstream effectors or to direct targeting of intracellular signalling molecules [131]. It has been shown that EGCG inhibits the activation of the EGFR and HER2 and multiple downstream signalling pathways such as Akt, ERK, and NFkB in various tumors [131, 159]. Recent studies have shown that the inhibitory effect of EGCG on tumor cell proliferation might be transduced through its binding to the 67-kDa laminin receptor (67LR) overexpressed on the cell surface of various tumor cells [160]. More studies are needed to better understand the cell signalling pathways and molecular events leading to EGCG's antitumor effect.

1.7 Hypothesis, goals, and cellular models

1.7.1 Hypothesis

Unfortunately, even with the current standard of care management, GBM tend to recur in proximity of the primary site in the majority of patients [161], possibly due to the proliferation of cells that escaped the cytotoxic effects of radiotherapy. Although the detailed mechanisms underlying GBM radioresistance remain unknown, some molecular markers have attracted attention. Survivin, an intracellular molecule with anti-apoptotic function, is widely expressed in human malignancies and its expression correlates with radioresistance in several tumors, including GBM [104]. Rho proteins have been proposed to be involved in the radioresistance and IR-induced invasiveness of primary GBM [107, 108]. Also, the use of anticancerous molecules naturally found in dietary constituents as a complementary approach to conventional treatment is appealing. EGCG has been recognized as having anticancerous properties on malignant primary brain tumors. It is possible that EGCG could sensitize GBMs' response to radiation. If EGCG's radiosensitizing effect is documented on glioblastoma U-87 cells, it might be mediated by mechanisms dependant on Survivin and RhoA.

To verify these hypothesis, we will culture several cell lines of human high grade astrocytoma and expose them to a single increasing IR dose. Cell proliferation will be analyzed by nuclear cell counting. In order to determine if IR induces a caspase-medicated apoptotic cell death, we will perform a fluorimetric caspase-3 assay. Our study will continue using the most radioresistant cell line of those tested (U-87). For the following assays, we will irradiate cells with the IR dose that will

result in a significant decrease in cell proliferation without any caspase-3 activity. To assess if IR could modulate the protein expression of Survivin, RhoA and Caveolin-1, we will perform western blot assays with cell lysates. If IR induces the expression of Survivin, RhoA and Caveolin, we will partially mimic IR's effect by transfecting U-87 cells with cDNA constructs encoding Survivin, RhoA and Caveolin-1. To assess if the overexpression of these proteins could alter U-87 cells' sensitivity to IR exposure, we will irradiate the transfected cells and assess their proliferation rate. We will also assess how EGCG influences mock and transfected cells's proliferation. To verify if EGCG has the ability to modify the radioprotective effect of Survivin overexpression, we will pretreat mock and Survivin-transfected cells with EGCG, irradiated them, and assessed their proliferation rate. We will assess by Western immunobloting the impact of EGCG treatment and/or IR exposure on mock and Survivin-transfected cells to elucidate and underlying relationship between RhoA and Survivin.

Until recently tumor cells have been recognized as the sole cellular target of radiotherapy's anti-cancerous effects. However, studies have shown that ECs are also targeted by radiotherapy [104]. Interestingly, both apoptosis and necrosis have been documented in various cancer lines following IR exposure [104]. It is possible that similar cell death mechanisms may be in part responsible for IR's cytotoxic effect on tumor-associated ECs. Given the radiosensitizing effect of EGCG on tumor cells, this anti-angiogenic nutrient might potentiate IR's effect.

To verify these hypothesis we will assess HBMEC survival immediately and 48 hours after either IR with a single dose or EGCG treatment by nuclear cell counting

and Trypan blue exclusion methods. HBMEC survival will also be assessed 48 hours after the combined EGCG/IR treatment. In order to evaluate if the reduction of HBMEC survival following the combined EGCG/IR treatment is due to a modification of cell distribution throughout the cell cycle, we will perform a cell cycle assay by flow cytometry. Also, we will assess by Western immunoblotting the protein expression of the cyclin inhibitors p21 and p27, both known to arrest cell cycle progression, following EGCG +/- IR exposure. To evaluate if the combined treatment reduced cell survival by inducing apoptosis, we will analyse the pattern of DNA fragmentation in the sub-G1 population by flow cytometry and measure the expression of pro-apoptotic proteins caspase-3, caspase-9 and cytochrome C by Western immunoblotting. We will assess the relative importance of apoptosis and necrosis on overall cell death following EGCG and/or IR treatment by flow cytometry following annexin V-fluorescein isothiocyanate and propidium iodide.

The altered angiogenic functions and signalling pathways in radioresistant tumor-associated ECs have not yet been investigated. It is known that radioresistant tumor cells present an altered malignant behavior such as an enhanced invasive potential [104]. The Rho signalling pathway hase been proposed to be involved in IR-induced invasiveness of primary GBM [104]. IR possibly alters resistant ECs' capacity to migrate and form tubules. This might be related to radioresistant ECs' altered response to brain tumor-derived growth factors. Modulations of the RhoA/ROK pathway might be related to altered ECs functions.

To verify these hypothesis, HBMEC survival will be assessed 48 hours after IR with a single dose by nuclear cell counting and Trypan blue exclusion methods. Cell death, including apoptosis and necrosis, will be evaluated by Annexin-V/PI staining. Migration will be assessed in control and irradiated (10 Gy) HBMEC in response to VEGF, S1P, or brain tumor-derived growth factors (U-87 GF). We will examine if IR modifies the protein expression of RhoA, ROK, and Caveolin-1 by Western immunoblotting. If the RhoA/ROK is activated following IR, we will partially mimic IR's effect by transfecting HBMEC with a cDNA construct encoding for RhoA. We will document migration in mock, IR, and RhoA-transfected cells in response to agents that will have stimulated the most mock cells' migration. We will evaluate the effect of inhibiting the RhoA/ROK pathway on HBMEC migration by adding to previous conditions Y-27632, an inhibitor or ROK. We will evaluate the capacity of mock, IR, and RhoA-transfected cells to form capillary-like structure by Matrigel assays. The effect of inhibiting the RhoA/ROK on tubulogenesis will also be assessed.

1.7.2 Goals

The goals of this theses are:

1) To determine if EGCG, in synergy with radiotherapy, can sensitize GBM's response to radiation and whether specific molecular markers are involved.

2) To characterize the impact of ionizing radiation on HBMEC survival and to determine whether EGCG, also recognized for its anti-angiogenic properties, could optimize this effect.

3) To investigate the effects of ionizing radiation on HBMEC angiogenic responses, i.e. cell proliferation, migration in response to brain tumor-derived growth factors, and capacity for tubulogenesis.

1.7.3 Cellular models used

1.7.3.1 Brain tumor-derived cell lines

The U-118, U-138, U-87 glioblastoma-derived cell lines were used as a surrogate model for human glioblastomas. The DAOY medulloblastoma-derived cell line were used as a surrogate model for medulloblastoma. The DAOY cell line was derived from biopsy material taken from a tumor in the posterior fossa of a 4 year old boy. We chose to compare high grade astrocytomas response to radiotherapy with DAOY's given the recognized radiosensitivity of medulloblastomas[162].

1.7.3.2 Brain-derived endothelial cell line

The HBMEC model is a surrogate model that approximates the most tumor-derived ECs. Although ECs have been isolated from GBM specimens [76, 163], the primary cell cultures were found not to be suitable for long-term *in vitro* studies since they de-differentiated in culture and had inherently limited proliferative potential before senescence [164]. Although this model is not derived from glioma-associated ECs, the HBMEC model is, to our knowledge, the closest *in vitro* model that can approximate the human brain tumor-derived ECs phenotype in long term studies, maintaining both morphological and functional characteristics of brain ECs [165].

CHAPTER II

The survivin-mediated radioresistant phenotype of glioblastomas is regulated by RhoA and inhibited by the green tea polyphenol (-) epigallocatechin-3-gallate

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* N. McLaughlin is responsible for 80% of the experimentation and writing of this manuscript. M. Bouzeghrane helped with technical issues. A. Temme cloned and validated the WT-Survivin cDNA construct. JP Bahary and R Moumdjian have reviewed the manuscript. B. Annabi and R. Béliveau supervised the entire study.

ABSTRACT

Introduction : Glioblastoma multiforme's (GBM) aggressiveness is potentiated in radioresistant tumor cells. The combination of radiotherapy and chemotherapy has been envisioned as a therapeutic approach for GBM. The goal of this study is to determine if epigallocatechin-3-gallate (EGCg), a green tea-derived anti-cancer molecule, can modulate GBM's response to ionizing radiation (IR) and whether this involves mediators of intracellular signaling and inhibitors of apoptosis proteins.

Material and Methods : U-87 human GBM cells were cultured and transfected with cDNAs encoding for Survivin, RhoA or Caveolin-1. Mock and transfected cells were irradiated at sublethal single doses. Cell proliferation was analyzed by nuclear cell counting. Apoptosis was detected using a fluorimetric caspase-3 assay. Analysis of protein expression was accomplished by Western immunobloting.

Results : IR (10 Gy) reduced control U-87 cell proliferation by 40% through a caspase-independent mechanism. The overexpression of Survivin induced a cytoprotective effect against IR while the overexpression of RhoA conferred a cytosensitizing effect upon IR. Control U-87 cells pretreated with EGCg exhibited a dose-dependent decrease in their proliferation rate. The growth inhibitory effect of EGCg was not antagonized by overexpressed Survivin. However, Survivin-transfected cells pretreated with EGCg became sensitive to IR and their RhoA expression was downregulated. A potential therapeutic effect of EGCg targeting the prosurvival intracellular pathways of cancer cells is suggested to act synergistically with IR.

Conclusion : The radioresistance of GBM is possibly mediated by a mechanism dependent on Survivin in conjunction with RhoA. The combination of natural anti-

cancerous molecules such as EGCg with radiotherapy could improve the efficacy of IR treatments.

Abbreviations for this manuscript:

EGCg: Epigallocatechin-3-gallate

GBM: Glioblastoma multiforme

IR: Ionizing radiation

INTRODUCTION

Glioblastoma multiforme (GBM) represents the most aggressive and invasive primary cerebral neoplasm in the adult population. Median length of survival without further therapy is usually less than one year from the time of diagnosis [18, 28]. When surgical excision is considered, the goal should be gross total removal to prolong quality survival [32]. However, the effect of surgical resection on the time to tumor progression and the median length of survival can only be optimized when combined with other therapies. For instance, conventional local field radiotherapy has been shown by itself to prolong median survival for 6-8 months [23]. Unfortunately, 90% of patients receiving radiation therapy following GBM resection still develop tumor recurrence in the proximity of the primary site [30]. Neither whole brain irradiation nor high focal radiation doses can decrease the incidence or change the location of recurrences [45]. Ionizing radiation (IR)-induced alterations of the malignant behavior are not unique to astrocytic tumors and are still poorly understood [40]. Recent studies have reported that GBMs' recurrence following IR is partially mediated by an enhanced invasive character of radioresistant tumor cells, which makes them more difficult to treat [17, 59].

Chemotherapy is thus used either as an adjuvant, concurrent, or pre-irradiation treatment along with radiotherapy for malignant primary tumors. However, only modest benefits in survival have been reported [23, 34, 44]. More recently the combination of chemotherapeutic drugs such as temozolomide with molecules

exhibiting an anti-angiogenic activity have been shown to be safe and more effective with respect to survival than the administration of the chemotherapeutic agent alone [9, 55]. Also attention has been focused on identifying naturally occurring substances capable of inhibiting, retarding or reversing the multi-stage carcinogenesis process. Recent reports have proposed that some phytochemicals can function as sensitizers, augmenting the effectiveness of conventional radiotherapy [15, 25]. Epigallocatechin-3-gallate (EGCg), a major anti-oxydative green tea polyphenol, has been recognized for its anti-mutagenic and anti-carcinogenic properties [20, 48]. More recently, we have shown that EGCg also possessed anti-angiogenic properties as it suppressed vascular endothelial growth factor receptors functions in endothelial cells [33]. We have also demonstrated that EGCg efficiently targeted endothelial cells that escaped IR-induced apoptosis [6]. Whether this natural polyphenol can be used to target pro-survival pathways involved in GBM radioresistant phenotype is unknown.

The identification of the molecular mechanisms underlying GBM radioresistance thus becomes essential for the development of combination therapies against this lethal condition. Survivin, along with other markers, has been proposed as a major factor for radioresistance in glioblastoma [13]. Survivin, belonging to the family of inhibitor of apoptosis proteins, is involved in the modulation of apoptosis [3, 16, 36, 46] in the regulation of cell growth [3, 49, 52], in the regulation of mitotic events such as chromosomal segregation and cytokinesis [54, 57], and in the process of angiogenesis [12, 39]. Its expression has been associated with enhanced malignant potential of gliomas, increased cell viability after IR exposure and adverse clinical

prognosis [14, 31]. Rho proteins, which belong to a family of small GTPases, are also involved in the control of key cellular processes such as modulation of the cytoskeleton, receptor internalization, or cell adhesion [7, 22, 58]. More specifically, farnesylated RhoB pathway has been suggested as a key factor in glioblastoma resistance to IR. Indeed, overexpression of RhoB in radiosensitive cells increased cell survival after IR [2]. Conversely, inhibition of RhoB led to the appearance of multinucleated cells and induced a post-mitotic cell death that led to decreased glioma cell survival [1]. Interestingly, the same effect was observed in glioma cells transduced with a $p34^{cdc2}$ phosphorylation-defective SurvivinT34A, suggesting a link between Survivin and Rho proteins [53]. Although studies have shown that the expression of RhoA and RhoB were similar in brain tumors of grades II to IV [24], the specific role of RhoA, in conjunction with Survivin, in glioma radioresistance remains to be investigated. Caveolin-1, a protein associating RhoA to caveolae-enriched membrane domains [26, 42], is proposed to participate in cell survival and angiogenesis [37, 41]. The molecular implication of Caveolin-1 in glioma radioresistance has also not yet been investigated. Hence, the goal of this study is to determine whether EGCg can sensitize GBMs' response to radiation and whether specific molecular markers are involved.

MATERIALS AND METHODS

Materials: Agarose, (-)-epigallocatechin 3-gallate (EGCg), sodium dodecylsulfate (SDS), gelatin, and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). FUGENE-6 transfection reagent was from Roche Diagnostics Canada (Laval, QC). Mouse anti-Survivin monoclonal antibody was from Cell Signaling Technology (Beverly, MA), mouse anti-RhoA monoclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-Caveolin-1 monoclonal antibody was from BD Pharmingen (Mississauga, ON) and mouse β-actin monoclonal antibody was from Sigma. Horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). BCA protein assay kit was purchased from Pierce (Rockford, Il) and enhanced chemiluminescence (ECL)-Western blot kit from Chemicon International (Temecula, CA). Products for electrophoresis were bought from Bio-Rad (Mississauga, ON) and polyvinylidene difluoride (PVDF) membranes from Boehringer Mannheim. Trypsin was from INVITROGEN (Burlington, ON).

Cell culture and cDNA transfection : The U-118, U-138, and U-87 human glioblastoma cell lines were purchased from the American Type Culture Collection (Manassas, VA) and were maintained in Eagle's minimum essential medium (MEM) containing 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, and were cultured at 37°C under a humidified atmosphere containing 5% CO_2. U-87 cells were transiently transfected with cDNA constructs using the non-liposomal FUGENE-6

transfection reagent. The EGFP-tagged WT-Survivin cDNA construct has been described previously [54]. The Myc-tagged WT-RhoA cDNA construct was provided by Dr Allan Hall (London, UK). GFP-tagged WT-Caveolin-1 was provided by Dr Sung-Soo Yoon (Sung Kyon Kwan University, Korea). Transfection efficiency was analysed by Western blotting and fluorescent microscopy. All experiments involving these cells were performed 36 hrs following transfection. Mock transfection of U-87 cultures with the empty pcDNA (3.1+) expression vector was used as controls.

EGCg and irradiation treatment: Cells were treated in serum-free MEM supplemented (or not) with EGCg (3-30 μM) for 6 hours and were overlaid with sufficient medium to subsequently provide efficient build up doses. Cells were irradiated with a 6 MV photon beam from an Elekta SL75 linear accelerator. The delivered radiation doses were measured using a thermoluminescence dosimetry (TLD) system with an accuracy of 7%. After irradiation, MEM containing 20% FBS was added and cultures were left to recuperate for 18 hours. Non-irradiated control cells were handled similarly to the cells which were subjected to IR.

Cell proliferation assay : The extent of cell proliferation was assessed 18 hours after irradiation. Cells were collected by gentle scraping and were resuspended in the overlaying medium. From each probe, 150 μl homogenate was saved for nuclear cell counting using an automatic nuclear counter and its specific reagents (New Brunswick Scientific Co., Edison, NJ) and for cell number determination using Trypan blue for exclusion of dead cells.

Fluorimetric caspase-3 activity assay : U-87 cells were grown to about 60% confluence and treated with EGGg or IR doses. Cells were collected and washed in ice-cold PBS pH 7.0. Cells were subsequently lysed in Apo-Alert lysis buffer (Clontech, Palo Alto, CA) for one hour at 4°C and the lysates were clarified by centrifugation at 16,000g for 20 minutes. Caspase-3 activity was determined by incubation with 50 μM of the caspase-3-specific fluorogenic peptide substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) in assay buffer [50 mM Hepes-NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0,1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 5 mM DTT and 1 mM EDTA] in 96-well plates. The release of AFC was monitored for at least 30 minutes at 37 °C on a fluorescence plate reader (Molecular Dynamics) (λ_{ex}= 400nm, λ_{em}=505nm).

Immunoblotting procedures: Total protein lysates from control cells and treated cells were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes which were then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mM Tris, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were further washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in TBST containing 3% bovine serum albumin, followed by a 1 hr incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1/10,000 dilution) in TBST containing 5% non-fat dry milk. The secondary

antibodies were visualized by enhanced chemiluminescence and quantified by densitometry.

Statistical data analysis : Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired t-test and was used to compare the relative proliferation rates. Probability values of less than 0.05 were considered significant. In each figure statistically significant differences are identified by an asterisk (*) for ECGg or IR treatment compared to control, while double asterisk (**) is to show significance between combined EGCg/IR treatment and either EGCg or IR treatment alone.

RESULTS

Sublethal, low, single dose IR partially inhibits U-87 and DAOY cell proliferation. We examined the proliferation rates of several human high grade astrocytoma (U-138, U-118, U-87) and medulloblastoma (DAOY) cell lines in response to increasing doses of IR (Fig.1). We observed a dose-dependent decrease of the cell proliferation rate at increasing IR doses, up to 30 Gy. DAOY was the most radiosensitive cell line, as the proliferation rate was decreased by 70% after 10 Gy and by 95% after 30 Gy exposures. U-87 cells were the most radioresistant cell line when compared to the other astrocytoma cell lines; their proliferation rate was decreased by only 40% after 10 Gy and by 50% after 30 Gy exposures.

Sublethal, low, single dose IR inhibits U-87 cell proliferation by a caspase-independent mechanism. In order to assess whether the decrease in cell proliferation was due to IR-induced caspase-mediated apoptosis, we measured caspase-3 activity. In all human astrocytoma cell lines analysed, radiation exposure up to 10 Gy did not induce caspase-3 activity (Fig.2). However, at 30 Gy, the caspase-3 activity was twofold increased in U-138 and U-118 cells, whereas in U-87 cells no significant increase in caspase-3 activity was detectable. In contrast, IR induced significant caspase-3 activity in DAOY cells even at doses as low as 3 Gy. These results prompted us to further investigate the possible apoptosis-independent mechanisms underlying the observed radioresistance of U-87 cells.

Low dose IR induces the expression of prosurvival proteins in U-87 cells. We investigated the protein expression of Survivin, RhoA, and Caveolin-1 in irradiated U-87 cells. We selected 10 Gy as the delivered IR dose since it was associated with a significant decrease in cell proliferation and absence of caspase-3 activity (Fig.1 and 2). After exposure to IR, Survivin expression in U-87 control cells increased 2-fold over basal expression, while that of RhoA increased 4-fold (Fig.3a and 3b). Interestingly, the expression of Caveolin-1, a protein regulating RhoA functions, also increased by 4.5-fold in irradiated U-87 cells. β-actin and total Erk protein levels were not affected by IR (Fig.3a).

Overexpression of Survivin and RhoA exhibit antagonistic effects on the radioresistant phenotype of U-87 cells. U-87 cells were cultured to 50% confluence and transfected with cDNA constructs encoding Survivin, RhoA, or Caveolin-1 proteins. Transfection efficiencies were assessed by the presence of GFP positive cells in Survivin and Caveolin-1 transfectants (Fig.4a) and by immunodetection for ectopic RhoA protein in RhoA-transfected cells (Fig.4b). Surprisingly, ectopic expression of Survivin induced RhoA, whereas recombinant Caveolin-1 did not (Fig.4b). Mock-transfected controls and cells either expressing Survivin, RhoA or Caveolin-1, were then exposed to increasing doses of IR. In mock-transfected U-87 cells, we observed a dose-dependent decrease in the cell proliferation rate (open circles) when cells were irradiated with doses up to 30 Gy. Interestingly, the proliferation rate of U-87 cells overexpressing Survivin (closed circles) did not decrease upon IR treatment, and maintained a proliferation rate similar to non-irradiated cells (Fig.4c). This clearly

demonstrates that Survivin confers resistance to irradiation. Strikingly, cells overexpressing RhoA (closed squares) exhibited a significantly decreased cell proliferation rate in comparison to mock U-87 cells (Fig.4c). Overexpression of RhoA thus seems to render U-87 cells more sensitive to IR resulting in a decreased proliferation capacity. Overexpression of Caveolin-1 (open squares) had no influence on the U-87 cell proliferation rate (Fig 4c).

Survivin overexpression does not antagonize EGCg's antiproliferative effect. Recent studies have demonstrated that EGCg, a green tea-derived polyphenol, inhibits the growth of various human cancer cell lines [8, 43, 51], particularly human glioblastoma cells [61]. We further investigated the effect of EGCg on U-87 glioma cell proliferation. We show that EGCg indeed inhibited cell proliferation in a dose-dependent manner with an optimal inhibitory effect at 25 μM of more than 70% (Fig.5a). Caspase-3 activity was measured in parallel and found not to be significantly induced by EGCg (Fig.5a) suggesting that the inhibition in cell proliferation was not due to apoptosis. Cells were cultured to 50% confluence and transfected with cDNAs encoding for Survivin, RhoA, or Caveolin-1, treated with EGCg, and left to recuperate overnight. Maximal inhibition of proliferation (35-40% of untreated cells) was achieved with 25 μM EGCg (Fig.5b). The growth of U-87 cells overexpressing RhoA, Survivin, and Caveolin-1 was also decreased by EGCg, similarly to the mock U-87 cells. We conclude that neither RhoA, Survivin, nor Caveolin-1 were able to fully reverse EGCg's antiproliferative effect. Individual, maximally inhibitory treatments

with EGCg (25 μM) and IR (10 Gy) were also compared to show that only Survivin was able to reverse the inhibitory effect that IR has on cell proliferation.

Effect of combined EGCg and low dose IR on U-87 glioma cells proliferation. We studied the effect of a combined approach consisting of pre-treating U-87 cells with EGCg followed by exposure to sublethal single IR doses on the protein expression levels of Survivin, RhoA, and Caveolin-1. EGCg did not affect the steady state protein levels of Survivin, RhoA and Caveolin-1 in non-irradiated cells (Fig.6a). Interestingly, while IR induced the expression of Survivin, RhoA and Caveolin-1, EGCg pre-treated cells showed a dose-dependent decrease in the IR-induced protein expression of Survivin and of RhoA (Fig.6a). The protein expression levels of Caveolin-1 were increased in cells treated with both EGCg and irradiation. The effect of this combined treatment was further investigated with respect to RhoA expression. U-87 cells were transfected with the Survivin cDNA, which induced RhoA expression (Fig.4b and Fig.6b). Interestingly, this effect was reversed when Survivin-transfected cells were treated with EGCg but not when they were exposed to IR (Fig.6b). Consequently, the cytoprotective effect that Survivin has towards IR may, in part, be caused by RhoA signalling, which is significantly abrogated when cells are treated with EGCg. Most importantly, cell proliferation assays showed that the combined EGCg/IR treatment was able to significantly decrease cell proliferation when compared to individual EGCg or IR treatments (Fig.6c, left panel). Furthermore, the combined EGCg/IR treatment in U-87 cells overexpressing recombinant Survivin was able to reverse the cytoprotective effect that Survivin had towards IR (Fig.6c, right

panel). Prosurvival intracellular pathways might thus be targeted by EGCg, a phytochemical that could be efficiently used as an adjunct to radiotherapy.

DISCUSSION

Glioblastomas multiforme (GBM) are highly invasive primary tumors of the adult central nervous system. Although radiotherapy is routinely prescribed in the management of high grade gliomas, its efficacy remains limited because of tumor cell radioresistance, enhanced invasive character following radiation and resultant tumor recurrence [17, 59]. In our study, we first analyzed the sensitivity of various human high-grade astrocytoma- and medulloblastoma-derived cell lines to ionizing radiation (IR). U-87 glioma cells were found to be the most radioresistant cell line, while DAOY medulloblastoma cells were the most radiosensitive cell line tested. This is in accordance with the reported radioresistance of glioblastoma cells [13, 17] and radiosensibility of medulloblastoma cells [35]. Studies have indeed shown that p53-mediated apoptosis is an important response of medulloblastomas to radiotherapy [19]. In contrast, p53 mutations are present in as much as 40-60% of GBMs [60], suggesting that either pro-survival mechanisms or p53-independent events may regulate their therapeutic response to IR [29].

In light of the emergence of several markers of glioma malignant progression, the molecular characterization of GBMs' radioresistance was next investigated. Among these markers, Survivin has been recently related to the malignant phenotype of gliomas as its expression is associated with glioma progression from low- to high-grade [14, 31]. Furthermore, Survivin expression is well recognized as a predictor of adverse clinical prognosis for patients with gliomas [14], as it increases survival of primary GBM cell lines through its capacity to suppress caspase-mediated apoptosis

[13] by directly binding to caspase-9 or by interacting with smac-DIABLO [38, 47]. The effects of Survivin in our study support the lack of IR-induced growth inhibition that we observed in GBM. Furthermore, Survivin is also implicated in the regulation of cell division [3, 49] as it is localized in multiple components of the mitotic apparatus and centrosomes and participates in cell cytokinesis [54, 57], a process that involves RhoA [4]. Novel functions of Survivin have been proposed to emerge following IR such as double-strand DNA break repair and enhancement of tumor cell metabolism potentially mediating radioresistance [13]. In addition, some studies have suggested that overexpression of Survivin may facilitate evasion from checkpoint mechanisms of growth arrest and promote resistance to chemotherapeutic regiments targeting the mitotic spindle [27]. Whether these mechanisms also regulate the radioresistant phenotype of GBM remains to be confirmed.

Our study shows that IR exposure increases the basal expression of Survivin in U-87 glioma cells, possibly through RhoA-mediated intracellular signaling, and that this overexpression subsequently confers radioprotection against IR. Rho GTPases function as molecular switches that modulate the activation of several enzymes involved in different biological processes related to tumor progression, such as cell growth, transcriptional regulation, and apoptosis [7, 22, 58]. Interestingly, RhoA-induced apoptosis is independent of p53 but dependent on modulation of anti-apoptotic proteins [21]. In our study, whether RhoA expression was induced by IR or through cDNA cell transfection, a cytosensitizing effect was observed in U-87 cells which resulted in a significant decrease in cell proliferation following IR. On the other

hand, although the overexpression of recombinant Survivin also induced RhoA expression, the resultant was rather a cytoprotective effect on cell proliferation following IR. This paradoxal effect might be explained by post-transcriptional modifications that would be induced by IR and that may alter RhoA's function or cellular distribution to specialized membrane domains. We previously reported that RhoA is associated with caveolae-enriched membrane domains, possibly through physical interaction with Caveolin-1 [26]. Although caveolae regulate cell survival processes [37], we show that overexpression of Caveolin-1 induced either by IR or cDNA cell transfection did not influence cell proliferation but may potentially have regulated RhoA's functions or localization. The interaction between RhoA and Caveolin-1 in the context of IR is currently under investigation.

One of the major features characterizing high-grade radioresistant gliomas is their infiltrating character. Recently, we showed that this phenotype might be linked to RhoA/Rok-mediated CD44 cell surface shedding which could be targeted and inhibited by EGCg, a green tea-derived polyphenol with anticancerous properties [5]. In light of this evidence and of recent studies which have shown that EGCg could serve as an IR enhancer on cancer cell lines [8], we investigated whether EGCg pretreatment prior to IR could reverse the cytoprotective effect of Survivin. We observed that the combined EGCg/IR treatment was able to significantly decrease cell proliferation when compared to individual EGCg or IR treatments. Furthermore, the combined EGCg/IR treatment in U-87 cells overexpressing recombinant Survivin was able to downregulate RhoA expression and reverse the cytoprotective effect that

Survivin exhibited towards IR. Although the exact molecular mechanism of EGCg is not completely understood, recent studies have shown that the inhibitory effect of EGCg on tumor cell proliferation might be transduced through its binding to the 67-kDa laminin receptor (67LR) [50], a protein whose expression is strongly correlated with tumor aggressiveness [11]. Interestingly, studies have shown that through its binding to the 67LR, EGCg may also reduce the phosphorylation of myosin II regulatory light chain, resulting in an increase in cells in the G2/M phase of cell cycle and ultimately in an inhibition of cell growth [56] possibly through increased cell radiosensitivity [10].

In conclusion, we demonstrate that the growth inhibitory effect of EGCg pretreatment efficiently antagonized both the IR-induced expression of Survivin, as well as that of RhoA induced by overexpression of recombinant Survivin. A potential therapeutic impact of EGCg in targeting pro-survival and RhoA-mediated intracellular pathways in cancer cells is suggested to act synergistically with IR. We propose that EGCg might potentiate IR's inhibitory effect on tumor cell proliferation by increasing the proportion of cells in a radiosensitive state. This natural phytochemical might thus be an important molecule to consider in combined adjuvant chemotherapy and radiotherapy.

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REFERENCES

[1] I Ader, C Delmas, J Bonnet, P Rochaix, G Favre, C Toulas, E Cohen-Jonathan-Moyal, Inhibition of Rho pathways induces radiosensitization and oxygenation in human glioblastoma xenografts, Oncogene. 22 (2003) 8861-8869.

[2] I Ader, C Toulas, F Dalenc, C Delmas, J Bonnet, E Cohen-Jonathan, G Favre, RhoB controls the 24kDa FGF-2 induced radioresistance in HeLa cells by preventing post-mitotic cell death, Oncogene. 21 (2002) 5998-6006.

[3] DC Altieri, Survivin versatile modulation of cell division and apoptosis in cancer, Oncogene. 22 (2003) 8581-8589.

[4] M Amano, M Ito, K Kimura, Y Fukata, K Chihara, T Nakano, Y Matsuura, K Kaibuchi, Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase), J Biol Chem. 271 (1996) 20246-9.

[5] B Annabi, M Bouzeghrane, R Moumdjian, A Moghrabi, Beliveau R, Probing the infiltrating character of brain tumors: inhibition of RhoA/ROK-mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCg, J Neurochem. 94 (2005) 906-16.

[6] B Annabi, YT Lee, C Martel A Pilorget, JP Bahary, R Béliveau, Radiation-induced-tubulogenesis in endothelial cells is antagonized by the antiangiogenic properties of green tea polyphenol (-) epigallocatechin-3-gallate, Cancer Biol Ther. 2 (2003) 642-649.

[7] S Aznar, JC Lacal, Rho signals to cell growth and apoptosis, Cancer Lett. 165 (2001) 1-10.

[8] S Baatout, P Jacquet, H Derradji, D Ooms, A Michaux, M Mergeay, Study of the combined effect of X-irradiation and epigallocatechin-gallate (a tea component) on the growth inhibition and induction of apoptosis in human cancer cell lines, Oncol Rep. 12 (2004) 159-167.

[9] F Baumann, M Bjeljac, SS Kollias, BG Baumert, S Brandner, V Rousson, Y Yonekawa, RL Bernays, Combined thalidomide and temozolomide treatment in patients with glioblastoma multiforme, J Neurooncol. 67 (2004) 191-200.

[10] A Bernand, J Bourhis, Radiotherapy. Radiobiological aspects, principal secondary effects, Rev Prat. 51 (2001) 1265-71.

[11] V Berno, D Porrini, F Castiglioni, M Campiglio, P Casalini, SM Pupa, A Balsari, S Menard, E Tagliabue, The 67 kDa laminin receptor increases tumor aggressiveness by remodeling laminin-1, Endocr Relat Cancer. 12 (2005) 393-406.

[12] OP Blanc-Brude, M Mesri, NR Wall, J Plescia, T Dohi, DC Altieri, Therapeutic targeting of the survivin pathway in cancer: initiation of mitochondrial apoptosis and suppression of tumor-associated angiogenesis, Clin Cancer Res. 9 (2003) 2683-2692.

[13] A Chakravarti, GG Zhai, M Zhang, R Malhotra, DE Latham, MA Delaney, P Robe, U Nestler, Q Song, J Loeffler, Survivin enhances radiation resistance in primary human glioblastoma cells via caspase-independent mechanisms, Oncogene. 23 (2004) 7494-7506.

[14] A Chakravarti, E Noll, PM Black, DF Finkelstein, DM Finkelstein, NJ Dyso, JS Loeffler, Quantitatively determined survivin expression levels are of pronostic value in human gliomas, J Clin Oncol. 20 (2002) 1063-1068.

[15] D Chendil, R.S. Ranga, D Meigooni, S Sathishkumar, MM Ahmed, Curcumin confers radiosensitizing effect in prostate cancer cell line PC-3, Oncogene 23 (2004) 1599-1607.

[16] EM Conway, S Pollefeyt, J Cornelissen, I DeBaere, M Steiner-Mosonyi, K Ong, M Baens, D Collen, AC Schuh, Three differentially expressed survivin cDNA variants encode proteins with distinct antiapoptotic functions, Blood. 95 (2000) 1435-1442.

[17] N Cordes, B Hansmeier, C Beinke, V Meineke, D van Beuningen, Irradiation differentially affects substratum-dependent survival, adhesion, and invasion of glioblastoma cell lines, Br J Cancer. 89 (2003) 2122-2132.

[18] FG Davis, BJ McCarthy, S Freels, Kupelian V, ML Bondy, The conditional probability of survival of patients with primary malignant brain tumors: surveillance, epidemiology, and end results (SEER) data, Cancer. 85 (1999) 485-491.

[19] S Dee, DA Haas-Kogan, MA Israel, Inactivation of p53 is associated with decreased levels of radiation-induced apoptosis in medulloblastoma cell lines. Cell Death Differ, 2 (1995) 267-275.

[20] M Demeule, J Michaud-Levesque, B Annabi, D Gingras, D Boivin, J Jodoin, S Lamy, Y Bertrand, R Béliveau, Green tea catechins as novel antitumor and antiangiogenic compounds, Curr. Med Chem – Anti-cancer Agents. 2 (2002) 441-463.

[21] P Esteve, N Embade, R Perona, B Jimenez, L del Peso, J Leon, M Arends, T Miki, JC Lacal, Rho-regulated signals induce apoptosis in vitro and in vivo by a p53-independent, but Bcl2 dependent pathway, Oncogene. 17 (1998) 1855-1869.

[22] S Etienne-Manneville, A Hall, Rho GTPases in cell biology, Nature. 420 (2002) 629-35.

[23] HA Fine, KB Dear, JS Loeffler, PM Black, GP Canellos, Meta-analysis of radiation therapy with and without adjuvant chemotherapy for malignant gliomas in adults, Cancer. 71 (1993) 2585-2597.

[24] MA Forget, RR Desrosiers, RF Del Maestro, R Moumdjian, D Shedid, F Berthelet, R Béliveau, The expression of Rho proteins decreases with human brain tumor progression: Potential tumor markers, Clin Exp Metastasis. 19 (2002) 9-15.

[25] S Fulda, K.M. Debatin, Sensitization for tumor necrosis factor-related apoptosis inducing ligand induced apoptosis by the chemopreventive agent resveratrol, Cancer Res. 64 (2004) 337-346.

[26] D Gingras, F Gauthier, S Lamy, RR Desrosiers, R Beliveau, Localization of RhoA GTPase to endothelial caveolae-enriched membrane domains, Biochem Biophys Res Commun. 247 (1998) 888-893.

[27] A Giodini, MJ Kallio, NR Wall, GJ Gorbsky, S Tognin, PC Marchisio, M Symons, DC Altieri, Regulation of microtubule stability and mitotic progression by survivin, Cancer Res. 62 (2002) 2462-7.

[28] Glioma Meta-analysis Trialists (GMT) Group, Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials, Lancet. 359 (2002) 1011-1018.

[29] DA Haas-Kogan, P Dazin, L Hu, DF Deen, MA Israel, p53-Independent Apoptosis: A Mechanism of Radiation-Induced Cell Death of Glioblastoma Cells, Cancer J Sci Am. 2 (1996) 114.

[30] C Hess, J Schaaf, R Kortmann, M Schabet, M Bamberg, Malignant glioma: patterns of failure following individually tailored limited volume irradiation. Radiother. Oncol. 30 (1994) 146-149.

[31] Y Kajiwara, F Yamasaki, S Hama, K Yahara, H Yoshioka, K Sugiyama, K Aritz, K Kurisu, Expression of survivin in astrocytic tumors: correlation with malignant grade and prognosis, Cancer. 97 (2003) 1077-1083.

[32] GE Keles, B Anderson, MS Berger, The effect of extent of resection on time to tumor progression and survival in patients with glioblastoma multiforme of the cerebral hemisphere, Surg Neurol. 52 (1999) 371-379.

[33] S Lamy, D Gingras, R Béliveau, Green tea catechins inhibit vascular endothelial growth factor receptor phosphorylation, Cancer Res. 62 (2002) 381-385.

[34] G Lanzetta, C Campanella, A Rozzi, M Nappa, A Costa, F Fedele, G Innocenzi, FM Gagliardi, M Salvati, G Minniti, A Frati, L Frati, Temozolomide in radio-chemotherapy combined treatment for newly-diagnosed glioblastoma multiforme: phase II clinical trial, Anticancer Res. 23 (2003) 5159-5164.

[35] JT Leith, S Cook, P Chougule, P Calabresi, L Wahberg, C Lindquist, M Epstein, Intrinsic and extrinsic characteristics of human tumors relevant to radiosurgery: comparative cellular radiosensitivity and hypoxic percentages, Acta Neurochir Suppl. 62 (1994) 18-27.

[36] F Li, G Ambrosini, EY Chu, J Plescia, S Tognin, PC Marchisio, DC Altieri, Control of apoptosis and mitotic spindle checkpoint by surviving, Nature. 396 (1998) 580-584.

[37] ML Massimino, C Griffoni, E Spisni, M Toni, V Tomasi, Involvement of caveolae and caveolae-like domains in signalling, cell survival and angiogenesis, Cell Signal. 14 (2002) 93-98.

[38] DS O'Connor, D Grossman, J Plescia, F Li, H Zhang, A Villa, S Tognin, PC Marchisio, DC Altieri, Regulation of apoptosis at cell division by p34cdc2 phosphorylation of surviving, Proc Natl Acad Sci U S A. 97 (2000) 13103-13107.

[39] DS O'Connor, JS Schechner, C Adida, M Mesri, AL Rothermel, F Li, AK Nath, JS Pober, Altieri DC, Control of apoptosis during angiogenesis by survivin expression in endothelial cells, Am J Pathol. 156 (2000) 398-399.

[40] K Ohuchida, K Mizumoto, M Murakami, LW Qian, N Sato, E Nagai, K Matsumoto, T Nakamura, M Tanaka, Radiation to stromal fibroblasts increases invasiveness of pancreatic cancer cells through tumor-stromal interactions, Cancer Res. 64 (2004) 3215-3222.

[41] B Razani,, A Schlegel, J Liu, MP Lisanti, Caveolin-1, a putative tumour suppressor gene, Biochem Soc Trans. 29 (2001) 494-499.

[42] A Regina, J Jodoin, P Khoueir, Y Rolland, F Berthelet, R Moumdjian, L Fenart, R Cecchelli, M Demeule, R Béliveau, Down-regulation of Caveolin-1 in glioma vasculature: Modulation by radiotherapy, J Neurosci Res. 75 (2004) 291-299.

[43] RJ Rosengren, Catechins and the treatment of breast cancer: possible utility and mechanistic targets, IDrugs. 6 (2003) 1073-1078.

[44] JN Scott, NB Rewcastle, PM Brasher, D Fulton, JA MacKinnon, M Hamilton, JG Cairncross, P Forsyth, Which glioblastoma multiforme patient will become a long-term survivor? A population-based study, Ann Neurol. 46 (1999) 183-188.

[45] RR Sharma, DP Singh, A Pathak, N Khandelwal, CM Sehgal, R Kapoor, S Ghoshal, FD Patel, SC Sharma, Local control of high-grade gliomas with limited volume irradiation versus whole brain irradiation, Neurol India. 51 (2003) 512-517.

[46] S Shin, BJ Sung, YS Cho, HJ Kim, NC Ha, JI Hwang, CW Chung, YK Jung, BH Oh, An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and –7, Biochemistry. 40 (2001) 1117-1123.

[47] Z Song, X Yao, M Wu, Direct interaction between survivin and Smac/DIABLO is essential for the anti-apoptotic activity of survivin during taxol-induced apoptosis, J Biol Chem. 278 (2003) 23130-23140.

[48] YJ Surh, Molecular mechanisms of chemopreventive effects of selected dietary and medicinal phenolic substances, Mutation research. 428 (1999) 305-327.

[49] A Suzuki, M Hayashida, T Ito, H Kawano, T Nakano, M Miura, K Akahane, K Shiraki, Survivin initiates cell cycle entry by the competitive interaction with Cdk4/p16(INK4a) and Cdk2/cyclin E complex activation, Oncogene. 19 (2000) 3225-3234.

[50] H Tachibana, K Koga, Y Fujimura, K Yamada, A receptor for green tea polyphenol EGCG, Nat Struct Mol Biol. 11 (2004) 380-1.

[51] M Takada, Y Ku, K Habara, T Ajiki, Y Suzuki, Y Kuroda, Inhibitory effect of epigallocatechin-3-gallate on growth and invasion in human biliary tract carcinoma cells, World J Surg. 26 (2002) 683-686.

[52] A Temme, P Diestelkoetter-Bachert, M Schmitz, A Morgenroth, B Weigle, MA Rieger, A Kiessling, EP Rieber, Increased p21(ras) activity in human fibroblasts

transduced with survivin enhances cell proliferation, Biochem Biophys Res Commun. 327 (2005) 765-773.

[53] A Temme, E Herzig, B Weigle, A Morgenroth, M Schmitz, A Kiessling, MA Rieger, HK Schackert, EP Rieber, Inhibition of malignant glioma cell growth by a survivin mutant retrovirus, Hum Gene Ther. 16 (2005) 209-222.

[54] A Temme, M Rieger, F Reber, D Lindemann, B Weigle, P Diestelkoetter-Bachert, G Ehninger, M Tatsuka, Y Terada, EP Rieber, Localization, dynamics, and function of survivin revealed by expression of functional survivinDsRed fusion proteins in the living cell, Mol Biol Cell. 14 (2003) 78-92.

[55] J Tuettenberg, R Grobholz, T Korn, F Wenz, R Erber, P Vajkoczy, Continuous low-dose chemotherapy plus inhibition of cyclooxygenase-2 as an antiangiogenic therapy of glioblastoma multiforme, J Cancer Res Clin Oncol. 131 (2005) 31-40.

[56] D Umeda, H Tachibana, K Yamada, Epigallocatechin-3-O-gallate disrupts stress fibers and the contractile ring by reducing myosin regulatory light chain phosphorylation mediated through the target molecule 67 kDa laminin receptor, Biochem Biophys Res Commun. 333 (2005) 628-35.

[57] SP Wheatley, A Carvalho, P Vagnarelli, WC Earnshaw, INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis, Curr Biol. 11 (2001) 886-890.

[58] AP Wheeler, AJ Ridley, Why three Rho proteins? RhoA, RhoB, RhoC, and cell motility, Exp Cell Res. 301 (2004) 43-49.

[59] C Wild-Bode, M Weller, A Rimner, J Dichgans, W Wick, Sublethal irradiation promotes migration and invasiveness of glioma cells: implications for radiotherapy of human glioblastoma, Cancer Res. 61 (2001) 2744-2750.

[60] JK Wu, Z Ye, BT Darras, Frequency of p53 tumor suppressor gene mutations in human primary brain tumors, Neurosurgery. 33 (1993) 824-830.

[61] S Yokoyama, H Hirano, N Wakimaru, KP Sarker, J Kuratsu, Inhibitory effect of epigallocatechin-gallate on brain tumor cell lines in vitro. Neuro-oncol., 3 (2001) 22-28.

Fig.1

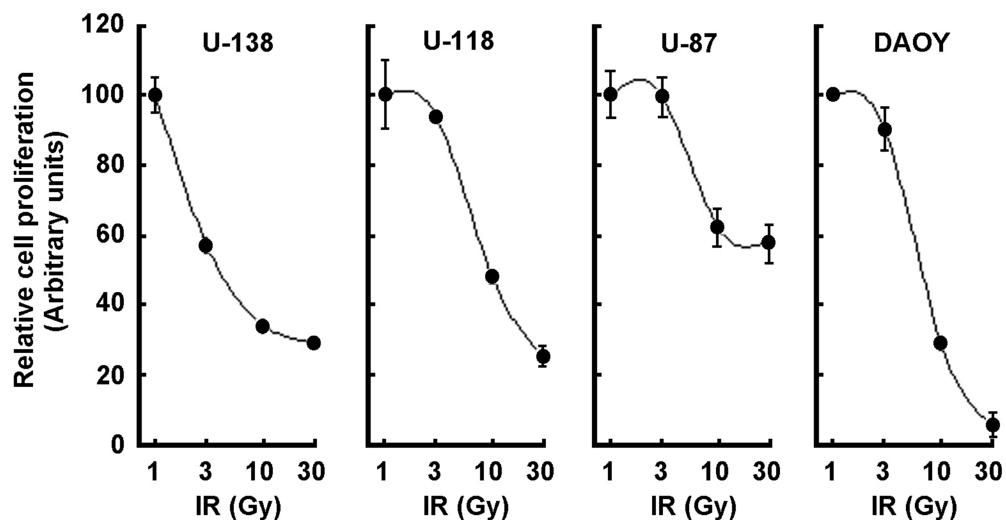


Fig.1 : **Effects of ionizing radiation on the proliferation rates of malignant glioma and medulloblastoma cell lines.** Single dose ionizing radiation (IR) was applied to subconfluent cells and cell proliferation rate was assessed 18 hours after IR using an automatic nuclear counter and Trypan blue staining. Cell proliferation is expressed as a percent of the non-irradiated (control) cell proliferation.

Fig.2

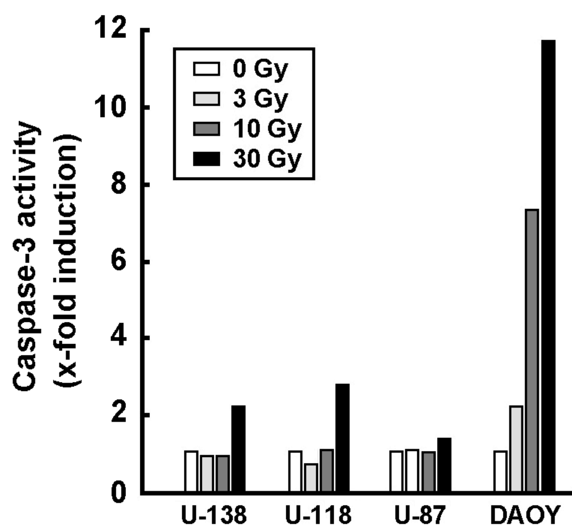


Fig.2 : **Ionizing radiation effects on caspase-3 activity in malignant glioma and medulloblastoma cell lines.** Cells were grown to 60% confluence and were exposed to a single dose of IR. A representative caspase-3 activity profile for each cell line tested is presented and was assessed as described in the Methods section.

Fig.3

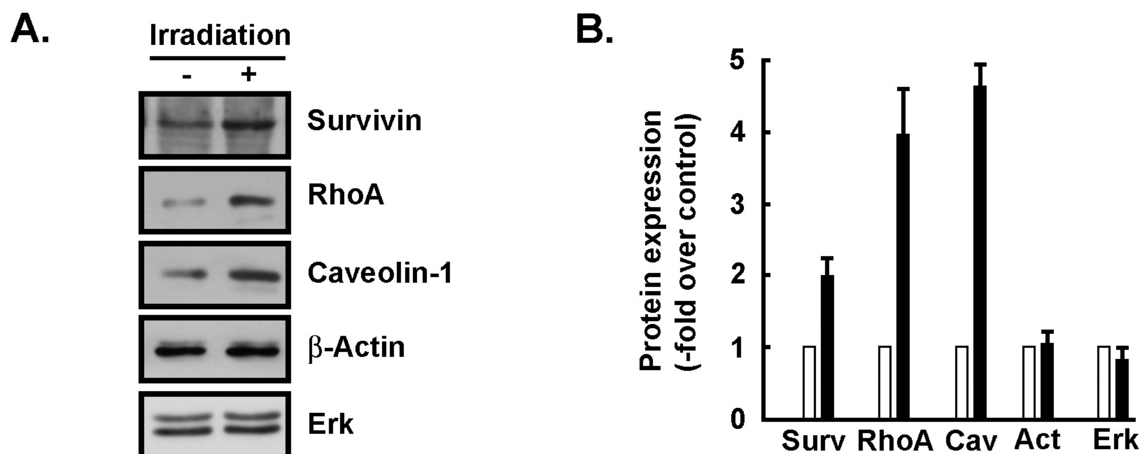
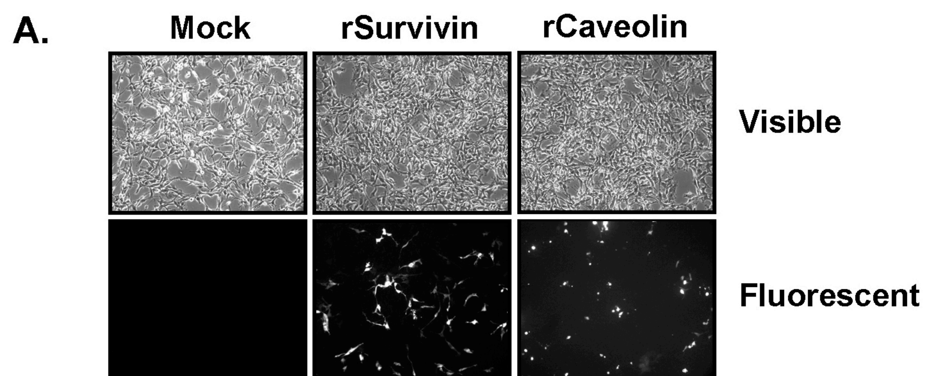
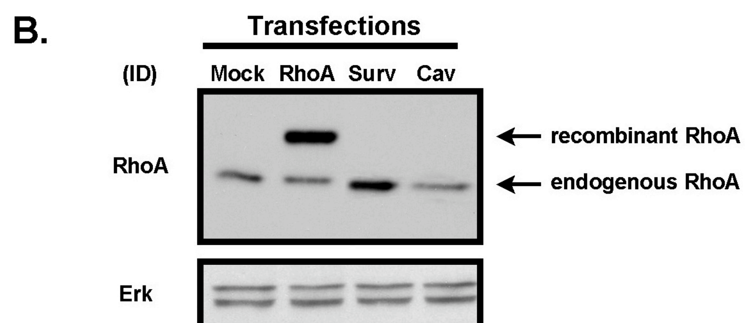


Fig.3 : **Expression of prosurvival proteins in malignant glioma cells exposed to ionizing radiation.** U-87 cells were cultured and exposed to 10 Gy IR. Cell lysates isolated from control and irradiated conditions were electrophoresed on SDS-gels, and transferred to PVDF membranes. (A) Immunodetection of the specific proteins was carried out as described in the Methods section. (B) Quantification of protein expression was performed by scanning densitometry of control, non-irradiated U-87 cells (white bars) and of irradiated cells (black bars).

Fig.4





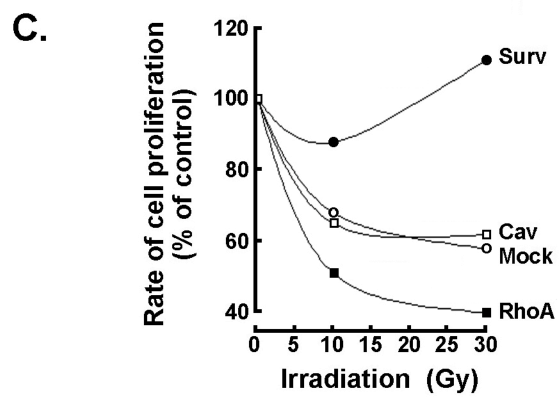


Fig.4 : **Effect of IR exposure on the proliferation rates of malignant glioma cells transfected with proteins involved in radioresistance.** U-87 cells were transfected with cDNA constructs encoding for recombinant Survivin, Caveolin-1 or RhoA. (A) Transfection efficiency was confirmed by fluorescent microscopy in cells transfected with Survivin and Caveolin-1 and (B) by Western blotting in cells transfected with RhoA. (C) A dose-dependent decrease in the mock U-87 (open circles) proliferation rate is observed as cells are irradiated with increasing sublethal doses. The proliferation rate of U-87 cells transfected with Survivin (closed circles), RhoA (closed squares), and Caveolin-1 (open squares) is also shown.

Fig.5

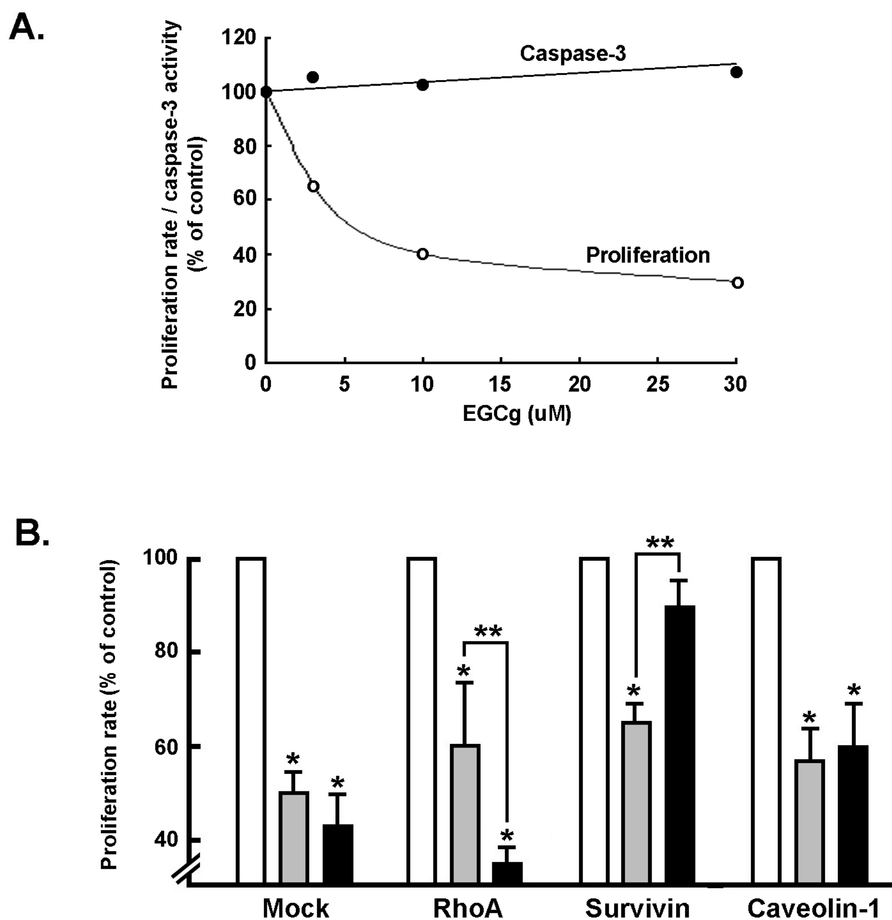


Fig.5 :**Survivin overexpression does not antagonize EGCg's antiproliferative effect.** Subconfluent cells were exposed for 6 hours to increasing concentrations of EGCg (0-30 μM) and cell proliferation rate (white circle) and caspase-3 activity (black circle) were assessed following EGCg treatment as described in the Methods section. Results are expressed as a percent of the control untreated Mock cells (A). The proliferation rate was also assessed in U-87 cells transfected with Survivin, RhoA or Caveolin-1 cDNAs and further

exposed to IR (black bars) or EGCg treatment (grey bars). Cell proliferation rate is expressed as a ratio of control untreated Mock cells (white bars).

Fig.6

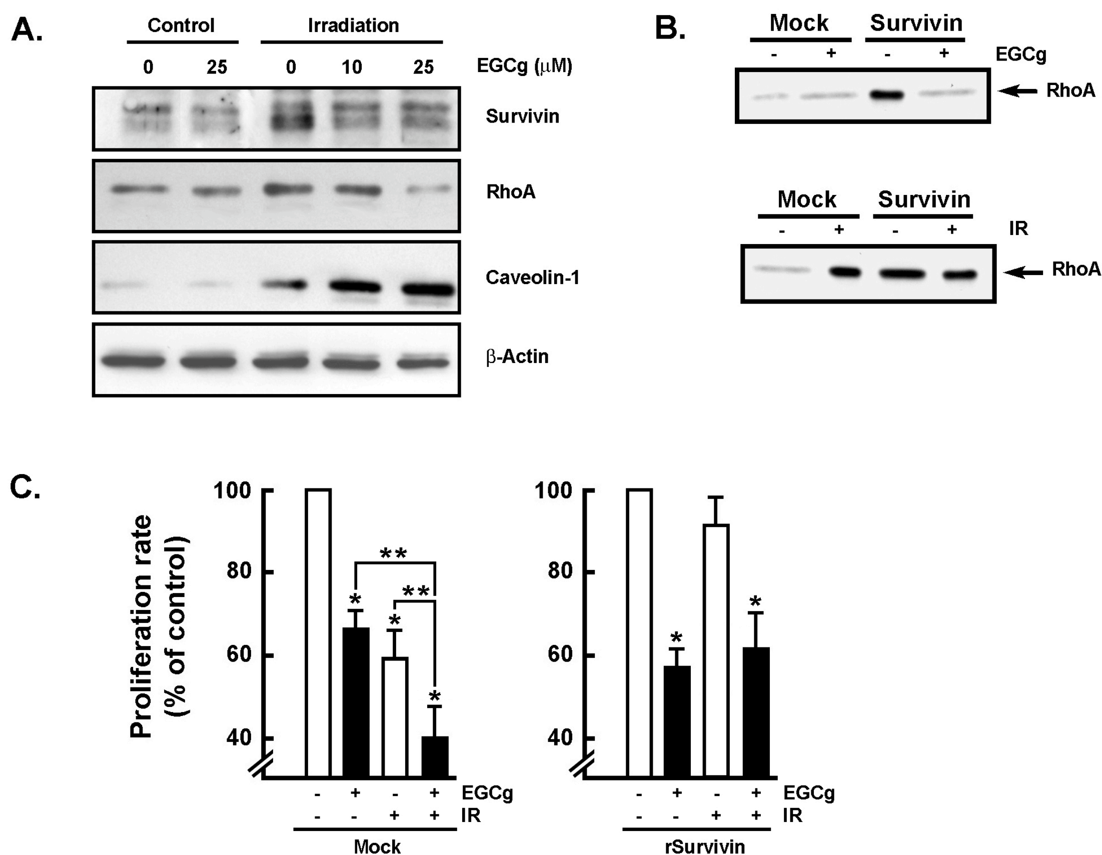


Fig.6 : **Effect of combined EGCg and low dose IR on U-87 glioma cell proliferation.** U-87 cells were cultured and treated with EGCg (0-25 μM) for 6 hours before IR (10 Gy) and left to recuperate overnight at 37°C. (A) Cell lysates of each condition were electrophoresed on SDS-gels and immunodetection was carried out as described in the Methods section. (B) Mock or Survivin-transfected U-87 cells were also treated with either EGCg (upper panel) or IR (lower panel) and RhoA immunodetection performed in cell lysates. (C) Cell proliferation was performed in Mock and Survivin-transfected cells that were either treated with EGCg, irradiated (IR), or which were submitted to combined EGCg/IR treatment.

CHAPTER III

Combined low dose ionizing radiation and green tea-derived epigallocatechin-3-gallate treatment induces human brain endothelial cells death

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ABSTRACT

The microvasculature of brain tumors has been proposed as the primary target for ionizing radiation (IR)-induced apoptosis. However, the contribution of low dose IR-induced non-apoptotic cell death pathways has not been investigated. This study aimed to characterize the effect of IR on human brain microvascular endothelial cells (HBMEC) and to assess the combined effect of epigallocatechin-3-gallate (EGCg), a green tea-derived anti-angiogenic molecule. HBMEC were treated with EGCg, irradiated with a sublethal (≤10Gy) single dose. Cell survival was assessed 48 hours later by nuclear cell counting and Trypan blue exclusion methods. Cell cycle distribution and DNA fragmentation were evaluated by flow cytometry (FC), cell death was assessed by fluorimetric caspase-3 activity, FC and immunoblotting for pro-apoptotic proteins. While low IR doses alone reduced cell survival by 30%, IR treatment was found more effective in EGCg pretreated-cells reaching 70% cell death. Analysis of cell cycle revealed that IR induced cell accumulation in G2-phase. Expression of cyclin-dependent kinase inhibitors p21(CIP/Waf1) and p27(Kip) were increased by EGCg and IR. Although random DNA fragmentation increased by approximately 40% following combined EGCg/IR treatments, the synergistic reduction of cell survival was not related to increased pro-apoptotic caspase-3, caspase-9 and cytochrome C proteins. Cell necrosis increased five-fold following combined EGCg/IR treatments while no changes in early or late apoptosis were observed. Our results suggest that the synergistic effects of combined EGCg/IR treatments may be related to necrosis, a non-apoptotic cell death pathway. Strategies

sensitizing brain tumor-derived EC to IR may enhance the efficacy of radiotherapy and EGCg may represent such a potential agent.

Abbreviations for this manuscript:

EC: Endothelial cells

EGCg: epigallocatechin-3-gallate

GBM: Glioblastoma multiforme

HBMEC: Human brain microvascular endothelial cells

HUVEC: Human umbilical vein endothelial cells

IR: Ionizing radiation

VEGF: Vascular endothelial growth factor

INTRODUCTION

Malignant gliomas, especially glioblastomas multiforme (GBM), are among the tumors most resistant to radiotherapy and chemotherapy [1]. Even in the group with the best prognosis, the median survival rarely passes 1 year [2, 3]. Malignant gliomas are also amongst the most intensively vascularized solid tumors [4]. The process of angiogenesis is essential for tumor progression from microscopic cell clusters, receiving nutrients and oxygen by passive diffusion, to macroscopic foci supplied by blood vessels [5]. Furthermore, malignant gliomas become more angiogenic with increasing tumor grade, suggesting that the vascular component plays an important role in their malignant progression [6]. Indeed, GBMs' cell proliferation, invasiveness and necrosis, three facets of gliomas' malignant phenotype, are directly related to brain tumor microvasculature [7].

Radiotherapy is a widely used adjuvant therapy for malignant brain tumors [1] and is believed to exert its anti-cancerous effects by targeting tumor cells [8, 9]. However, recently, it has been proposed that IR might prevent tumor growth or cause tumor regression by targeting tumor vasculature [10]. Indeed, studies have shown that radiotherapy can selectively target endothelial cells (EC) and associated angiogenesis of residual tumor [7]. Tumor-associated EC have a proliferation rate up to 20 times greater than the proliferation rate of normal vasculature [11], rendering them more radiosensitive than non-dividing cells [12]. In addition, EC are generally well oxygenated, which makes them more radiosensitive than poorly oxygenated tumor

cells because the oxygen present at the time of radiation may contribute to the formation of cytotoxic molecules and prevent the reversal of some chemical changes [12, 13]. Many investigators have found that IR exposure induces apoptosis in EC [14-17], and that this event precedes tumor cell apoptosis [18]. The occurrence of EC death following IR is a critical factor for both tumor survival following IR and for tumor growth. While toxic insults or physical damage have been found to initiate cellular necrosis [19], the impact of IR-induced cellular damage resulting in non-apoptotic pathways such as necrosis has not been yet investigated in EC.

Targeting tumor-associated EC as part of cancer treatments is an appealing prospect. Antiangiogenic agents have been used successfully in combination with IR to increase the therapeutic efficacy of radiation exposure [20, 21]. Only recently have the EC been considered not only the target of antiangiogenic agents but also of IR and therefore a powerful new potential treatment target in highly vascularized tumours such as glioblastoma [4]. Recent reports have proposed that some naturally occurring phytochemicals can function as sensitizers, augmenting the effectiveness of conventional radiotherary [22-24]. Epigallocatechin-3-gallate (EGCg), a green tea-derived molecule, has been recognized as having many biochemical functions including inhibition of tumor cell growth [8, 24], cell cycle arrest [25-27], induction of apoptosis [24, 28, 29], inhibition of invasion and metastasis [30, 31] and inhibition of angiogenesis [29, 32]. Moreover, we have shown that EGCg suppressed vascular endothelial growth factor (VEGF) receptor function in EC [33] and that EGCg efficiently targeted human EC that escaped IR-induced damage [34]. Whether this

natural polyphenol can be used to potentiate IR effects on human brain EC is unknown. Although the human brain microvascular endothelial cells (HBMEC) model, used in this study, is a surrogate model that only approximates tumor-derived EC, it is to our knowledge, the closest *in vitro* model that can approximate the human brain tumor-derived EC phenotype in long term studies. The goal of this study is thus to characterize the impact of irradiation on HBMEC and to determine whether EGCg can optimize this effect.

MATERIALS AND METHODS

Materials: (-)-epigallocatechin 3-gallate (EGCg), sodium dodecylsulfate (SDS) and bovine serum albumin (BSA) were purchased from Sigma (Oakville, ON). Mouse anti-p21 monoclonal antibody, rabbit anti-p27 polyclonal antibody and rabbit anti-Caspase-9 polyclonal antibody were from Cell Signaling Technology (Beverly, MA), rabbit anti-Caspase-3 polyclonal and mouse anti-cytochrome C monoclonal antibody were from BD Pharmingen (Mississauga, ON) and mouse anti-GAPDH monoclonal antibody was from Advanced Immunochemical Inc. (Long Beach, CA). Horseradish peroxidase-conjugated donkey anti-rabbit or anti-mouse IgG were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). BCA protein assay kit was purchased from Pierce (Rockford, IL) and enhanced chemiluminescence (ECL)-Western blot kit from Chemicon International (Temecula, CA). Products for electrophoresis were bought from Bio-Rad (Mississauga, ON) and polyvinylidene difluoride (PVDF) membranes were from Boehringer Mannheim. Trypsin was from Invitrogen (Burlington, ON).

Cell culture: Human brain microvascular endothelial cells (HBMEC) were generated and characterized by Dr Kwang Sik Kim from the John Hopkins University School of Medicine (Baltimore, MD). These cells were positive for factor VIII-Rag, carbonic anhydrase IV, Ulex Europeus Agglutinin I, took up fluorescently labelled acetylated low-density lipoprotein and expressed gamma glutamyl transpeptidase, demonstrating their brain EC specific phenotype [35]. HBMEC were immortalized by

transfection with simian virus 40 large T antigen and maintained their morphologic and functional characteristics for at least 30 passages [36]. HBMEC were maintained in RPMI 1640 (Gibco, Burlington, ON) supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 10% (v/v) NuSerum (BD Bioscience, Mountain View, CA), modified Eagle's medium nonessential amino acids (1%) and vitamins (1%) (Gibco), heparin (5 U/ml) (Gibco), sodium pyruvate (1 mM), L-glutamine (2 mM) (Gibco), EC growth supplement (30 μg/ml), 100 units/ml penicillin and 100 μg/ml streptomycin. Culture flasks were coated with 0.2% type-I collagen to support the growth of HBMEC monolayers. Cells were cultured at 37°C under a humidified atmosphere containing 5% CO_2. All experiments were performed using passages 15 to 28.

EGCg and irradiation treatment: Cells were treated in serum-free RPMI supplemented (or not) with 3-30 μM EGCg for 6 hours and were overlaid with medium. Cells were irradiated with a 6 MV photon beam from an Elekta SL75 linear accelerator. The delivered radiation doses were measured using a thermoluminescence dosimetry (TLD) system with an accuracy of 7%. After irradiation, serum free medium was replaced by RPMI containing 10% FBS and 10% NuSerum and cultures were left to recuperate for 48 hours. Non-irradiated control cells were handled similarly to the cells which were subjected to EGCg and/or IR treatments.

Cell survival assay: The extent of cell survival was assessed at either 1 or 48 hours after irradiation. Cells were collected by gentle scraping and were resuspended in the overlaying medium. From each experimental sample, 150 μl homogenate were reserved for nuclear cell counting using an automatic cell counter (New Brunswick Scientific Co., Edison, NJ). Viable cell number determination was also assessed using Trypan blue dye solution for exclusion of dead cells.

Analysis of cell cycle by flow cytometry: Distribution of HBMEC throughout the cell cycle was assessed by flow cytometry 48 hours after EGCg and/or IR treatments. No serum-fasting preparation was performed prior to analysis and therefore the cell populations were asynchronous. Cells were harvested by gentle scraping, pelleted by centrifugation, washed with ice-cold PBS + EDTA (5 mM), then resuspended in 1 volume PBS/EDTA and fixed with 100% ethanol overnight. Three volumes of staining solution, containing propidium iodide (50 μg/ml) (Sigma) and DNAse-free RNAse (20 μg/ml), were added. The fraction of the population in each phase of the cell cycle was determined as a function of the DNA content using a Becton Dickinson FACS Calibur flow cytometer equipped with CellQuest Pro software. In particular, the characteristics of cell distribution in the sub-G1 region were studied on the DNA histogram.

Fluorimetric caspase-3 activity assay: HBMECs were grown to 60% confluence and treated with EGGg and/or exposed to IR. Cells were collected and washed in ice-cold PBS pH 7.0. Cells were subsequently lysed in Apo-Alert lysis

buffer (Clontech, Palo Alto, CA) for one hour at 4°C and the lysates were clarified by centrifugation at 16,000g for 20 minutes. Caspase-3 activity was determined by incubation with 50 μM of the caspase-3-specific fluorogenic peptide substrate acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) in assay buffer [50 mM Hepes-NaOH (pH 7.4), 100 mM NaCl, 10% sucrose, 0,1% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 5 mM DTT and 1 mM EDTA] in 96-well plates. The release of AFC was monitored for at least 30 minutes at 37°C on a fluorescence plate reader (Molecular Dynamics) (λ_{ex}= 400nm, λ_{em}=505nm).

Immunoblotting procedures: Cell lysates were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred onto polyvinylidene difluoride membranes which were then blocked overnight at 4°C with 5% non-fat dry milk in Tris-buffered saline (150 mM Tris, 20 mM Tris–HCl, pH 7.5) containing 0.3% Tween-20 (TBST). Membranes were then washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in TBST containing 3% bovine serum albumin, followed by a 1 hr incubation with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG (1/2500 dilution) in TBST containing 5% non-fat dry milk. The secondary antibodies were visualized by ECL and quantified by densitometry.

Analysis of apoptosis by flow cytometry: Cell death was assessed 48 hours after irradiation by flow cytometry. Cells floating in the supernatant and adherent cells harvested by trypsin solution were gathered to produce a single cell suspension. The

cells were pelleted by centrifugation and washed with PBS. Then, $2x10^5$ cells were pelleted and suspended in 200 μL of buffer solution and stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) as described by the manufacturer (BD Bioscience). The cells were diluted by adding 300 μL of buffer solution and processed for data acquisition and analysis on a Becton Dickinson FACS Calibur flow cytometer using CellQuest Pro software. The X- and Y-axes indicated the fluorescence of annexin-V and PI respectively. It was possible to detect and quantitatively compare the percentages of gated populations in all of the four regions delineated. In the early stages of apoptosis, phosphatidylserine is translocated to the outer surface of the plasma membrane, which still remains physically intact. As annexin-V binds to phosphatidylserine but not to PI, and the die is incapable of passing the plasma membrane, it is excluded in early apoptosis (annexin-V^+/PI^-). Cells in late apoptosis are stained with annexin-V and PI (annexin-V^+/PI^+). Necrotic cells have lost the integrity of their plasma membrane and are predominantly stained with PI (annexin-V^-/PI^+).

Statistical data analysis: Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired t-test and was used to compare the relative proliferation rates. Probability values of less than 0.05 were considered significant. In each figure the statistically significant differences are identified by an asterisk (*) for EGCg or IR treatment compared to control, while a double asterisk (**) shows significance between combined EGCg/IR treatment and either EGCg or IR treatment alone.

RESULTS

Combined EGCg treatment and ionizing radiation exposure reduce HBMEC survival. We examined HBMEC survival in response to either EGCg treatment or IR exposure. Cell survival was assessed immediately after EGCg treatment or 48 hours after recuperation in medium with serum. Immediately after EGCg treatment, there was a dose-dependent decrease in cell survival with increasing EGCg concentrations, reaching a maximum effect of 30% decrease at 30 μM EGCg (Fig.1A, left panel, white circles). In contrast, 48 hours after treatment, cells had recovered from the EGCg treatment and no effect could be observed at this time on cell survival (Fig.1A, left panel, black circles). HBMEC survival was also assessed after exposure to single IR doses, either immediately or after 48 hours recovery. We found that low IR doses had no effect on cell survival assessed 1 hour after IR (Fig.1A, right panel, white circles). On the contrary, 48 hours after IR exposure, cell survival was reduced by 30% at 10 Gy and by 40% at 30 Gy (Fig.1A, right panel, black circles). Interestingly, HBMEC pretreated with 10 μM EGCg and exposed to a 10 Gy single IR dose showed a 70% reduction in cell survival 48 hours after IR exposure (Fig.1B). Therefore, combined EGCg/IR treatments synergistically reduced cell survival.

The reduction of HBMEC survival following combined EGCg/IR treatment is not due to changes in cell cycle phases. Cell cycle distribution was assessed in HBMEC which had been treated with 10 μM EGCg and/or irradiated at single low doses (≤ 10 Gy) and then left to recuperate in medium with serum for 48 hours. We

employed a 10 μM EGCg treatment concentration and used 10 Gy as the delivered IR dose since the combination of these treatment levels was associated with a synergistic decrease in cell survival 48 hours after treatments (Fig.1B). Discrete modulations of cell cycle phase distribution were observed in cells pretreated for 6 hours with low concentrations of EGCg (Fig.2, lower left panel). On the opposite, cell cycle phase analysis of irradiated HBMEC showed a significant accumulation of cells in G2M phase (Fig.2, upper right panel) and identical results were found when cells were treated with combined EGCg/IR (Fig.2, lower right panel).

Expression of the cyclin kinase inhibitors p21 and p27 is separately induced by EGCg and IR treatments. We investigated the protein expression of the cyclin kinase inhibitors p21(CIP/Waf1) and p27(Kip), known to arrest cell cycle progression by inhibiting G1-S and G2-M transitions [37-39] (Fig. 3A and B). IR exposure (10 Gy) increased the expression of cyclin kinase inhibitors p21 and p27 2.4-fold over basal expression (Fig.3B). In addition, 10 μM EGCg treatments also induced a 2.4-fold increase in the p21 and p27 proteins (Fig.3B). However, the combination of EGCg/IR treatments did not further modulate p21 and p27 expression. GAPDH protein levels were not affected by EGCg treatment and/or IR exposure (Fig.3A).

IR-induced DNA random fragmentation increases following combined EGCg/IR treatment. Forty-eight hours after treatment with EGCg and/or IR, we assessed cell cycle distribution in HBMEC. The characteristics of cell distribution in the sub-G1 region were studied on the DNA histogram obtained by flow cytometry

(Fig.2). Instead of observing only a distinct peak in the sub-G1 region representing apoptotic cells, we found an increase evenly distributed across the same region, suggesting the presence of randomly degraded DNA, a phenomenon representative of necrosis as established in the literature [40]. Quantification of the gated population found in the sub-G1 region revealed that IR and combined EGCg/IR treatments induced a 2-fold and almost 3.5-fold increase, respectively, in the sub-G1 population as characterized above (Fig.4). These results prompted us to further investigate whether apoptosis might contribute to the decrease in cell survival.

IR-induced caspase-dependant mechanisms are not increased by EGCg pretreatment. In order to assess whether the decrease in cell survival was due to increased cell death through apoptosis, we measured the expression of pro-apoptotic proteins caspase-3, caspase-9 and cytochrome C. No significant increases in the expression of these pro-apoptotic proteins or in that of the housekeeping protein GAPDH were observed following combined EGCg/IR treatments as compared to basal expression (Fig.5A). We also assessed apoptosis by measuring caspase-3 activity 48 hours after EGCg treatment and/or IR exposure. Although exposure to EGCg slightly induced caspase-3 activity at 10 μM, IR induced a significant dose-dependent increase in caspase-3 activity (Fig.5B, left panel). EGCg pre-treatment did not modulate the IR-induced caspase-3 activity (Fig.5B, right panel). We next examined whether the decrease in cell survival was rather due to increased cell death through necrosis.

Combined EGCg/IR treatments increase HBMEC necrosis. HBMEC treated with EGCg and/or IR and left to recuperate for 48 hours were harvested and stained with annexin-V and propidium iodide as described in the Methods section. Quantification of the population distribution throughout the four areas revealed that no significant increase occurred in early (Fig.6A, lower right quadrant) or late apoptosis (Fig.6A, upper right quadrant) in EGCg-treated, irradiated cells as compared to control cells. However, cell necrosis (Fig.6A, upper left quadrant) increased 5-fold following combined EGCg/IR treatments (Fig.6B). These results suggest that non-apoptotic pathways such as necrosis might contribute to the decrease in cell survival following combined EGCg/IR treatments and could explain the decrease in HBMEC survival.

DISCUSSION

We have recently shown that IR decreases *in vitro* cell proliferation of malignant glioma cells derived from highly vascularized brain tumors [8]. Furthermore, we also demonstrated that EGCg can enhance the effects of IR on these same cells by potentiating the decrease in cell proliferation [8]. Interestingly, malignant gliomas' proliferation, invasiveness and necrosis are directly related to their microvasculature [7]. Therefore tumor-associated EC represent a potential new treatment target. IR is capable of inducing cellular injuries to EC *in vitro* and *in vivo* [10, 14-16, 34]. However, little is known about EC injury occurring after radiation exposure and even less is known regarding EGCg's capacity to enhance the effects of IR on HBMEC. In the present study, we therefore characterized the impact of IR on HBMEC and tested whether EGCg could optimize this effect.

The HBMEC model used in this study is a surrogate model that approximates tumor-derived EC. Although one must recognize that this model does not represent glioma-derived EC, it is to our knowledge the closest *in vitro* model that can approximate the human brain tumor-derived EC phenotype in long term studies. Until recently, molecular impacts on brain microvasculature functions were in fact extrapolated from studies mostly performed on human umbilical vein EC (HUVEC) [34] or bovine aortic EC (BAEC) [41]. However, microvascular EC, such as brain EC, display a selective phenotype which differs from macrovascular EC [35]. Recently, isolation of human EC from glioblastoma specimens was achieved [42, 43].

Unfortunately, primary cell cultures were found not to be suitable for long-term *in vitro* studies since they de-differentiated in culture and had inherently limited proliferative potential before senescence [44]. The HBMEC model thus represents a stable *in vitro* model since they maintain both morphological and functional characteristics of brain EC, as well as an increased proliferation rate due to their transformation with the simian virus 40 large T antigen [36]. Noteworthy, HUVEC immortalized with SV40 antigens and the catalytic subunit of human telomerase overexpress the tumor endothelial marker-1/endosialin which is regarded as the most differentially expressed molecule in tumor-derived endothelium versus normal-derived endothelium [45]. Therefore the fact that HBMEC were immortalized by transfection with SV40 allows us to further approximate the molecular impact of our study to those cells that would have acquired some transformed phenotype in the GBM tumor microenvironment.

To investigate the mechanism responsible for the synergistic EGCg/IR decrease in cell survival we first assessed changes in the cell cycle. It has been firmly established that IR results in prolongation of the cell cycle, including delays or arrests in the G1, S and G2 phases[46-49], and induction of cyclin inhibitor p21(cip/waf) [37]. Interestingly, p21(cip/waf) and p27(kip) have also been recognized as effectors of cell cycle arrest at the G1-S and G2-M phases of the cell cycle [37-39]. Similarly, EGCg has been shown capable of inducing G1-phase arrest of cell cycle in many cell lines [25-27], depending on the concentration and the duration of treatment. Several studies have indeed recognized that EGCg treatment induces p21 [25-27] and p27

expression [26, 50]. Interestingly, p21 may exhibit an antiapoptotic or proapoptotic effect depending on the conditions inducing its expression [51]. In our study, pre-treatment with low concentrations of EGCg (≤ 10 μM) during a short period of time (≤ 6 hours) did not affect the IR-induced G2-M and G1-S transition arrests nor the overexpression of cyclin kinase inhibitor proteins. Therefore the cell cycle changes cannot account for the synergistic survival decrease of cells pretreated with EGCg and subsequently irradiated.

Recently, a study of total body irradiation in mice showed that the proportion of EC undergoing apoptosis in a population of cells was 20% after 12 hours [16]. In light of these findings, we sought to determine whether apoptosis-mediated cell death, a late event which eliminates cells with irreparable DNA damage, could account for the synergistic EGCg/IR effect. We showed that low dose IR induced only moderate caspase-3 activity, and that HBMEC exposure to EGCg did not induce significant caspase-3 activity nor did it modulate IR-induced, caspase-dependent mechanisms. Although the late stage of apoptosis is associated with secondary necrotic cell death, primary necrotic cell death may occur in the absence of apoptotic parameters [52]. The possible contribution of non-apoptotic pathways, such as necrosis, was next investigated. We found that random DNA fragmentation, assessed by sub-G1 cell distribution, increased significantly following IR exposure and, more importantly, this effect was enhanced by combined EGCg/IR treatments. Although no significant increase in cell necrosis was observed in HBMEC treated with either EGCg or IR, the combined treatments did increase cell necrosis. These results suggest that the cell

death pathways leading to necrosis may explain the synergistic effect of EGCg/IR treatments.

It is well recognized that IR is capable of inducing both apoptosis and necrosis in various tumor cell lines [52, 53]. Many factors may influence the importance of necrosis following IR. Studies have shown that necrosis is induced in tumor cells following exposure to high irradiation doses [53-55]. However, it has also been shown that human promyeloctytic leukemia cells, irradiated with 50 Gy, died early, primarily by apoptosis while cells irradiated with 10 Gy died later, predominantly by necrosis [56]. The effect of time after radiation is controversial and probably depends on many additional factors. Some authors report that rapid cell death occurs primarily through a non-apoptotic pathway [57, 58] while others observe a dose-related increase in necrosis with some delay after exposure [23]. Furthermore, human lymphocytes exposed to X-rays for up to 20 Gy displayed no necrosis at 4 hours post-IR. Although cell death was explained through apoptosis in short-term culture (4 hours), the proportion of apoptotic cells did not match the increase in cell death with increasing radiation. The authors explained this discrepancy as being due to prolongation of the apoptotic process [59]. In long-term cultures, a combination of apoptosis and necrosis was apparent and explained the decrease in viability [59]. The kinetics of appearance of both apoptotic and necrotic cells in irradiated tumors is most probably influenced by cell cycle progression and by cell loss. In addition, treatment of cancer cells with chemotherapeutic agents and IR resulted in an increase in the necrotic component of cell death [60]. For example, combined treatment of a melanoma human cell line with

radiation and with camptothecin, a topoisomerase I inhibitor with significant anticancer activities, induced significantly more necrotic than apoptotic cell death [60]. Interestingly, simultaneous exposure of a human leukaemic cell line, EOL-1, to high concentrations of EGCg (50 μM) and IR (0-8 Gy) resulted in a synergistic decrease in cell proliferation and a synergistic increase in apoptosis and necrosis[23].

Interestingly, EGCg bears recognized antiangiogenic properties. Many mechanisms have been proposed for this, including decreased production of VEGF by tumor cells, interfering with VEGF receptor activity [33] and expression [32], and altering endothelial morphogenesis and cellular functions [33]. Therefore, VEGF survival signalling might be inhibited by EGCG, which could partially explain the enhanced results of combining radiotherapy and antiangiogenesis agents [61]. Furthermore, EGCg has been shown to sensitize various tumor cell lines to radiation and to augment the effectiveness of radiotherapy [8, 23, 24]. EGCg has also been shown to induce apoptosis in a variety of cell lines including EC lines, but at doses ranging from 20-100 μM over various time periods [29, 34, 62]. In our study, because EGCg pre-treatment did not, by itself, induce apoptosis or potentiate IR-induced apoptosis, increased apoptosis cannot explain the synergistic EGCg/IR decrease in cell survival.

In summary, brain tumor-associated EC represent a powerful cancer treatment target especially in highly vascularized tumors such as GBM [4]. Although the HBMEC model used in the present study does not represent glioma-derived EC, it is

to our knowledge the closest *in vitro* model that can approximate the human brain tumor-derived EC phenotype in long term studies. The combination of EGCg and IR treatments resulted in a synergistic delayed anti-survival effect that could be accounted for by an increase in necrosis. It can be envisioned that strategies sensitizing brain tumor vasculature to low dose IR may thus enhance the efficacy of radiotherapy. Therefore, EGCg represents a potential agent for sensitizing brain EC to radiotherapy, possibly by potentiating IR's necrotic effect.

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REFERENCES

1. Laperriere N, Zuraw L, Cairncross G, Cancer Care Ontario Practice Guidelines Initiative Neuro-Oncology Disease Site Group: Radiotherapy for newly diagnosed malignant glioma in adults: a systematic review. *Radiother Oncol* 64: 259-73, 2002

2. Davis FG, McCarthy BJ, Freels S, Kupelian V, Bondy ML. The conditional probability of survival of patients with primary malignant brain tumors: surveillance, epidemiology, and end results (SEER) data. *Cancer* 85: 485-491, 1999

3. Glioma Meta-analysis Trialists (GMT) Group, Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. *Lancet* 359: 1011-1018, 2002

4. Brem S, Cotran R, Folkman J. Tumor angiogenesis: a quantitative method for histologic grading. *J Natl Cancer Inst* 48: 347-56, 1972

5. Folkman J. Tumor angiogenesis: therapeutic implications. *N Eng J Med* 285: 1182-1186, 1971

6. Chaudhry IH, O'Donovan DG, Brenchley PE, Reid H, Roberts IS. Vascular endothelial growth factor expression correlates with tumour grade and vascularity in gliomas. *Histopathology* 39: 409-15, 2001

7. Brem S. Angiogenesis and brain tumors. In: Youmans Neurological Surgery, fifth edition. Saunders, Philadelphia (PA), 2003, p.771-789

8. McLaughlin N, Annabi B, Bouzeghrane M, Temme A, Bahary JP, Moumdjian R, Beliveau R. The survivin-mediated radioresistant phenotype of glioblastomas is regulated by RhoA and inhibited by the green tea polyphenol (-) epigallocatechin-3-gallate. *Brain Research* 1071: 1-9, 2006

9. Hendry JH and West CM. Apoptosis and mitotic cell death: their relative contributions to normal tissue and tumor radiation response. *Int J Radiat Biol* 71: 709-19, 1997

10. Garcia-Barros M, Paris F, Cordon-Cardo C, Lyder D, Rafii S. Haimovitz-friedman A, Fuks Z, Kolesnick R. Tumor response to radiotherapy regulated by endothelial cell apoptosis. *Science* 300: 1155-9, 2003

11. Denekamp J. Endothelial cell proliferation as a novel approach to targeting tumour therapy. *Br J Cancer* 45: 136-9, 1982

12. Bolus NE. Basic review of radiation biology and terminology. *J Nucl Med Technol* 29: 67-73, 2001

13. Coleman CN. Hypoxia in tumors: a paradigm for the approach to biochemical and physiologic heterogeneity. *J Nat Cancer Inst* 80: 310-7, 1988

14. Maj JG, Paris F, Haimovitz-Friedman A, Venkatraman E, Kolesnick R, Fuks Z. Microvascular function regulates intestinal crypt response to radiation. *Cancer Res* 63: 4338-41, 2003

15. Paris F, Fuks Z, Kang A, Capodieci P, Juan G, Ehleiter D, Haimovitz-Friedman A, Cordon-Cardo C, Kolesnick R. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. *Science* 293: 293-7, 2001

16. Pena LA, Fuks Z, Kolesnick RN. Radiation-induced apoptosis of endothelial cells in the murine central nervous system: protection by fibroblast growth factor and sphingomyelinase deficiency. *Cancer Res* 60: 321-7, 2000

17. Fuks Z. Persaud RS, Alfieri A, McLoughlin M, Ehleiter D, Schwartz JP,Seddon AP, Cordon-Cardo C, Haimovitz-Friedman A. Basic fibroblast growth factor protects endothelial cells against radiation-induced programmed cell death in vitro and in vivo. *Cancer Res* 54: 2582-90, 1994

18. Browder T, Butterfield CE, Kraling BM, Shi B, Marshall B, O'Reilly MS, Folkman J. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. *Cancer Res* 60: 1878-86, 2000

19. Edinger AL, Thompson CB. Death by design: apoptosis, necrosis and autophagy. *Curr Opin Cell Biol* 16: 663-9, 2004

20. Stupp R, Mason WP, van der Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, Curschmann J, Janzer RC, Ludwin SK, Gorlia T, Allgeier A, Lacombe D, Cairncross JG, Eisenhauer E, Mirimanoff RO, European Organisation for Research and Treatment of Cancer Brain Tumor and Radiotherapy Groups; National Cancer Institute of Canada Clinical Trials Group. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 352: 987-96, 2005

21. Schuuring J, Bussink J, Bernsen HJ, Peeters W, van Der Kogel AJ. Irradiation combined with SU5416: microvascular changes and growth delay in a human xenograft glioblastoma tumor line. *Int J Radiat Oncol Biol Phys* 61: 529-34, 2005

22. Chendil D, Ranga RS, Meigooni D, Sathishkumar S, Ahmed MM. Curcumin confers radiosensitizing effect in prostate cancer cell line PC-3. *Oncogene* 23: 1599-607, 2004

23. Baatout S, Derradji H, Jacquet P, Mergeay M. Increased radiation sensitivity of an eosinophilic cell line following treatment with epigallocatechin-gallate, resveratrol and curcuma. *Int J Mol Med* 15: 337-52, 2005

24. Baatout S, Jacquet P, Derradji H, Ooms D, Michaux A, Mergeay M. Study of the combined effect of X-irradiation and epigallocatechin-gallate (a tea component) on the growth inhibition and induction of apoptosis in human cancer cell lines. *Oncol Rep* 12: 159-167, 2004

25. Kim CH, Moon SK. Epigallocatechin-3-gallate causes the p21/WAF1-mediated G(1)-phase arrest of cell cycle and inhibits matrix metalloproteinase-9 expression in TNF-alpha-induced vascular smooth muscle cells. *Arch Biochem Biophys* 435: 264-72, 2005

26. Gupta S, Hussain T, Mukhtar H. Molecular pathway for (-)-epigallocatechin-3-gallate-induced cell cycle arrest and apoptosis of human prostate carcinoma cells. *Arch Biochem Biophys* 410: 177-85, 2003

27. Ahmad N, Cheng P, Mukhtar H. Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate. *Biochem Biophys Res Commun* 275: 328-34, 2000

28. Yokoyama S, Hirano H, Wakimaru N, Sarker KP, Kuratsu J. Inhibitory effect of epigallocatechin-gallate on brain tumor cell lines in vitro. *Neuro-oncol* 3: 22-28, 2001

29. Fassina G, Vene R, Morini M, Minghelli S, Benelli R, Noonan DM, Albini A. Mechanisms of inhibition of tumor angiogenesis and vascular tumor growth by epigallocatechin-3-gallate. *Clin Cancer Res* 10: 4865-73, 2004

30. Annabi B, Bouzeghrane M, Moumdjian R, Moghrabi A, Beliveau R. Probing the infiltrating character of brain tumors: inhibition of RhoA/ROK-mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCg. *J Neurochem* 94: 906-16, 2005

31. Pilorget A, Berthet V, Luis J, Moghrabi A, Annabi B, Beliveau R. Medulloblastoma cell invasion is inhibited by green tea (-)epigallocatechin-3-gallate. *J Cell Biochem* 90: 745-55, 2003

32. Kojima-Yuasa A, Hua JJ, Kennedy DO, Matsui-Yuasa I. Green tea extract inhibits angiogenesis of human umbilical vein endothelial cells through reduction of expression of VEGF receptors. *Life Sci* 73: 1299-313, 2003

33. Lamy S, Gingras D, Béliveau R. Green tea catechins inhibit vascular endothelial growth factor receptor phosphorylation. *Cancer Res* 62: 381-5, 2002

34. Annabi B, Lee YT, Martel C, Pilorget A, Bahary JP, Beliveau R. Radiation induced-tubulogenesis in endothelial cells is antagonized by the antiangiogenic properties of green tea polyphenol (-) epigallocatechin-3-gallate. *Cancer Biol Ther* 2: 642-9, 2003

35. Stins MF, Gilles F, Kim KS. Selective expression of adhesion molecules on human brain microvascular endothelial cells. *J Neuroimmunol* 76: 81-90, 1997

36. Greiffenberg L, Goebel W, Kim KS, Weiglein I, Bubert A, Engelbrecht F, Stins M, Kuhn M. Interaction of Listeria monocytogenes with human brain microvascular endothelial cells: InlB-dependent invasion, long-term intracellular growth, and spread from macrophages to endothelial cells. *Infect Immun* 66: 5260-7, 1998

37. Kim HS, Cho HJ, Cho HJ, Park SJ, Park KW, Chae IH, Oh BH, Park YB, Lee MM. The essential role of p21 in radiation-induced cell cycle arrest of vascular smooth muscle cell. *J Mol Cell Cardiol* 37: 871-80, 2004

38. Maeda T, Chong MT, Espino RA, Chua PP, Cao JQ, Chomey EG, Luong L, Tron VA. Role of p21(Waf-1) in regulating the G1 and G2/M checkpoints in ultraviolet-irradiated keratinocytes. *J Invest Dermatol* 119: 513-21, 2002

39. Toyoshima H, Hunter T. p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21. *Cell* 78: 67-74, 1994

40. Ormerod MG. Investigating the relationship between the cell cycle and apoptosis using flow cytometry. *J Immunol Methods* 265: 73-80, 2002

41. Tanaka T. Effect of adenoviral-mediated thymidine kinase transduction and ganciclovir therapy on tumor-associated endothelial cells. *Neurol Med Chir (Tokyo)* 37: 730-7, 1997.

42. Bian XW, Jiang XF, Chen JH, Bai JS, Dai C, Wang QL, Lu JY, Zhao W, Xin R, Liu MY, Shi JQ, Wang JM. Increased angiogenic capabilities of endothelial cells from microvessels of malignant human gliomas. *Int Immunopharmacol* 6: 90-9, 2006

43. Charalambous C, Hofman FM, Chen TC. Functional and phenotypic differences between glioblastoma multiforme-derived and normal human brain endothelial cells. *J Neurosurg* 102: 699-705, 2005.

44. Bian C, Zhao K, Tong GX, Zhu YL, Chen P. Immortalization of human umbilical vein endothelial cells with telomerase reverse transcriptase and simian virus 40 large T antigen. *J Zhejiang Univ Sci B* 6: 631-6, 2005.

45. Tentori L, Vergati M, Muzi A, Levati L, ruffini F, Forini O, Vernole P, Lacal PM, Graziani G. Generation of an immortalized human endothelial cell line as a model of neovascular proliferating endothelial cells to assess chemosensitivity to anticancer drugs. *Int J Oncol* 27: 525-535, 2005.

46. Zhao H, Spitz MR, Tomlinson GE, Zhang H, Minna JD, Wu X. Gamma-radiation-induced G2 delay, apoptosis, and p53 response as potential susceptibility markers for lung cancer. *Cancer Res* 61: 7819-24, 2001

47. Hwang A, Muschel RJ. Radiation and the G2 phase of the cell cycle. *Radiat Res*. 150 (Suppl): 52-9, 1998

48. Schwartz D, Almog N, Peled A, Goldfinger N, Rotter V. Role of wild type p53 in the G2 phase: regulation of the gamma-irradiation-induced delay and DNA repair. *Oncogene* 15: 2597-607, 1997

49. Bernhard EJ, Maity A, Muschel RJ, McKenna WG. Effects of ionizing radiation on cell cycle progression. A review. *Radiat Environ Biophys* 34: 79-83, 1995

50. Liang YC, Lin-Shiau SY, Chen CF, Lin JK. Inhibition of cyclin-dependent kinases 2 and 4 activities as well as induction of Cdk inhibitors p21 and p27 during growth arrest of human breast carcinoma cells by (-)-epigallocatechin-3-gallate. *J Cell Biochem* 75: 1-12, 1999

51. Nakamura K, Arai D, Fukuchi K. Identification of the region required for the antiapoptotic function of the cyclin kinase inhibitor, p21. *Arch Biochem Biophys* 431: 47-54, 2004

52. Vanden Berghe T, Denecker G, Brouckaert G, Vadimovisch Krysko D, D'Herde K, Vandenabeele P. More than one way to die: methods to determine TNF-induced apoptosis and necrosis. *Methods Mol Med* 98: 101-26, 2004

53. Shimamura H, Sunamura M, Tsuchihara K, Egawa S, Takeda K, Matsuno S. Irradiated pancreatic cancer cells undergo both apoptosis and necrosis, and could be phagocytized by dendritic cells. *Eur Surg Res* 37: 228-34, 2005

54. Rainaldi G, Ferrante A, Indovina PL, Santini MT. Induction of apoptosis or necrosis by ionizing radiation is dose-dependent in MG-63 osteosarcoma multicellular spheroids. *Anticancer Res* 23: 2505-18, 2003

55. Nagata S, Obana A, Gohto Y, Nakajima S. Necrotic and apoptotic cell death of human malignant melanoma cells following photodynamic therapy using an amphiphilic photosensitizer, ATX-S10(Na). *Lasers Surg Med* 33: 64-70, 2003

56. Dynlacht JR, Earles M, Henthorn J, Roberts ZV, Howard EW, Seno JD, Sparling D, Story MD. Degradation of the nuclear matrix is a common element during radiation-induced apoptosis and necrosis. *Radiat Res* 152: 590-603, 1999

57. Kurita H, Ostertag CB, Baumer B, Kopitzki K, Warnke PC. Early effects of PRS-irradiation for 9L gliosarcoma: characterization of interphase cell death. *Minim Invasive Neurosurg* 43: 197-200, 2000

58. Olive PL, Vikse CM, Vanderbyl S. Increase in the fraction of necrotic, not apoptotic, cells in SiHa xenograft tumours shortly after irradiation. *Radiother Oncol* 50: 113-9, 1999

59. Payne CM, Bjore CG Jr, Schultz DA. Change in the frequency of apoptosis after low- and high-dose X-irradiation of human lymphocytes. *J Leukoc Biol* 52: 433-40, 1992

60. Quto b SS, Ng CE. Comparison of apoptotic, necrotic and clonogenic cell death and inhibition of cell growth following camptothecin and X-radiation treatment in a human melanoma and a human fibroblast cell line. *Cancer Chemother Pharmacol* 49: 167-75, 2002

61. Siemann DW, Horsman MR. Targeting the tumor vasculature: a strategy to improve radiation therapy. *Expert Rev Anticancer Ther* 4: 321-7, 2004

62. Yoo HG, Shin BA, Park JC, Kim HS, Kim WJ, Chay KO, Ahn BW, Park RK, Ellis LM, Jung YD. Induction of apoptosis by the green tea flavonol (-)-epigallocatechin-3-gallate in human endothelial ECV 304 cells. *Anticancer Res* 22: 3373-8, 2002

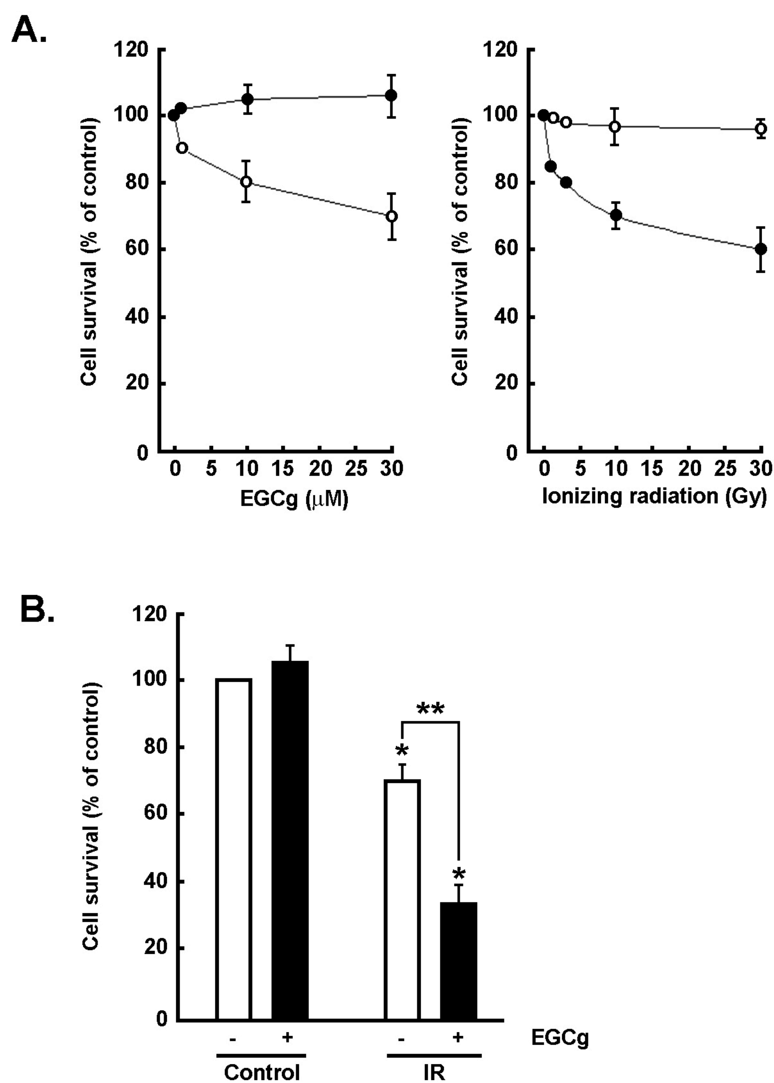


Fig.1 : **Effects of EGCg treatment or ionizing radiation exposure on HBMECs' survival.** A) Subconfluent HBMEC were either treated with EGCg for 6 hours in serum-free medium or exposed to a single dose of ionizing radiation (IR). Cell survival was assessed immediately after (white circles) and 48 hours after (black circles) individual treatments using an automatic nuclear cell counter

and Trypan blue staining. Cell survival is expressed as percent of the non-treated and non-irradiated (control) cell survival. B) HBMEC survival assessed 48 hours after combined EGCg (10 μM)/IR (10 Gy) treatments. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance against untreated control. A double asterisk (**) identifies significant probability values of less than 0.05 in irradiated conditions.

Fig.2

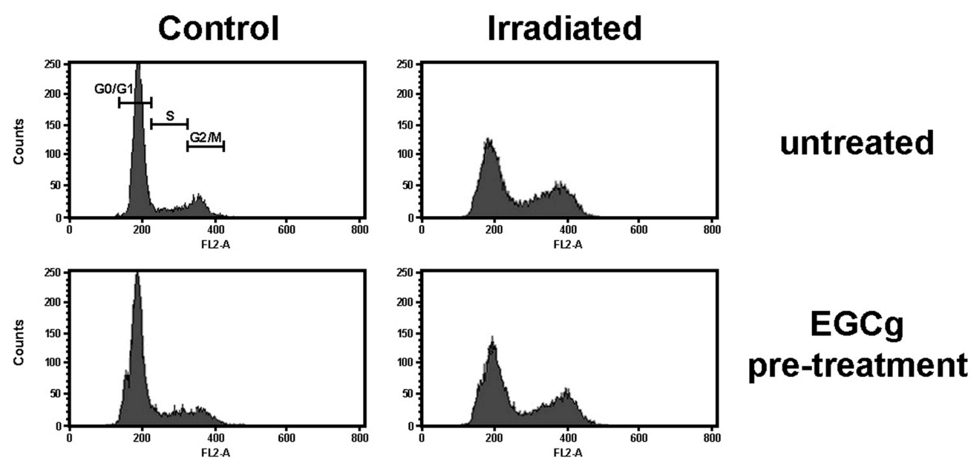


Fig.2 : **Ionizing radiation's influence on HBMEC cell cycle phase distribution is not modulated by EGCg pre-treatment.** Cells were harvested 48 hours following 10 _M EGCg and/or 10 Gy IR treatments and cell cycle was analyzed by flow cytometry as described in the Methods section.

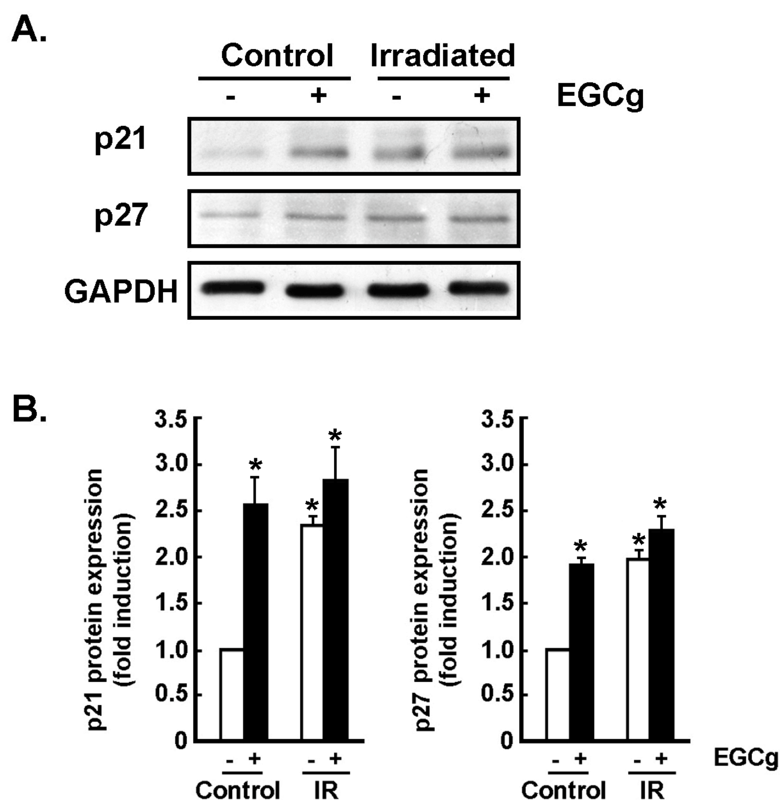


Fig.3 : **Induction of cyclin kinase inhibitors p21 and p27 by EGCg treatment and IR.** A) Subconfluent HBMEC were treated with EGCg (10 _M) and/or irradiated at 10 Gy, left to recuperate at 37°C for 48 hours and then harvested. Cell lysates of each condition were electrophoresed on SDS-gels and immunodetection was carried out as described in the Methods section. B) Results from a representative experiment were normalized to GAPDH expression. Quantification of p21 and p27 protein expression was performed by scanning densitometry of samples from control cells and from HBMEC

treated with EGCg and/or IR. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance against untreated control.

Fig.4

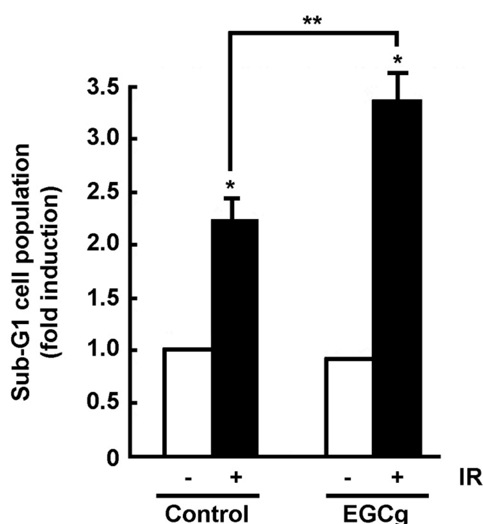


Fig.4 : **Combined EGCg and ionizing radiation treatements increased sub-G1 population.** Forty-eight hours after EGCg and/or IR treatments, cell cycle was assessed by flow cytometry, with specific attention to the sub-G1 region. Although IR (10 Gy) treatment induced 2.25-fold induction of the sub-G1 region content, combined EGCg (10 _M)/IR (10 Gy) treatments induced a 3.25-fold induction, suggesting that cell death is increased. Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance against the respective non-irradiated conditions. A double asterisk (**) identifies significant probability values of less than 0.05 in irradiated conditions.

Fig.5

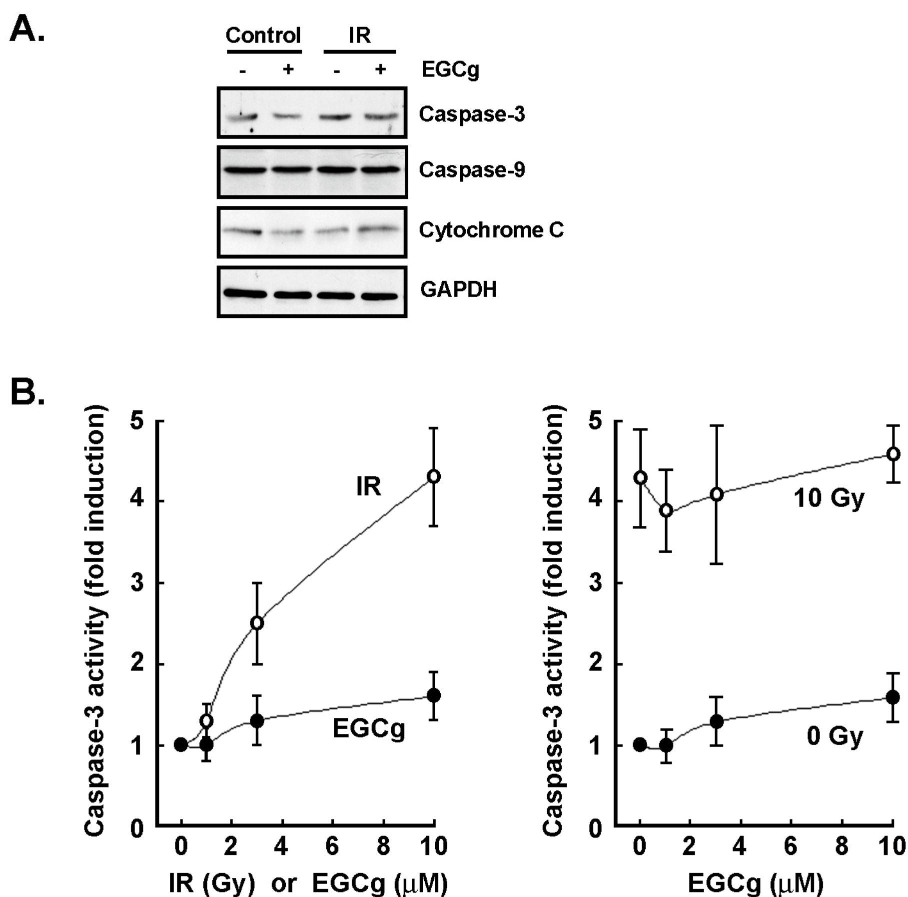
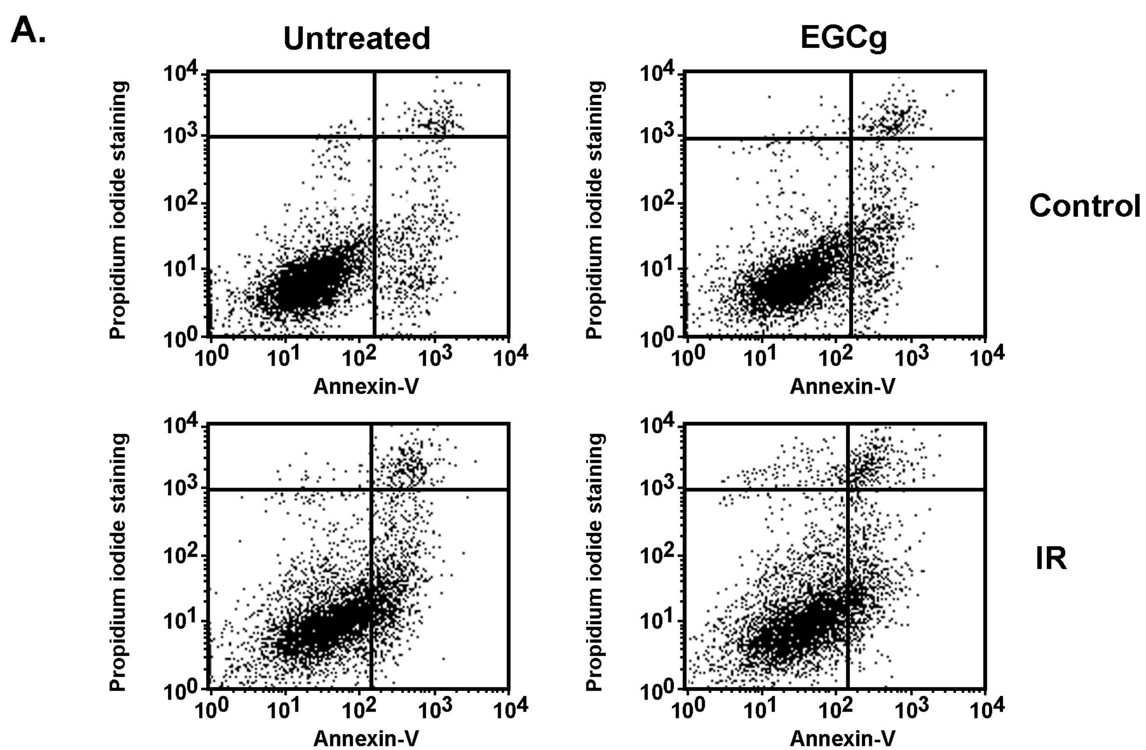


Fig.5 : **IR-induced caspase-dependant mechanisms are not increased by EGCg pre-treatment.** A) HBMEC were treated with 10 _M EGCg and/or exposed to a single 10 Gy IR dose. Cells were left to recuperate for 48 hours and were harvested. Cell lysates were electrophoresed on SDS-gels and immunodetection was carried out for caspase-3, caspase-9, and cytochrome C as described in the Methods section. B) In the left panel, caspase-3 activity was measured as described in the Methods section, on harvested cells after EGCg

treatment (black circles) or single radiation exposure (white circles). In the right panel, caspase-3 activity is shown for cells treated with various doses of EGCg and then followed (white circles) or not (black circles), with 10 Gy IR exposure.

Fig.6

A.



B.

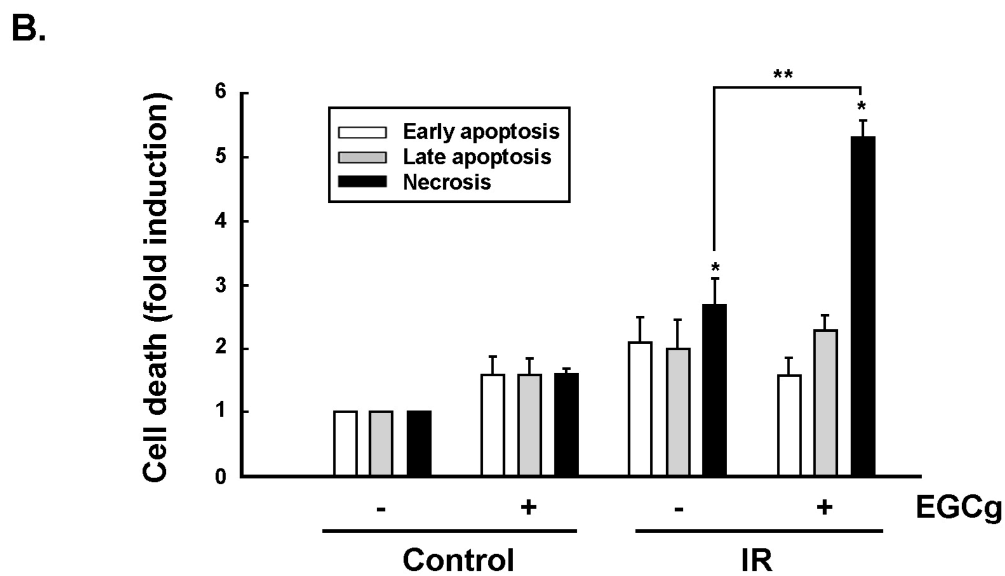


Fig.6 : **Cell necrosis significantly increases following EGCg/IR combined treatments.** Cell apoptosis and necrosis were analyzed, 48 hours after 10 μM EGCg and/or 10 Gy IR treatments, by flow cytometry after double staining with Annexin V and propidium iodide as described in the Methods section. A) Lower left quadrant represents viable cells; lower right quadrant, early apoptosis; upper right quadrant, late apoptosis; upper left quadrant, necrosis. B) Bar graph illustrates the quantification performed on each quadrant from A). Probability values of less than 0.05 were considered significant, and an asterisk (*) identifies such significance against untreated control. A double asterisk (**) identifies significant probability values of less than 0.05 in irradiated conditions.

CHAPTER IV

The response to brain tumor-derived growth factors is altered in radioresistant human brain endothelial cells

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* N. McLaughlin is responsible for 90% of the experimentation and writting of this manuscript. KS Kim generously provided the human brain microvascular endothelial cells. JP Bahary and R Moumdjian have reviewed the manuscript. B. Annabi and R. Béliveau supervised the entity of the study.

ABSTRACT

Introduction: Radioresistant brain tumor vasculature is thought to hamper the efficiency of adjuvant cancer therapies. However, little is known regarding the signalling pathways involved in the angiogenic response to brain tumor-derived growth factors in irradiated human brain microvascular endothelial cells (HBMEC). The goal of this study is to assess the effect of ionizing radiation (IR) on HBMEC survival, migration and tubulogenesis.

Methods: HBMEC were cultured and irradiated at sublethal single doses. Cell survival was assessed by nuclear cell counting and flow cytometry. HBMEC migration in response to brain tumor-derived growth factors (U-87 GF) and tubulogenesis were assayed using modified Boyden chambers and Matrigel, respectively.

Results: We observed that single administration of 3-10 Gy IR doses only reduced cell survival by 30%. Radioresistant HBMEC overexpressed RhoA, a small GTPase protein regulating cellular adhesion and migration, and Rho-kinase (ROK), a serine-threonine protein kinase and one of RhoA's major targets. HBMEC migration was induced by vascular endothelial growth factor (VEGF), but even more so in response to sphingosine-1-phosphate (S1P) and to U-87 GF. Following IR exposure, HBMEC basal migration increased more than two-fold, whereas the response to S1P and to U-87 GF was significantly diminished. Similarly, the inhibitor of ROK Y-27632 decreased HBMEC migration in response to S1P and U-87 GF. Overexpression of RhoA decreased tubulogenesis, an effect also observed in irradiated HBMEC.

Conclusion: Our results suggest that radioresistant HBMEC migration response to tumor-secreted growth factors and tubulogenesis are altered following IR. The RhoA/ROK signalling pathway is involved in the IR-altered angiogenic functions and may represent a potential molecular target for enhancing the impact of radiotherapy on tumor-associated endothelial cells.

Abbreviations for this manuscript:

EC: Endothelial cells;

GBM: Glioblastoma multiforme

HBMEC: Human brain microvascular endothelial cells

HUVEC: Human umbilical vein endothelial cells

IR: Ionizing radiation

PDGF: Platelet-derived growth factor

ROK: Rho-kinase

S1P: Sphingosine-1-phosphate

U-87 GF: Brain tumor-secreted growth factors

VEGF: Vascular endothelial growth factor

INTRODUCTION

Glioblastoma multiform (GBM) represents the most common but also the most lethal primary cerebral neoplasm in the adult population.[1] In fact, median length of survival rarely exceeds one year after diagnosis.[1,2] From the highly cellular rim of viable tumor which surrounds the necrotic core, GBM cells infiltrate the adjacent cerebral tissue.[3] Although single invasive glioma cells do not solely depend on tumor-associated angiogenesis, the growth of primary GBM and the development of a recurrent mass critically depends on neovascularisation. This explains why malignant gliomas are amongst the most intensively vascularized solid tumors.[4]

The process of tumor angiogenesis begins with increased vessel permeability via degradation of basement membrane by proteases secreted by activated endothelial cells (EC), which migrate and proliferate, leading to the formation of solid EC sprouts into the stroma.[5,6] This process is regulated by a host of growth factors such as the vascular endothelial growth factor (VEGF) or the platelet-derived growth factor (PDGF). These are produced either by the tumor itself or by infiltrating inflammatory cells.[5,7,8] It is noteworthy that several studies have recently defined platelet phospholipids as potential angiogenic factors, most notably sphingosine-1-phosphate (S1P), which is released by activated platelets as well as by glioblastoma cells[9] and which is a very potent EC chemoattractant.[10,11]

Radiotherapy is generally believed to exert its anti-cancerous effects by targeting tumor cells.[12] To this end, we have shown that ionizing radiation (IR) decreases *in vitro* cell proliferation of malignant glioma cells.[13] Paradoxically, the glioma cells that resist the acute cytotoxic effects of radiation may acquire an increased migratory potential and enhanced invasiveness.[14,15] It was recently suggested that IR prevents tumor growth by directly targeting tumor vasculature and inducing pro-apoptotic and necrotic processes.[16-20] It is recognized that both single-dose and fractionated radiotherapy induce EC damage.[21] Interestingly, endothelial apoptosis and microvascular dysfunction contribute more significantly to tumor cell lethality by the single-dose approach (8-10 Gy) than the low-dose exposures to fractionated therapy (1-3 Gy) since adaptive responses are simultaneously induced in the latter regiment.[21] Surprisingly, EC can also survive single radiation exposure similarly to cancer cells, in part due to of the tumor-derived microenvironment in which the phenotypic and functional properties of the EC differ from those of normal brain EC.[22] The signaling pathways and altered angiogenic functions in radioresistant human brain EC have not yet been investigated.

The Rho signalling pathway has been proposed to be involved in the radioresistance and IR-induced invasiveness of primary GBM.[13,23,24] Rho GTPase proteins function as molecular switches that modulate the activation of enzymes involved in different biological processes related to tumor progression, such as cell proliferation, apoptosis, cytoarchitecture, adhesion, migration, cell polarity, and transcriptional regulation.[25,26] The roles of Rho proteins in the cell motility and cell

morphology of EC have only recently begun to be uncovered.[26,27] The role(s) of this pathway in the functional properties of radioresistant EC has not yet been assessed. To our knowledge the human brain microvascular endothelial cell (HBMEC) model used in this study is the closest *in vitro* model for approximating the human brain tumor-derived EC phenotype in long term studies.

The goal of our study is to investigate the effects of IR on HBMEC angiogenic responses, i.e. cell survival, 3D tubulogenesis, and migration in response to VEGF, S1P and to human glioblastoma cell-derived growth factors. The RhoA/ROK signalling pathway was also investigated in relation to the adaptive properties of radioresistant HBMEC.

MATERIALS AND METHODS

Materials: Sodium dodecyl sulfate (SDS), bovine serum albumin (BSA) and S1P were purchased from Sigma (Oakville, ON). Lipofectamine-2000 transfection reagent and trypsin were from Invitrogen (Burlington, ON). Mouse anti-RhoA and anti-ROK monoclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-Caveolin-1 monoclonal antibody was from BD Pharmingen (Mississauga, ON) and mouse anti-GAPDH monoclonal antibody was from Advanced Immunochemical (Long Beach, CA). Horseradish peroxidase-conjugated anti-mouse IgG was obtained from Jackson Immunoresearch Laboratories (West Grove, PA). BCA protein assay kit was purchased from Pierce (Rockford, IL) and enhanced chemiluminescence (ECL)-Western blot kit from Chemicon International (Temecula, CA). Products for electrophoresis were obtained from Bio-Rad (Mississauga, ON) and polyvinylidene difluoride (PVDF) membranes were from Boehringer Mannheim (Laval, QC). Human recombinant VEGF (isoform 165) was produced and purified as previously described.[28] Y-27632 was purchased from Calbiochem (San Diego, CA).

Cell culture and cDNA transfection method: Human brain microvascular endothelial cells (HBMEC) were obtained from Dr Kwang Sik Kim (John Hopkins University School of Medicine, Baltimore, MD). These cells were positive for factor VIII-Rag, carbonic anhydrase IV, Ulex Europeus Agglutinin I, took up fluorescently labelled, acetylated low-density lipoprotein and expressed gamma glutamyl transpeptidase, demonstrating their brain EC specific phenotype.[29] HBMEC were

immortalized by transfection with simian virus 40 large T antigen and maintained their morphological and functional characteristics for at least 30 passages.[30] HBMEC were maintained in RPMI 1640 (Gibco, Burlington, ON) supplemented with 10% (v/v) fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 10% (v/v) NuSerum (BD Bioscience, Mountain View, CA), modified Eagle's medium nonessential amino acids (1%) and vitamins (1%), heparin (5 U/ml), sodium pyruvate (1 mM), L-glutamine (2 mM), EC growth supplement (30 μg/ml), 100 units/ml penicillin and 100 μg/ml streptomycin. Culture flasks were coated with 0.2 % type-I collagen to support the growth of HBMEC monolayers. Cells were cultured at 37°C under a humidified atmosphere containing 5% CO_2. All experiments were performed between cell passages 16 and 28. HBMEC were transiently transfected with the Myc-tagged WT-RhoA cDNA construct generously provided by Dr. Allan Hall (University College London, London, UK). All experiments involving these cells were performed 36 hours following transfection. Mock transfection of HBMEC cultures with empty pcDNA (3.1+) expression vector was used as a control.

Irradiation treatment: Cells were irradiated with a 6 MV photon beam from an Elekta SL75 linear accelerator. The delivered radiation doses were measured using a thermoluminescence dosimetry (TLD) system with an accuracy of 7%. During irradiation, cells were in RPMI containing 10% FBS and 10% NuSerum. Radioresistant cells were allowed to recuperate for 48 hours. Non-irradiated control cells were handled similarly to those which were subjected to IR treatment.

Cell survival assay: Cells were collected by gentle scraping and were resuspended in the overlaying medium. From each experimental sample, 150 μl of cell suspension were saved for nuclear cell counting using an automatic cell counter (New Brunswick Scientific Co., Edison, NJ). Viable cell number determination was also assessed using Trypan blue dye exclusion. Cells stained dark blue were not considered viable. Cell survival data are expressed as a mean value for at least four independent experiments.

Analysis of apoptosis/necrosis by flow cytometry: Cell death was assessed 48 hours after irradiation by flow cytometry. Cells floating in the supernatant and adherent cells harvested by trypsin solution were gathered to produce a single cell suspension. The cells were pelleted by centrifugation and washed with PBS. Then, $2x10^5$ cells were pelleted and suspended in 200 μL of buffer solution and stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) as described by the manufacturer (BD Bioscience). The cells were diluted by adding 300 μL of buffer solution and processed for data acquisition and analysis on a Becton Dickinson FACS Calibur flow cytometer using CellQuest Pro software. The X- and Y-axes indicated the fluorescence of annexin-V and PI respectively. It was possible to detect and quantitatively compare the percentages of gated populations in all of the four regions delineated. In the early stages of apoptosis, phosphatidylserine is translocated to the outer surface of the plasma membrane, which still remains physically intact. As annexin-V binds to phosphatidylserine but not to PI, and the die is incapable of passing the plasma membrane, it is excluded in early apoptosis (annexin-V^+/PI^-). Cells

in late apoptosis are stained with annexin-V and PI (annexin-V^+/PI^+). Necrotic cells have lost the integrity of their plasma membrane and are predominantly stained with PI (annexin-V^-/PI^+).

Analysis of HBMEC migration: HBMEC migration was assessed using modified Boyden chambers. The lower surfaces of Transwells (8-_m pore size; Costar, Acton, MA) were pre-coated with 0.2% type-I collagen for 2 hours at 37°C. The Transwells were then assembled in a 24-well plate (Fisher Scientific Ltd, Nepean, ON). The lower chamber was filled with serum-free HBMEC medium or growth factor-enriched conditioned medium isolated from 48 hours serum starved-U-87 human glioblastoma cells (U-87 GF). Control HBMEC or cells exposed to IR were collected by trypsinization, washed and resuspended in serum-free medium at a concentration of 10^6 cells/ml and 10^5 cells were then inoculated onto the upper side of each modified Boyden chamber. The plates were placed at 37°C in 5% CO_2/95% air for 30 minutes after which VEGF (50 ng/ml), S1P (10 μM), or Y-27632 (10 μM) (an inhibitor of Rho-kinase (ROK)) were added to the lower chambers of the Transwells. Migration then proceeded for 20 hours at 37°C in 5% CO_2/95% air. Cells that had migrated to the lower surface of the filters were fixed with 10% formalin phosphate and stained with 0.1% crystal violet-20% methanol (v/v). Images of at least five random fields per filter were digitized (100X magnification). The average number of migrating cells per field was quantified using Northern Eclipse software (Empix Imaging Inc., Mississauga, ON). Migration data are expressed as a mean value of at least four independent experiments.

Endothelial cell morphogenesis assay: Tubulogenesis was assessed using Matrigel aliquots of 50 _L, plated into individual wells of 96-well tissue culture plates (Costar, Amherst, MA) and allowed to polymerize at 37°C for 30 minutes. After brief trypsination, control or irradiated HBMEC were washed and resuspended at a concentration of 10^6 cells/ml in serum-free medium. Next, 25 _L of cell suspension (25,000 cells/well) and 75 _L of serum with medium were added into each culture well. Cells were allowed to form capillary-like tubes at 37°C in 5% CO_2/95% air for 20 hours. The structures formed within the Matrigel were digitized and quantified (100X magnification). For each experiment, four randomly chosen areas were quantified by counting the number of tubes formed. Tubulogenesis data are expressed as a mean value of at least three independent experiments.

Immunoblotting procedures: Cell lysates were separated by SDS–polyacrylamide gel electrophoresis (PAGE). After electrophoresis, proteins were electrotransferred onto polyvinylidene difluoride membranes which were then blocked overnight at 4°C with 5 % non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris–HCl, pH 7.5) containing 0.3 % Tween-20 (TBST). Membranes were then washed in TBST and incubated with the primary antibodies (1/1,000 dilution) in TBST containing 3 % BSA, followed by a 1 hr incubation with horseradish peroxidase-conjugated anti-mouse IgG (1/2,500 dilution) in TBST containing 5 % non-fat dry milk. The secondary antibodies were visualized by ECL and quantified by densitometry.

Statistical data analysis: Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired t-test and was used to compare migration and extent of capillary-like structure formation to mock non-irradiated HBMEC migration and tube formation. Probability values of less than 0.05 were considered significant and are indicated by an asterisk (*).

RESULTS

Ionizing radiation reduces HBMEC survival. HBMEC survival to increasing doses of IR was first examined. Subconfluent cells were exposed to single doses of IR and the cell survival was assessed 48 hours after exposure to IR using flow cytometry (Fig.1A) and trypan blue dye exclusion (Fig.1B). We observed a dose-dependent decrease in cell survival (lower left quandrants in Fig.1A) that was quantified by up to 40% at 30 Gy. (Fig.1B, left panel). Induction of significant cell death was also observed with increasing IR doses, as 10-30 Gy induced a combined necrosis/apoptosis of 4-10-fold over control (Fig.1B, right panel). The appearance of a heterogeneous cellular morphology (100X magnification) was also apparent at 10-30 Gy confirming some cell death (Fig.1C). These observations suggest that approximately 60-70% cells escaped the IR-induced cytotoxic effect and potentially exhibited some radioresistant phenotype. This sub-population was further analyzed for the rest of our study.

HBMEC migration in response to pro-angiogenic factors is diminished in radioresistant cells. EC migration in response to pro-angiogenic stimuli is a feature of angiogenesis. Several reports have previously shown that both tumor-derived VEGF and the angiogenic, platelet–derived S1P stimulated brain EC migration.[9,31,32] Therefore we assessed cell migration of control and irradiated HBMEC. A single 10 Gy radiation dose was found to trigger a significant 2.15-fold increase in HBMEC migration (Fig.2). As expected, basal migration of control HBMEC was increased by

1.75-fold in response to VEGF (50 ng/ml), 2.5-fold with S1P (10 μM) and 4.4-fold with U-87 GF. Interestingly, the pro-migratory effect of S1P tended to decrease in irradiated HBMEC, while it was significantly abrogated in response to U-87 GF (Fig.2).

Single dose IR induces the expression of RhoA, ROK and Caveolin-1. We previously found that a single IR dose induced the expression of RhoA, a small GTPase protein regulating cellular adhesion, migration and invasion,[25] in radioresistant U-87 malignant glioma cells.[13] Therefore we examined, in irradiated HBMEC, expression of the RhoA protein, expression of ROK (a serine-threonine protein kinase known to be one of RhoA's major targets) and the expression of Caveolin-1, a protein associating RhoA to endothelial caveolin-enriched membrane domains[33], which are proposed to participate in cell survival and angiogenesis.[34] After exposure to 10 Gy IR, a 2-fold increase was observed in RhoA expression, while that of ROK increased by 1.5-fold (Fig.3A&B). Interestingly, the expression of caveolin-1 also significantly increased by 1.6-fold after IR, while that of the house-keeping protein GAPDH remained unaffected.

Inhibition of RhoA signalling pathway diminishes HBMECs' response to pro-angiogenic factors. In order to partially mimic the RhoA/ROK-activated signalling pathway of irradiated cells, HBMEC were transfected with the cDNA construct encoding for RhoA protein. Transfection efficiency was confirmed by immunodetection for ectopic RhoA protein in RhoA-transfected cells (Fig.4A). Mock,

irradiated and RhoA-transfected HBMEC were inoculated onto the upper side of each Transwell and migration proceeded as described in the Methods section. Both IR and overexpression of RhoA significantly increased basal HBMEC migration by 1.75-fold and 2.6-fold, respectively (Fig.4B). We further studied the migration of RhoA-transfected HBMEC in the presence of the two most potent pro-angiogenic conditions used, namely, S1P and U-87 GF. The migration of HBMEC which overexpressed recombinant RhoA increased respectively 3.2-fold and 6.4-fold in response to S1P and U-87 GF (Fig.4B, black bars), relatively to mock cells in the absence of added stimulatory agent. Whereas RhoA-transfected HBMEC exhibit increased migration, as reflected with a RhoA-transfected/Mock ratio of 2.6, their migration response was lower showing a ratio of 1.26 for S1P and 1.46 for U-87 GF. Indeed, RhoA-transfected HBMEC present a weaker migration response to pro-angiogenic agents but irradiated HBMEC's migration response is abrogated. (Fig.2 and 4). This suggests that other signalling pathways, in addition to the RhoA pathway, may be implicated in mediating the altered angiogenic functions of radioresistant HBMEC. We also assessed the effect of inhibiting the RhoA/ROK signalling pathway on HBMEC migration. The addition of Y-27632, an inhibitor of ROK, into the lower chamber of the Transwells did not affect the migration of control HBMEC but significantly decreased that of RhoA-transfected cells as well as decrease the migration of irradiated HBMEC as compared to cells untreated with the ROK inhibitor. The addition of Y-27632 to S1P and CM U-87 significantly inhibited mock, irradiated and RhoA-transfected HBMECs' migration response to these angiogenic agents.

IR and RhoA overexpression decrease HBMEC in vitro tubulogenesis. We next investigated HBMEC's capacity to form tube-like structures on Matrigel. Matrigel is a reconstituted basement membrane containing various growth factors including basis fibroblast growth factor, platelet-derived growth factor, transforming growth factor beta, epidermal growth factor and insulin-like growth factor 1.[35] In a first series of experiments, irradiated and RhoA-transfected HBMEC were seeded on Matrigel and allowed to form capillary-like tubes. While non-irradiated HBMEC were capable of forming an extensive network of thin tubules (Fig.5A), these capillary-like structures were decreased in irradiated and RhoA-transfected HBMEC by 50% and 42%, respectively (Fig.5B). Tubules formed under these conditions presented aggregated cells with heterogeneous tubule thickness and diameter (Fig.5A). Therefore HBMEC that overexpress RhoA or that survive IR present a decreased tubulogenesis potential. Moreover, we assessed the inhibitor of ROK, Y-27632, on tubulogenesis. Mock, irradiated and RhoA-transfected HBMEC were resuspended in medium containing Y-27632 and were seeded on Matrigel. In the presence of Y-27632, mock HBMEC's capacity to form tubes was completely abolished (Fig.5A&B). Furthermore, although the inhibitor of RhoA/ROK pathway completely disrupted the abnormal tubes formed by radioresistant HBMEC, it partially reversed the abnormal appearance of tubes formed by RhoA-transfected HBMEC (Fig.5A&B). Indeed, small diameter tube-like structures were observed in HBMEC overexpressing recombinant RhoA and treated with the RhoA/ROK inhibitor (Fig.5A&B).

DISCUSSION

Until recently, research on the effects of IR on malignant gliomas was primarily focused on the cancer cells themselves. Although IR suppressed neoplastic glial cell proliferation,[13,36,37] inhibited cell cycle progression[36] and triggered cell death,[38] radiotherapy not only still fails to eradicate all tumour cells, but paradoxically enhances glioblastoma cells invasive potential.[14,15] On the other hand, the effects of IR on the cerebral vascular compartment have been far less documented. It has been suggested that the microvascular damage induced by IR might regulate the tumor response to further radiation and therefore mediate the sensitivity of tissues to radiotherapy, especially in single-dose regiments.[21,39] In fact, IR is thought to exert antiangiogenic effects on EC of various origins such as human umbilical vein EC (HUVEC).[19,40,41] It should be noted that HUVEC immortalized with SV40 antigens and the catalytic subunit of human telomerase overexpress the tumor endothelial marker-1/endosialin, which is regarded as the most differentially expressed molecule in tumor-derived endothelium versus normal endothelium.[42] Therefore, the fact that HBMEC were immortalized by transfection with SV40 allows us to further approximate the molecular impact of our study on cells that would have acquired some transformed phenotype in the GBM tumor microenvironment. The HBMEC model used in this study is a surrogate model that approximates tumor-derived EC. To our knowledge, it is the closest *in vitro* model that can approximate the human brain tumor-derived EC phenotype in long term studies.[20]

We have recently shown that IR decreases *in vitro* HBMEC cell survival through altered cell cycle progression and induction of apoptosis and necrosis.[20] In fact, that study was the first to report a dose response to IR in HBMEC.[20] Although EC dysfunction has been observed following fractionated 1-3 Gy therapy, the endothelial damage induced by single 8-10 Gy dose radiotherapy has been reported to induce greater tumor cell lethality.[21] Given the variability of radiation regiments applied in clinical practice, one can envision EC to be exposed to different total radiation doses administered in a single or multiple fractions.[43] Noteworthy, EC response to IR in mouse and human tumor specimens displayed an apparent threshold at 8-10 Gy[21] supporting the doses range of our current study. Therefore, the ability of IR to damage HBMEC at doses relevant to clinical radiotherapy suggests that targeting EC may contribute to the overall anti-cancerous effect of radiotherapy.[16-20]

Since each tumor capillary vascularizes hundreds of tumor cells, targeting tumor vasculature should potentiate the anti-tumorigenic effect of radiation. However, similar to tumour cells, EC can escape IR effects. The signalling pathways through which EC are protected against IR have only begun to be investigated and the activation of the phosphoinositide-3-kinase (PI3-K)-Akt-Bcl-2 survival pathway has been recently suggested.[44,45] The impact of IR exposure on the angiogenic functions of surviving human brain tumor-derived EC has not previously been investigated.

Here we investigated the specific impact of IR on HBMEC, which approximate human brain tumor-derived EC, with a focus on migration and tubulogenesis. Initial

characterization of HBMEC migration revealed induction by VEGF and S1P. However, the strong migration response induced by S1P, in comparison to VEGF, suggests that this factor induces agonist-specific regulation of EC angiogenic responses.[46] The HBMEC migration response was even greater in the presence of U-87 GF, which contains numerous pro-angiogenic growth factors and cytokines secreted by malignant brain tumors. Radioresistant HBMEC had an increased basal migration potential in comparison to non-irradiated HBMEC consistent with our previous observations on irradiated HUVEC.[19] The IR-induced migration of HBMEC correlates with their increased expression of RhoA and caveolin-1, both known to regulate EC migration.[19,47]

Interestingly, although radioresistant HBMEC have an increased migration potential, their response to angiogenic factors, including VEGF, S1P and U-87 GF, was attenuated, especially in the presence of U-87 GF. Furthermore, tubulogenesis capacity was also decreased in irradiated HBMEC. The reported effects of IR on tubulogenesis vary widely as some studies reported that IR increased tube formation[19], while others reported decreased tube formation.[40,48] This discrepancy may reflect cell type specificity and differences in experimental conditions, notably the maximum IR dose and the time elapsed from radiation to the time that tubulogenesis was assessed.

It is established that tumors can regulate the responsiveness of their associated EC by secreting growth factors, cytokines and mitogens.[39] The induction of angiogenic factors has also been proposed to be part of the tumors-associated EC

response to IR-induced stress.[49,50] It has been documented that IR of the tumor cell compartment may induce the co-expression of VEGF[40,51,52] and of its receptors[51] and stimulate the expression of PDGF.[53] Irradiated HUVEC and human dermal microvascular EC showed up-regulation of VEGF receptor-2.[40] IR may also result in increased phosphorylation of tyrosine residues in the cytoplasmic domain of receptor tyrosine kinases, which serve as docking sites for signalling entities of downstream pathways.[54,55] Therefore, radioresistant HBMEC angiogenic functions, namely migration response to chemoattractants and tubulogenesis, may be attenuated due to alterations in downstream signaling pathways. Although some EC escape radiation's cytotoxicity, they respond weakly to growth factors required for tumor expansion and angiogenesis. This probably contributes to IR's capacity to stabilize or reduce tumor cell burden.

The Rho signalling pathway has been reported to be involved in the radioresistance of glioblastomas.[13,23,24] Rho, Rac and Cdc42 have been shown, *in vitro*, to affect multiple aspects of cell behavior relevant to tumorigenicity.[25] RhoGTPases are critical not only for tumor cells but also for EC shape changes and for the adhesion dynamics that drive migration.[26,56,57] Although the RhoA/ROK signaling pathway may be activated in radioresistant HBMEC, a balance in the RhoA/ROK pathway may be crucial in regulating EC functions.[27,57] Tubulogenesis in control and irradiated HBMEC may be diminished by ROK inhibitors if the resultant balance of RhoA/ROK expression is negative. However, tube formation of RhoA-transfected cells may be partially re-established by RhoA/ROK inhibitors if the balance tends toward

equilibrium. Other pathways may also be involved in mediating the altered angiogenic functions of radioresistant HBMEC as one can acknowledge the limitations of the well characterized and specific pharmacological inhibitors used in our study. Specific gene silencing using siRNA, combined to dominant negative molecule approaches, will ultimately provide further evidence on the alternate pathways involved. Our data however strongly suggest that the RhoA/ROK signaling pathway plays an important role in the response of tumor-associated EC to IR.

Collectively, our data suggest that IR significantly alters crucial steps of angiogenesis, namely cell proliferation, migration and tubulogenesis in radioresistant HBMEC. Response to growth factors important for tumor expansion and angiogenesis is significantly attenuated in radioresistant HBMEC. These functional alterations probably contribute to IR's capacity to stabilize or reduce tumor cell burden. We propose that the RhoA/ROK signalling pathway may be involved in mediating the IR-induced altered angiogenic functions. This pathway might represent a potential molecular target for enhancing the impact of radiotherapy on tumor-associated EC.

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REFERENCES

1. Davis FG, McCarthy BJ, Freels S, et al. The conditional probability of survival of patients with primary malignant brain tumors: surveillance, epidemiology, and end results (SEER) data. Cancer. 1999; 85: 485-491.

2. Glioma Meta-analysis Trialists (GMT) Group. Chemotherapy in adult high-grade glioma: a systematic review and meta-analysis of individual patient data from 12 randomised trials. Lancet. 2002; 359: 1011-1018.

3. Giese A, Bjerkvig R, Berens ME, et al. Cost of migration: invasion of malignant gliomas and implications for treatment. J Clin Oncol. 2003; 21: 1624-36.

4. Brem S, Cotran R, Folkman J. Tumor angiogenesis: a quantitative method for histologic grading. J Natl Cancer Inst. 48: 347-56, 1972.

5. Ferrara N. Vascular endothelial growth factors as a target for anticancer therapy. Oncologist. 2004; 9 Suppl 1: 2-10.

6. Folkman J. Role of angiogenesis in tumor growth and metastasis. Semin Oncol. 2002; 29: 15-18.

7. Aonuma M, Iwahana M, Nakayama Y, et al. Tumorigenicity depends on angiogenic potential of tumor cells: dominant role of vascular endothelial growth factor and/or fibroblast growth factors produced by tumor cells. Angiogenesis. 1998; 2: 57-66.

8. Harrigan MR. Angiogenic factors in the central nervous system. Neurosurgery. 2003; 53: 639-60.

9. Van Brocklyn JR, Young N, Roof R. Sphingosine-1-phosphate stimulates motility and invasiveness of human glioblastoma multiforme cells. Cancer Lett. 2003; 199: 53-60.

10. Wang F, Van Brocklyn JR, Hobson JP, et al. Sphingosine 1-phosphate stimulates cell migration through a G(i)-coupled cell surface receptor. Potential involvement in angiogenesis. J Biol Chem. 1999; 274: 35343-50.

11. English D, Welch Z, Kovala AT, et al. Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. FASEB J. 2000; 14: 2255-65.

12. Laperriere N, Zuraw L, Cairncross G, Cancer Care Ontario Practice Guidelines Initiative Neuro-Oncology Disease Site Group. Radiotherapy for newly diagnosed malignant glioma in adults: a systematic review. Radiother Oncol. 2002; 64: 259-73.

13. McLaughlin N, Annabi B, Bouzeghrane M, et al. The Survivin-mediated radioresistant phenotype of glioblastomas is regulated by RhoA and inhibited by the green tea polyphenol (-)-epigallocatechin-3-gallate. Brain Research. 2005; 1071: 1-9.

14. Wick W, Wick A, Schulz JB, et al. Prevention of irradiation-induced glioma cell invasion by temozolomide involves caspase 3 activity and cleavage of focal adhesion kinase. Cancer Res. 2002; 62: 1915-9.

15. Wild-Bode C, Weller M, Rimner A, et al. Sublethal irradiation promotes migration and invasiveness of glioma cells: implications for radiotherapy of human glioblastoma. Cancer Res. 2001; 61: 2744-2750.

16. Maj JG, Paris F, Haimovitz-Friedman A, et al. Microvascular function regulates intestinal crypt response to radiation. Cancer Res. 2003; 63: 4338-41.

17. Paris F, Fuks Z, Kang A, et al. Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice. Science. 2001; 293: 293-7.

18. Pena LA, Fuks Z, Kolesnick RN. Radiation-induced apoptosis of endothelial cells in the murine central nervous system: protection by fibroblast growth factor and sphingomyelinase deficiency. Cancer Res. 2000; 60: 321-7.

19. Annabi B, Lee YT, Martel C, et al. Radiation induced-tubulogenesis in endothelial cells is antagonized by the antiangiogenic properties of green tea polyphenol (-) epigallocatechin-3-gallate. Cancer Biol Ther. 2003; 2: 642-649.

20. McLaughlin N. Annabi B, Lachambre MP, et al. Combined low ionizing radiation and green tea-derived epigallocatechin-3-gallate treatment induces human brain endothelial cells death. J Neurooncol. 2006; (In press).

21. Fuks Z, Kolesnick R. Engaging the vascular component of the tumor response. Cancer Cell. 2005; 8: 89-91.

22. Charalambous C, Hofman FM, Chen TC. Functional and phenotypic differences between glioblastoma multiforme-derived and normal human brain endothelial cells. J Neurosurg. 2005; 102: 699-705.

23. Zhai GG, Malhotra R, Delaney M, et al. Radiation Enhances the Invasive Potential of Primary Glioblastoma Cells via Activation of the Rho Signaling Pathway. J Neurooncol. 2006; 76: 227-37.

24. Ader I, Toulas C, Dalenc F, et al. RhoB controls the 24kDa FGF-2 induced radioresistance in HeLa cells by preventing post-mitotic cell death. Oncogene. 2002; 21: 5998-6006.

25. Aznar S, Fernandez-Valeron P, Espina C, et al. Rho GTPases: potential candidates for anticancer therapy. Cancer Lett. 2004; 206: 181-91.

26. Fryer BH, Field J. Rho, Rac, Pak and angiogenesis: old roles and newly identified responsibilities in endothelial cells. Cancer Lett. 2005; 229: 13-23.

27. Tkach V, Bock E, Berezin V. The role of RhoA in the regulation of cell morphology and motility. Cell Motil Cytoskeleton. 2005; 61: 21-33.

28. Labrecque L, Lamy S, Chapus A, et al. Combined inhibition of PDGF and VEGF receptors by ellagic acid, a dietary-derived phenolic compound. Carcinogenesis. 2005; 26: 821-826.

29. Stins MF, Gilles F, Kim KS. Selective expression of adhesion molecules on human brain microvascular endothelial cells. J Neuroimmunol. 1997; 76: 81-90.

30. Greiffenberg L, Goebel W, Kim KS, et al. Interaction of Listeria monocytogenes with human brain microvascular endothelial cells: InlB-dependent invasion, long-term intracellular growth, and spread from macrophages to endothelial cells. Infect Immun. 1998; 66: 5260-5267.

31. Radisavljevic Z, Avraham H, Avraham S. Vascular endothelial growth factor up-regulates ICAM-1 expression via the phosphatidylinositol 3 OH-kinase/AKT/Nitric oxide pathway and modulates migration of brain microvascular endothelial cells. J Biol Chem. 2000; 275 : 20770-4.

32. Waeber C, Blondeau N, Salomone S. Vascular sphingosine-1-phosphate S1P1 and S1P3 receptors. Drug News Perspect. 2004; 17: 365-82.

33. Gingras D, Gauthier F, Lamy S, et al. Localization of RhoA GTPase to endothelial caveolae-enriched membrane domains. Biochem Biophys Res Commun. 1998; 247: 888-893.

34. Razani B, Schlegel A, Liu J, et al. Caveolin-1, a putative tumor suppressor gene. Biochem Soc Trans. 2001; 29: 494-499.

35. Vukicevic S, Kleinman HK, Luyten FP, et al. Identification of multiple active growth factors in basement membrane Matrigel suggests caution in interpretation of cellular activity related to extracellular matrix components. Exp Cell Res. 1992; 202: 1-8.

36. Yao KC, Komata T, Kondo Y, et al. Molecular response of human glioblastoma multiforme cells to ionizing radiation: cell cycle arrest, modulation of the expression of cyclin-dependent kinase inhibitors, and autophagy. J Neurosurg. 2003; 98: 378-84.

37. Gliemroth J, Feyerabend T, Gerlach C, et al. Proliferation, migration, and invasion of human glioma cells exposed to fractionated radiotherapy in vitro. Neurosurg Rev. 2003; 26: 198-205.

38. Hara S, Nakashima S, Kiyono T, et al. p53-Independent ceramide formation in human glioma cells during gamma-radiation-induced apoptosis. Cell Death Differ. 2004; 11: 853-61.

39. Garcia-Barros M, Paris F, Cordon-Cardo C, et al. Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science. 2003; 300: 1155-1159.

40. Abdollahi A, Lipson KE, Han X, et al. SU5416 and SU6668 attenuate the angiogenic effects of radiation-induced tumor cell growth factor production and amplify the direct anti-endothelial action of radiation in vitro. Cancer Res. 2003; 63: 3755-63.

41. Gupta VK, Jaskowiak NT, Beckett MA, et al. Vascular endothelial growth factor enhances endothelial cell survival and tumor radioresistance. Cancer J. 2002; 8: 47-54.

42. Tentori L, Vergati M, Muzi A, et al. Generation of an immortalized human endothelial cell line as a model of neovascular proliferating endothelial cells to assess chemosensitivity to anticancer drugs. Int J Oncol. 2005; 27: 525-535.

43. Wakchsberger P, Burd R, Dicker AP. Tumor response to ionizing radiation combined with antiangiogenesis or vascular targeting agents: exploring mechanisms of interaction. Clin Cancer Res. 2003; 9: 1957-71.

44. Kumar P, Benedict R, Urzua F, et al. Combination treatment significantly enhances the efficacy of antitumor therapy by preferentially targeting angiogenesis. Lav Invest. 2005; 85: 756-67.

45. Tan J, Hallahan DE. Growth factor-independent activation of protein kinase B contributes to the inherent resistance of vascular endothelium to radiation-induced apoptotic response. Cancer Res. 2003; 63: 7663-7.

46. Liu F, Verin AD, Wang P, et al. Differential regulation of sphingosine-1-phosphate- and VEGF-induced endothelial cell chemotaxis. Involvement of G(ialpha2)-linked Rho kinase activity. Am J Respir Cell Mol Biol. 2001; 24: 711-9.

47. Regina A, Jodoin J, Khoueir P, et al. Down-regulation of caveolin-1 in glioma vasculature: modulation by radiotherapy. J Neurosci Res. 2004 ; 75 : 291-9.

48. Mao XW. A quantitative study of the effects of ionizing radiation on endothelial cells and capillary-like network formation. Technol Cancer Res Treat. 2006; 5: 127-34.

49. Weichselbaum RR, Hallahan D, Fuks Z, et al. Radiation induction of immediate early genes: effectors of the radiation-stress response. Int J Radiat Oncol Biol Phys. 1994; 30: 229-34.

50. Witte L, Fuks Z, Haimovitz-Friedman A, et al. Effects of irradiation on the release of growth factors from cultured bovine, porcine, and human endothelial cells. Cancer Res. 1989; 49: 5066-72.

51. Steiner HH, Karcher S, Mueller MM, et al. Autocrine pathways of the vascular endothelial growth factor (VEGF) in glioblastoma multiforme: clinical relevance of radiation-induced increase of VEGF levels. J Neurooncol. 2004; 66: 129-38.

52. Gorski DH, Beckett MA, Jaskowiak NT, et al. Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation. Cancer Res. 1999; 59: 3374-8.

53. Li M, gong P, Plathow C, et al. Small molecule receptor tyrosine kinase inhibitor of platelet-derived growth factor signaling (SU9518) modifies radiation response in fibroblasts and endothelial cells. BMC Cancer. 2006; 6: 79.

54. Ethier SP. Signal transduction pathways: the molecular basis for targeted therapies. Semin Radiat Oncol. 2002; 12: 3-10.

55. Goldkorn T, Balaban N, Shannon M, et al. EGF receptor phosphorylation is affected by ionizing radiation. Biochem Biophys Acta. 1997; 1358: 289-99.

56. Wojciak-Stothard B, Entwistel A, Garg R, et al. Regulation of TNF-alpha-induced reorganization of the actin cytoskeleton and cell-cell junctions by Rho, Rac, and Cdc42 in human endothelial cells. J Cell Physiol. 1998; 176: 150-65.

57. Nobes CD, Hall A. Rho GTPases control polarity, protrusion, and adhesion during cell movement. J Cell Biol. 1999; 144: 1235-44.

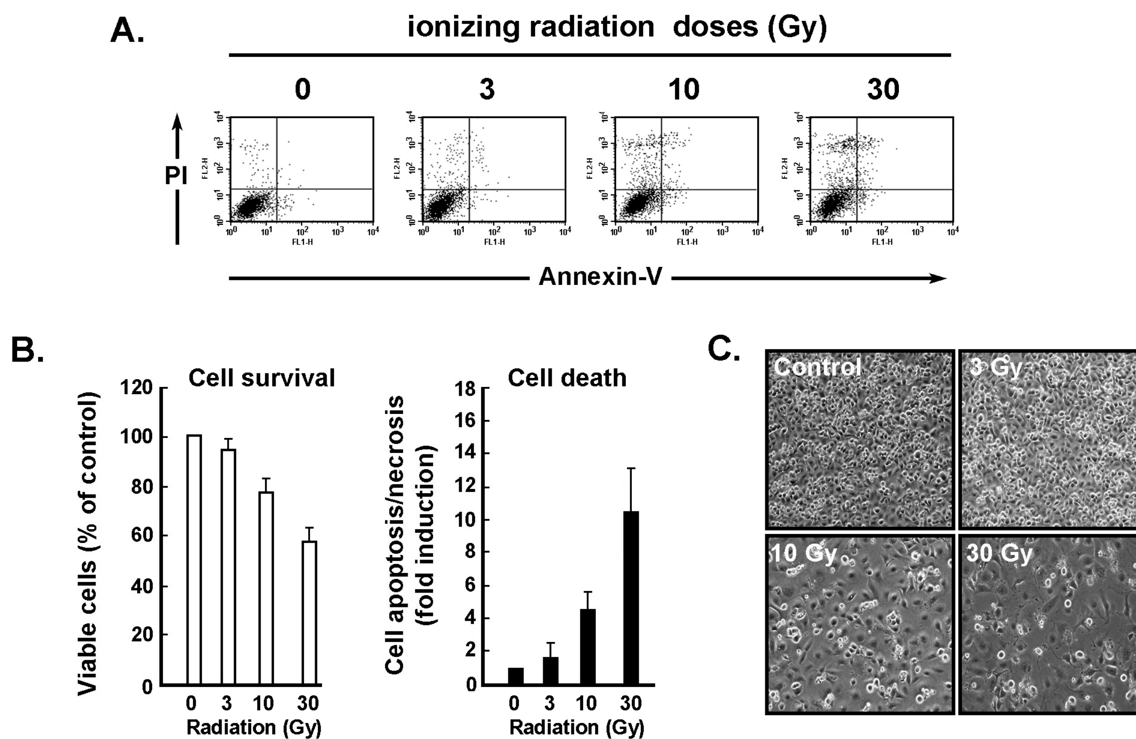

Fig.1 : **Ionizing radiation decreases HBMEC survival.** Subconfluent HBMEC were exposed to single doses of ionizing radiation of different strengths (IR). A) Cell survival was assessed 48 hours after IR exposure by using Annexin-V/PI staining and trypan blue exclusion (B) as described in the Methods section. Cell survival data are expressed as a mean value of at least three independent experiments. Error bars represent standard deviation of values. C) Pictures of adherent HBMEC were taken 48 hours after IR.

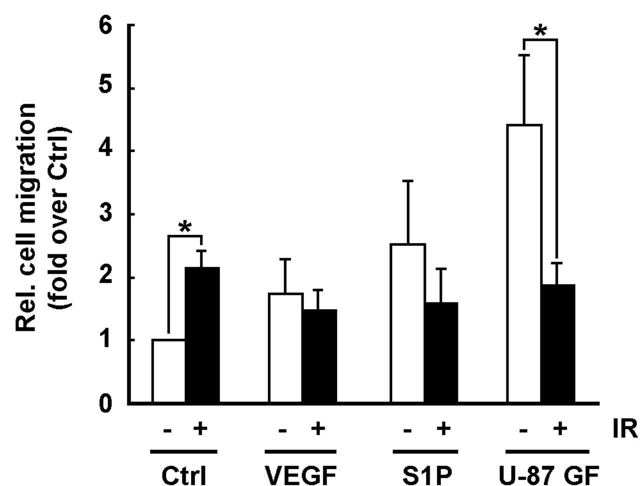

Fig.2 : **Ionizing radiation inhibits HBMECs' migratory response to brain tumor-derived growth factors.** Control and irradiated (10 Gy) HBMEC were seeded on type-1 collagen-coated filters, allowed to adhere for 30 min and migration performed as described in the Methods section in the presence or absence of VEGF (50 ng/ml), S1P (10 μM), or U-87 GF. Migration data are expressed as a mean value for the relative migration rate from at least four independent experiments. Error bars represent standard deviation of values.

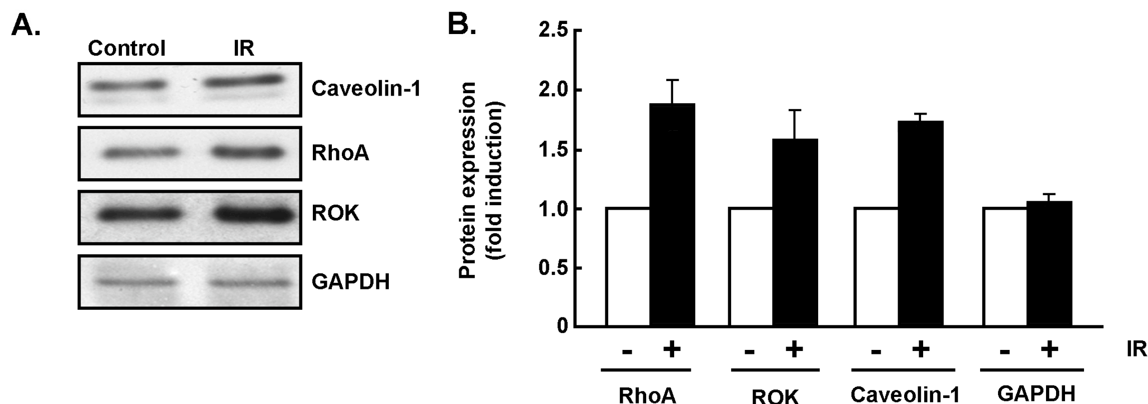

Fig.3 :**Ionizing radiation induces RhoA, ROK and Caveolin-1 expression in radioresistant HBMEC.** Subconfluent HBMEC were irradiated at 10 Gy, left to recuperate at 37°C for 48 hours and then radioresistant cells were harvested. Cell lysates from each condition were electrophoresed on SDS gels and (A) immunodetection was carried out as described in the Methods section. B) Quantification of RhoA, ROK and Caveolin-1 protein expression was performed by scanning densitometry of samples from control and from irradiated HBMEC. Protein expressions were normalized to GAPDH expression.

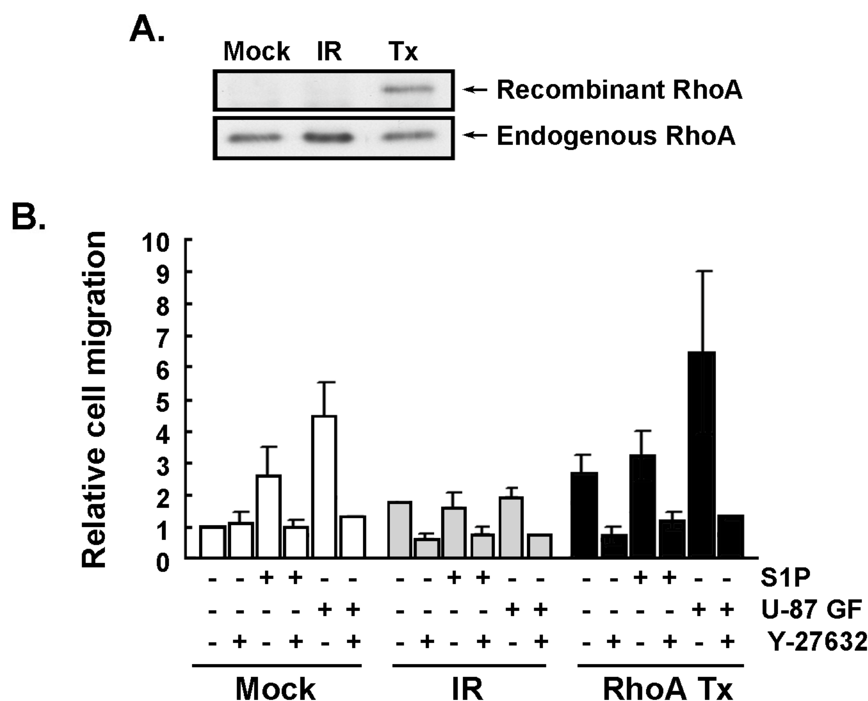

Fig.4 : **Inhibition of RhoA/ROK signalling pathway produces similar diminution in migration responses of RhoA-transfected and of irradiated HBMEC.** A) HBMEC cultured at 70% confluence were transfected with the Myc-tagged WT-RhoA cDNA construct. The transfection efficiency was confirmed by Western blotting and immunodetection for anti-RhoA as described in the Methods. Endogenous RhoA was detected after a 30 second exposition and recombinant RhoA after a 2 minute exposition. B) RhoA-transfected (RhoA Tx) and irradiated (IR, 10 Gy) HBMEC were seeded on type-1 collagen-coated filters and allowed to adhere for 30 min. Migration was performed for 20 hours

in the presence or absence of S1P (10 μM), U-87 GF or Y-27632 (10 μM). Migration data are expressed as a mean value of at least four independent experiments. Error bars represent standard deviation of values.

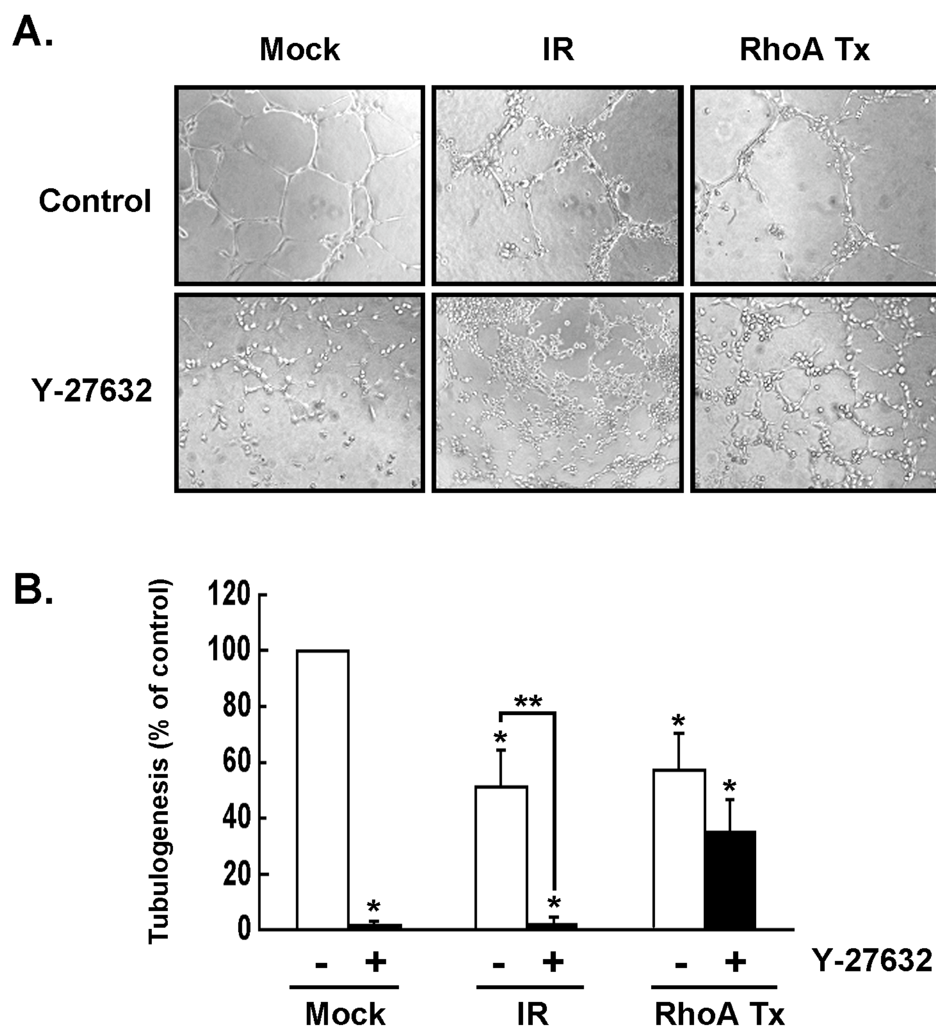

Fig.5 : **Ionizing radiation and RhoA overexpression decrease HBMEC capacity to form tubes *in vitro*.** HBMEC were seeded on Matrigel and allowed to form capillary-like tubes at 37°C for 20 hours in the presence or absence of Y-27632 (10 μM). A) The formation of capillary-like structures was examined microscopically and pictures (100X magnification) were taken. A

representative experiment is presented. B) Capillary-like structures were quantified as described in the Methods section. For each experiment, four randomly chosen areas were quantified by counting the total number of well formed tubes. Tubulogenesis data are expressed as a mean value of at least three independent experiments. Error bars represent standard deviation of values. (*) represents statistical significance with respect to untreated mock cells, while (**) statistical significance between untreated and Y-27632-treated cells in mock, IR-treated cells, and in RhoA-transfected cells.

CHAPTER V. DISCUSSION

5.1 Summary

In chapter II, we documented that IR induced the expression of Survivin and RhoA in U-87 human glioblastoma cells. U-87 cells transfected with the Survivin cDNA induced a cytoprotective effect against IR and induced RhoA expression. However, when Survivin-transfected cells were treated with EGCG, cells were rendered sensitive to IR and their RhoA expression was downregulated. The radioresistance of GBM may in part be mediated by a mechanism dependent on Survivin in conjunction with RhoA, which is abrogated with EGCG treatment.

In chapter III, we showed that although IR could reduce cell survival of HBMEC, radiation exposure was most effective in EGCG pre-treated cells. The synergistic effects of combined EGCG/IR treatments were related to increased cell necrosis, a non-apoptotic cell death mechanism. In chapter IV, we demonstrated that IR induced the expression of RhoA/ROK pathway in HBMEC. Irradiated or RhoA-transfected HBMEC presented an increased migration capacity. However, their response to angiogenic factors was decreased, especially in irradiated cells suggesting that other pathways, in addition to the RhoA/ROK pathway, may contribute to altered functions of radioresistant ECs. Regarding tubulogenesis, IR and RhoA-transfected cells presented a decreased capacity to form tubules. The decreased tubulogenesis was partially overcome in RhoA-transfected cells treated with Y-27632, an inhibitor of the RhoA-ROK pathway. This also suggests that other pathways possibly contribute to defective tubulogenesis following IR. Therefore, radioresistant HBMEC present

altered angiogenic functions and the RhoA-ROK pathway equilibrium may be involved in ECs' response to IR.

5.2 Discussion

5.2.1 Radioresistance of cells

Radiotherapy is routinely administered in the management of GBMs. However, its efficiency remains poor mostly because of tumor cell radioresistance, acquired enhanced invasive character following radiation and resultant tumor recurrence [101, 102]. Numerous signalling pathways have been proposed to contribute to GBM's radioresistance. Our studies showed that radioresistance of GBM may in part be mediated by a mechanism dependent on Survivin in conjunction with RhoA signalling. Following IR, Survivin and RhoA expression increased in U-87 cells. Rho proteins have been identified as an immediate-early gene activated following DNA damage [166]. Authors have documented a biphasic activation of the RhoA/ROK signalling pathway following IR [167]. The initial burst accounts for the rapid cytoskeletal remodelling. The second peak corresponds to the sustained contractile cell phenotype [167]. Activation of Rho proteins may be mediated through ligand interactions with growth factor or integrin receptors. Therefore, IR may induce RhoA by enhancing the production of growth factor [168, 169]. Rho signalling pathway may also be activated through IR-induced ROS that trigger signal transduction pathways leading to the activation of transcription factors [166]. Indeed, it has been documented that IR can activate the stress-activated protein kinases or c-

Jun N-terminal kinase (SAPK/JNK) and p38 mitogen-activated protein kinases (MAPK) through non-tyrosine kinase pathways [170].

RhoA has been recognized as a key regulator of cytosqueletal reorganization, cell motility, cell-cell and cell-extracellular matrix adhesion, cell cycle progression by promoting entry into G1 and progression to S phase, gene expression and regulator of apoptosis [171, 172]. RhoA may modulate Survivin's activity through various ways. RhoA may regulate Survivin's activity by acting on its promoter activity or on its cell cycle-regulated gene transcription. Also, RhoA pathways may modulate Survivin's function by altering its nuclear exportation which is dependent on Crm-1, a nuclear export receptor that is regulated by small GTPases [173]. Survivin has been found to directly interact with Ran-GTP, a small GTPase, in S phase and during mitosis [174]. Furthermore, Rho proteins, through their downstream pathways, may influence Survivin's intracellular regulation pathways including sequential ubiquitylation/deubiquitylation and multiple steps of phosphorylation [174]. Therefore, RhoA and Survivin probably interact at many levels given the extent of their cellular functions.

It has been shown that Rho proteins as well as Survivin may contribute to radioresistance. Overexpression of RhoB in radiosensitive cells increased cell survival after radiation exposure. The expression of RhoBN19, an inducible dominant negative form of Rho, as well as the treatment with farnesyltransferase inhibitors, led to the appearance of multinucleated cells and induced post-mitotic cell death in radiosensitive cells transfected with basic fibroblast growth factor-2 (bFGF-2). Therefore, it was proposed that RhoB, but not RhoA, might control the FGF-2-

induced radioresistance by protecting cells against radiation-induced post-mitotic cell death [107]. Farnesyltransferase inhibitors have had similar radiosensitizing effects on U-87 glioblastoma radioresistant cells [175]. Expression of RhoBN19 in U-87 cells induced a more important radiosensitizer effect in vivo than in vitro, due, according to the authors, to the control by Rho pathways of the tumor oxygenation and angiogenesis [108]. Also, it has been recently proposed that RhoA may be involved it the radiation-induced invasion [176]. Activation of RhoA may be mediated by EGFR and insulin-like growth factor-1 receptor (IGFR-1) through PI3K [176]. The extent of RhoA's implication in glioma radioresistance remains to be investigated. Survivin may also contribute to glioma radioresistance. Survivin may mediate double-strand DNA break repair and enhancement of tumor cell metabolism [104]. Recently, it was shown that suppression of Survivin by siRNA in p53 mutant malignant glioma cells enhanced tumor cells' radiosensitivity [177]. This supports that Survivin inhibition might be an attractive therapeutic target to overcome GBM's radioresistance.

Many other mechanisms have been proposed to contribute to glioblastomas' radioresistance. Potential radioresistance mechanisms may be grouped into three broad categories including extracellular growth factors or growth factor receptors, signal transduction cascades, and control of cell cycle. ErbBR have been identified as immediate early response gene products that are activated by IR as well as by their physiological growth factor ligands. However, the magnitude of activation following IR is much more significant than by growth factor ligand interaction. Activation of ErbBR induces a secondary stimulation of cytoplasmic protein kinase cascades leading to radioresistance by stimulating proliferation of surviving cells and by

inhibiting cell death [178]. Indeed, numerous reports suggest that antagonizing EGFR in irradiated cells induces a significant radiosensitizing effect [179]. However, not all EGFR-expressing GBMs are amenable to anti-EGFR radiosensitization [180]. Resistant GBM cells had an increased IGFR-1 expression and downstream signalling through PI3K-Akt was sustained [180].

The PI3K/Akt/mTOR pathway is a proposed mechanism for radioresistance. Ionizing radiation has been found to induce Akt in U-87 cells, which stimulates mammalian target of rapamycin (mTOR). mTOR inhibits autophagy, a programmed cell death, by activating p70S6K, a cytoprotective molecule. Inhibiting PI3K, Akt or mTOR may increase radiation response by enhancing autophagy. Furthermore, Akt is inactivated by PTEN. Authors have shown in PTEN-deficient cells that inhibition of PI3K/Akt signalling by treatment with a PI3K inhibitor or by conditional PTEN expression may lead to radiosensitization through the persistence of unrepaired DNA damage. These results suggest that PI3K/Akt may also modulate radioresistance by modulating response to radiation-induced DNA damage [181]. Since the PI3K/Akt pathway is implicated in many downstream pathways contributing to tumor formation and progression [40], their inhibition may possibly have many cellular effects influencing radioresistance.

Another pathway proposed to be implicated in GBMs' radioresistance is p53, a transcription factor that is induced in response to DNA damage. It can either induce cell cycle arrest and orchestrate DNA repair or promote apoptosis. When present and activated, p53 may induce arrest of cells in the G_1 phase of cell cycle and inhibit cell division. However, loss of p14/ARF, amplification of MDM2, or loss of p53 releases

cell cycle control and cell division from inhibition. IR cells lacking p53 may continue to replicated and accumulate additional oncogenic genetic alterations, contributing to radioresistance. However, if proapoptotic p53 targets are also induced with p53 activation, then apoptosis will occur [182]. Since approximately 30-60% of GBMs have inactivated p53 pathways, the lack of p53-mediated apoptosis may in itself contribute to GBMs' radioresistance.

Most probably, biological therapies trying to favourably modulate GBMs' radioresistance will have to be individualized to each patient given the marked genetic heterogeneity of GBMs and adjusted to the modifications occurring throughout radiotherapy.

Another important aspect when studying radioresistance is which cell within the tumor microenvironment is responsible for the tumor's radioresistance? Most studies have assessed the tumor cells' implication in radioresistance. More recently, it has been well documented that tumor cells may modulate their response to IR by stimulating angiogenesis through the release of numerous growth factors such as VEGF and SCF [62, 183-185], to assure their supply in oxygen and nutrients. Interestingly, this paracrine relationship may be modulated by radiation exposure [168, 169, 186]. Indeed, IR elicits a stress response in malignant cells which induces an up-regulation of VEGF-A expression [186]. IR has been found to significantly enhance the secretion of VEGF as well as hepatocyte growth factor (HGF), both growth factors are known to be implicated in malignant tumor cells' proliferation, invasion and angiogenesis [168, 169]. Therefore, IR-enhanced secretion of VEGF

may contribute to the protection of tumor blood vessels from IR-mediated cytotoxicity and thereby prevent tumor cell death [186]. However, because tumor vasculature is abnormal and because heterogeneous hypoxic regions are spread throughout the tumor, it is difficult to predict how tumor vasculature responds to radiation therapy in a given tumor. Interestingly, it has been documented that ECs can be directly targeted by IR. Investigators have found that IR exposure can induce apoptosis in ECs [187-190], even prior to tumor cell apoptosis [191]. On the other hand, ECs can also escape the cytotoxic effects of IR and survive single radiation exposure similarly to cancer cells. We demonstrate in the present thesis that the angiogenic properties of these radioresistant ECs in response to tumor-derived growth factors were altered. The signalling pathways through which ECs are protected against IR have only begun to be investigated and the activation of the PI3K-Akt-Bcl-2 survival pathway has been recently suggested [192, 193]. The impact of IR exposure on the angiogenic functions of surviving human brain tumor-derived ECs had not previously been investigated. Our results suggest that radioresistant HBMEC migration response to tumor-secreted growth factors and tubulogenesis are altered following IR. It is possible that these dysfunctional ECs contribute to the persistence of hypoxic microenvironments and subsequent radioresistance.

Recently, tumor stem cells within malignant gliomas have been proposed as the mediator of radioresistance. Bao and colleague showed that cancer stem cells expressing CD133, a marker for both neural stem cells and brain cancer stem cells, isolated from both human glioma xenografts and primary patient glioblastoma specimens preferentially activate the DNA damage checkpoint in response to

radiation, and repair radiation-induced DNA damage more effectively than CD133-negative tumor cells [194]. These results suggest that CD133-positive tumor cells may represent the cellular population that mediates glioma radioresistance. In addition, the radioresistance of CD133-positive glioma stem cells could be reversed with a specific inhibitor of Chk1 and Chk2 checkpoint kinases [194]. Therefore, DNA damage checkpoint response in cancer stem cells may be an interesting target to overcome GBM radioresistance.

In summary, resistance of GBM to radiotherapy implicates many players namely the tumor cells, the tumor-associated endothelial cells, and more recently recognized the brain tumor stem cells. This way of conceiving radioresistance also implies that new players in a complex phenomenon will need to be taken into consideration if tumor eradication is seeked.

5.2.2 Tumor-associated endothelial cells as a therapeutic target

Only recently have the ECs been considered a powerful new potential treatment target in highly vascularized tumors such as glioblastoma [2]. Tumor-associated ECs have a proliferation rate up to 20 times greater than the proliferation rate of normal vasculature [195], rendering them more radiosensitive than non-dividing cells [95]. ECs are generally well oxygenated, which makes them more radiosensitive than poorly oxygenated tumor cells because the oxygen present at the time of radiation may contribute to the formation of cytotoxic molecules which may prevent the reversal of some IR-induced changes [95, 196].

Tumor-associated ECs disruption and death following radiation exposure modifies the tumor vasculature. Consequently, oxygen and nutrient supply to tumor cells is altered. Following IR, tumor burden decreases, tumor vasculature reorganizes itself and the proportion of hypoxic tumor is decreased. This leads to a radiosensitization for subsequent treatment. Also, the generation of a more hypoxic tumor microenvironment after vessel disruption might subsequently induce death of tumor cells. On the other hand, this hypoxic microenvironment can also establish the conditions needed for selecting tumor cells that are more able to survive and grow in hypoxic environments and are less susceptible to apoptosis and eventually more radioresistant [197]. These observations suggest that a multimodal approach is necessary to optimize radiation efficiency and prevent the occurrence of highly resistant cells.

More recently, antiangiogenic agents have been successfully used in combination with IR to increase the therapeutic effectiveness of radiation exposure [124, 198]. Tumor-associated endothelial cells are an advantageous target regarding many aspects. Endothelial cells represent a homogeneous non-neoplastic cell compartment that is less likely to be subject to resistance. They are easily accessible for circulating agents. They proliferate rapidly which makes them vulnerable to radiation and cytotoxic agents. Furthermore, tumor-associated ECs can be distinguished from the resting vasculature, enabling a target specific treatment [199].

Many targets have been explored and the VEGF/VEGFR-2 signaling pathway has been suggested as an important target [199]. VEGF expression has been found predominantly in palissading cells adjacent to necrotic areas and in clusters of tumor

cells without obvious adjacent necrosis [200]. The areas of most pronounced angiogenesis and most rapid glioma growth within a malignant glioma is the invading border and this area corresponds to the highest SCF expression [62]. Recently, the SCF/c-kit pathway has been found to play an important role in tumor- and normal host cell-induced angiogenesis within the brain [62]. Therefore, it is possible that pro-angiogenic factors have complementary roles given their predominant effect in various regions of the tumor. Targeting multiple angiogenic signaling pathways by polyvalent inhibitors or combining antiangiogenic agents with distinct antiangiogenic properties might offer the best chance for clinical success.

The addition of a chemotherapeutic agent with antiangiogenic properties often has additive or synergistic effect when used in combination with IR or cytotoxic chemotherapy. Many hypotheses have been proposed to explain this sensitizing effect. Administration of an antiangiogenic agent can transiently "normalize" the altered structure and function of tumor vasculature to make it more efficient for oxygen and drug delivery. These agents can alleviate hypoxia and increase the efficiency of conventional therapies [201]. However, not all tumor cells are dependent on tumor vasculature and therefore remain unresponsive to antiangiogenic therapies. Tumor cells that have migrated away from the tumor bulk to form tumor cell islands probably receive adequate oxygen and nutrients either by passive diffusion or by normal blood vessels [202]. These cells are not targeted by any adjuvant therapies since radiotherapy is directed at the primary tumor site and angiogenic agents do not target normal brain vessels from which tumor clusters supply. Also, some viable tumor cells might be located in a microenvironment at low oxygen tensions. These

cells are radioresistant but also highly angiogenic given the hypoxic condition and are potentially resistant to antiangiogenic strategies [203]. Therefore, some tumor clusters at distance or within the primary tumor site constitute eligible candidates for recurrent GBM.

In addition to being in interaction with tumor cells, ECs have been identified as a critical component of the neural stem cell niche. Brain tumor stem cells may also rely on signalling interaction with nearby tumor vasculature to maintain their stem-like state. Folkins and collaborators have shown that only the combined treatment with antiangiogenic and cytotoxic drugs induced a reduction in sphere-forming units in the tumor xenografts [204]. They hypothesized that the synergetic effect of combined treatment may be due to a preferential eradication of tumor brain stem cell by the combined regiment. The angiogenic therapies might disrupt the glioma tumor brain stem cell vascular niche resulting in a reduction or loss of stem cell characteristics [204].

To assess basic mechanisms of angiogenesis in gliomas as well as the efficacy of antiangiogenic treatments, a variety of *in vitro* and *in vivo* models have been developed. *In vitro* systems using established EC lines have been shown to be reproducible and reliable. Until recently, most studies on brain microvasculature were extrapolated from assays performed on human umbilical vein endothelial cells (HUVEC) [154] or bovine aortic endothelial cells (BAEC) [205]. However, EC lines isolated from large vessels do not adequately reflect the phenotype of microvascular ECs [206]. Furthermore microvascular ECs vary from organ to organ and present species-specificity. Therefore, isolation and characterization of human cerebral

microvascular ECs was essential for the study of brain microvasculature. HBMEC have been isolated from a brain biopsy of a woman with epilepsy. The HBMEC were positive for factor VIII-Rag, carbonic anhydrase IV, Ulex Europeus Agglutinin I, took up fluorescently labeled acetylated low density lipoprotein and expressed gamma glutamyl transpeptidase, demonstrating their brain endothelial cell characteristics [206]. Their replicative senescence was bypassed by transformation with the viral oncogene SV40LT [165]. The major limitation of using HBMEC as a surrogate model surrogate model to approximate tumor-derived ECs resides in the fact that the microvascular endothelium of malignant gliomas has been shown to differ from normal brain endothelium. Recently, ECs have been isolated from glioblastoma specimens [76, 163]. Throughout the isolation process, different subfractions of EC have been separated depending on their antigen expression pattern [80]. This suggests the existence of different EC populations within the tumor, possibly each having specific functional properties. Isolated ECs adhered difficultly on culture plates, failed to proliferate easily *in vitro* and were sensitive to passaging [80]. Interestingly, authors also reported that cell culture and passaging influenced VE-cadherin, vWF and CD31 expression in EC isolated from gliomas [80]. Given the dynamic phenotype of isolated tumor ECs and their inherently limited proliferation potential, it has been recommended to use freshly isolated tumor EC rather than repeated passages [80]. This model may not be suitable for long-term *in vitro* studies requiring a homogeneous population from passage to passage to reproduce results.

Concerning *in vivo* models, no currently available animal model exactly simulates human brain tumor growth and angiogenesis. Orthotopic models have the

potential to give experimental intracerebral tumors that resemble glioblastoma invasion and angiogenesis. To date, no rat glioma model is an optimal model since the 9L cell line is not a glioma but rather a gliosarcoma and the C6 model has the unfortunate tendency to evoke a strong alloimmunogenic response, limiting rat survival [207]. Another option is xenografting human glioblastoma cells into immunocompromized hosts such as thymectomized, SCID, or *nu/nu* animals. In such models, the use of immunocompromized animals leads to a partially artificial proliferation and angiogenesis pattern, given the absence of tumor-host immunologic interaction. Furthermore, not all human glioma cells inoculated in animal cerebral parenchyma result in a tumor with glioblastomas' phenotype. The U-87 human glioblastoma cell line, for example, gives rise to tumors that develop a perivascular infiltration but not white matter infiltration as GBMs usually do [208]. Finally, models of spontaneous tumor formation in genetically engineered mice offer the opportunity to examine the pathophysiology of gliomas in immunocompetent models *in vivo*. However, in brain tumor modeling, authors have found that these tumor models are complicated by poor reproducibility, low tumor penetrance, prolonged tumor formation latency, and a need for *in vivo* imaging techniques [209]

Depending on the study's objectives, different model systems might be more or less suitable. Questions regarding EC cellular functions may be best answered by *in vitro* models. In the chapters III and IV, the HBMEC model has been used as a surrogate model to approximate tumor-derived ECs. The HBMEC model represents a stable and reliable *in vitro* model, maintaining both morphological and functional characteristics of brain ECs, as well as an increased proliferation rate due to their

transformation with the SV40-LT antigen [165]. Interestingly, transfection of tumor cell lines with SV40-LT has lead to the repression of tumor suppressor genes such as p53 and Rb. Also, VEGF expression was increased in SV40-LT transfected cells [210]. These modifications could contribute to regulate cellular proliferation and tumorigenesis [210]. Therefore, HBMEC immortalized with SV40-LT may have acquired a phenotype and genotype ressembling tumor-derived ECs. However, this specific comparison has not yet been performed.

Although one recognizes that this model does not exactly represent glioma-derived EC, it is to our knowledge the closest *in vitro* model that could approximate brain tumor-derived ECs for long term studies requiring phenotypically stable cell throughout the same passage and between passages. Depending on the experimental protocol, ECs might have been transfected and treated with EGCG +/-IR, and then submitted to various assays such as proliferation, cell cycle assays by FACS, migration, tubulogenesis. Endothelial cells isolated from the glioblastoma resected from a human or an animal in an orthotopic model might not be ideal for such research protocols given their difficulty to adhere, their limited proliferation rate and early senescence *in vitro,* and their heterogeneity as a whole and dynamic phenotype [80]. The conclusions of chapters II and III must be put into perspective given the inherent limitation of the HBMEC model as surrogate for tumor-derived EC and the absence of other flawless existing models.

5.2.3 Necrosis, an important goal of adjuvant therapies

In most cases, adjuvant therapies aim to kill residual tumor cells not resected during surgery. In response to a given toxic insult or physical damage there is often a continuum of apoptotic and non-apoptotic cell death mechanisms.

Apoptosis is described as a caspase-dependent active or programmed cell death characterized by nuclear condensation and fragmentation, membrane blebbing and formation of apoptotic bodies (Figure 1). Apoptotic cells can be cleared from the organism in the absence of inflammatory response. The late stage of apoptosis may be associated with secondary necrotic cell death [211].

Non-apoptotic cell death includes necrosis, mitotic cell death, autophagy and senescence [211]. Necrosis was initially considered as an unprogrammed default form of cell death but should be regarded as a regulated event displaying aspects of non-apoptotic programmed cellular suicide. Necrosis allows cells to actively recruit either a defensive or reparative response to damage. As such, programmed cell necrosis allows systems to give a warning that the integrity of the system as a whole is menaced. To do so, various proteins are released into the extracellular environment to trigger a host response. When the integrity of the plasma membrane is lost by damage to membrane lipids or loss of function of ion channels/pumps, the cells swell and rupture, releasing intracellular content in the microenvironment and subsequently inducing an inflammatory and immune response (Figure 1).

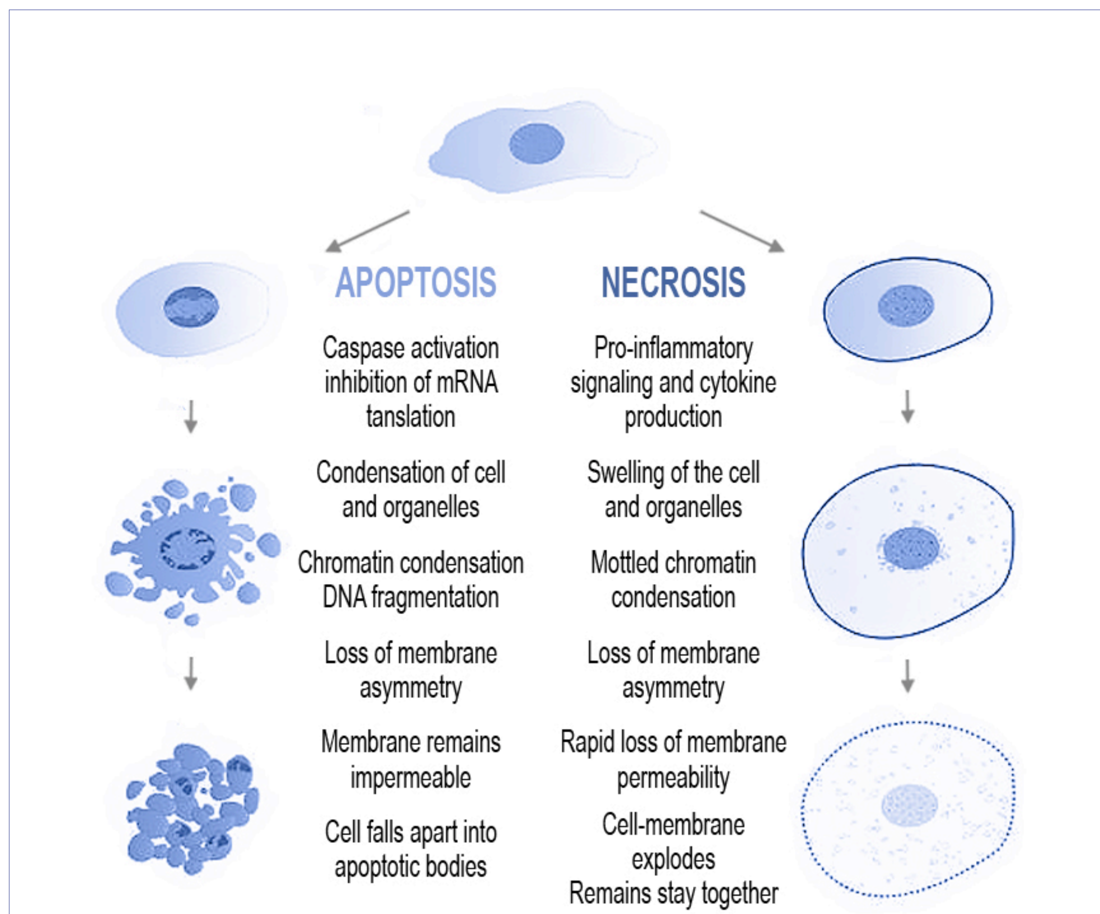

Figure 1. Differences between necrosis and apoptosis

Mitotic catastrophe refers to an aberrant mitosis which occurs when the cell cycle checkpoints are defective and cells enter mitosis prematurely before DNA damage has been repaired. This mode of cell death is preferentially seen in tumors with suppressed apoptotic pathways treated with DNA damaging treatments. Other than identification of multinucleated cells on histology, no specific in vitro or in vivo assays are available to document mitotic catastrophe [212].

Autophagy occurs in numerous stress conditions to degrade and recycle organelles and cytoplasmic proteins by lysosomal hydrolases. In periods of starvation, autophagy may enable cell survival by providing energy. When extreme situations exist, autophagy becomes a suicide mechanism by inducing digestion of essential cellular proteins and structures. In tumor cells, autophagy, as a cell death mechanism, may occur either in combination with or as a back-up of other deficient pathways, such as apoptosis. Autophagy is detected in tissues by electron microscopy, by immunoblotting-precipitation-fluorescence detection of microtubule-associated protein 1A/1B-light chain 3 on autophagosomes [212, 213] and by flow cytometry [214].

Senescence refers to a state of permanent loss of proliferative capacity. Replicative senescence is associated with aging and results from a progressive shortening of telomeres during cell division. Cells may also respond to certain types of DNA damaging stimuli by adopting a senescent phenotype [212]. Senescent cells undergo irreversible growth arrest and loose clonogenic potential, possibly representing an important component of tumor response to therapy [215]. Senescence is dectected in tissues by electron microscopy, by detecting the senescence-associated β-galactosidase by histochemical staining and by growth arrest assays [212].

IR is capable of activating numerous cell death pathways in numerous types of tumors [211, 216, 217]. Many factors may influence the balance between these mechanisms of cell death such as the radiation dose and timing of analysis after irradiation. Some authors report that rapid cell death occurs primarily through a non-apoptotic pathway [218] while others observe a dose-related increase in necrosis with

some delay after exposure [219]. The kinetics of appearance of both apoptotic and necrotic cells in irradiated tumors is most probably influenced by cell cycle progression and by cell death. In addition, combined treatment of cancer cells with chemotherapeutic agents and IR has resulted in an increase in the necrotic component of cell death [220]. For example, combined treatment of a melanoma human cell line with radiation and camptothecin, a topoisomerase I inhibitor with significant anticancer activities, induced significantly more necrotic than apoptotic cell death [220].

The development of resistance to apoptosis is an important step in carcinogenesis. Glioma cells present both important proliferation and neoangiogenesis processes and can actively migrate relatively long distances away from the tumor's primary site. Invasive malignant glioma cells show a decrease in their proliferation rates and a relative resistance to apoptosis compared to the highly cellular center of the tumor [221]. Reducing tumor cell's migration results in an increase of these cells' sensitivity to proapoptotic drugs [222]. Inhibiting the molecular pathways involved in apoptosis resistance and overexpressed in gliomas is an option to overcome malignant gliomas' resistance. Also, inducing other non-apoptotic cell death mechanisms such as necrosis or autophagy to kill tumor cells might be an appealing strategy to bypass the relative resistance to apoptosis [222].

Of the non-apoptotic cell death mechanisms, necrosis may be beneficial since it leads to a pro-inflammatory response. Depending on the activated signal-transduction pathway, tumor cells can express 'danger' and 'eat me' signals on the cell surface such as heat shock proteins or can release immunostimulatory factors such as

cytokines and high-mobility group box 1 to stimulate immune effectors [223]. Dexamethasone (DXM), a synthetic glucocorticoid, is commonly administered to reduce the vasogenic oedema and inflammatory reaction surrounding the glioblastoma [224]. DXM has also been reported to make GBM cells resistant to IR and chemotherapeutic agents that otherwise cause DNA damage [225, 226]. Most recently it has been found that pre-treatment of U87 cells with DXM blocked the temozolomide-induced apoptosis [227]. The exact mechanism of DXM mediated protection of glioblastoma cells from apoptosis remains unclear.

Therefore, should effective anticancer regiments be able to induce or potentiate non-apoptotic cell death pathways? For example, following IR exposure and administration of DNA-alkylating agents, DNA damage occurs to some extent. In the presence of moderate amounts of DNA damage, PARP-1 is thought to participate in the DNA repair process [228]. However, repair through poly (ADP-ribosyl)ation is an energetically expensive process. It results in a depletion of b-nicotinamide adenine dinucleotide (NAD) [229], failure of ATP production and, eventually, cell death [230]. Therefore PARP-1 activation by DNA-alkylating agents might induce necrosis by inducing energy failure [230]. Interestingly, in response to oxidative stress, PARP is also able to elicit ROS-induced autophagy that plays a prosurvival function. Indeed, autophagy induced in nutrient depletion or oxidative stress could produce the metabolic subtrates required to meet to the cells particular bioenergetic needs and prevent cell death [231]. However, induction of autophagy does not inevitably have a prosurvival function. Overexpression of autophagic proteins or radiation-induced inactivation of the mTOR pathway can lead to increased sensitivity to IR exposure

[232]. Therefore, tumor cells that have defective apoptotic mechanisms may respond to anticancer regiments by activating alternative non-apoptotic cell death mechanisms [232]. Treatments targeting tumor cells through alternative cell death mechanisms offer promising avenues to overcome cancer.

5.2.4 Nutritherapy as a complementary therapeutic modality

Population-based studies have suggested that green tea consumption is associated with reduced risk of several human malignancies [128, 233]. Indeed, green tea polyphenols' chemopreventive properties have been demonstrated in numerous target organs such as oesophagus, duodenum, large intestin, colon, liver, lung, mammary glands [233]. Of these, EGCG has been recognized as having many anticancer properties including inhibition of tumor cell growth [142, 234], cell cycle arrest [146, 235], induction of apoptosis [144, 234, 236], inhibition of invasion and metastasis [147] and inhibition of angiogenesis [151]. EGCG has been shown to sensitize various tumor cell lines and endothelial cell lines to radiation and to augment the effectiveness of radiotherapy [142, 219, 234]. EGCG has been recognized to have anti-inflammatory properties. Studies have shown that EGCG can regulate inflammatory cells' recrutement to damages or inflamed tissues [237], migration [238] and subsequently their infiltration into the sites of inflammation [239]. Also EGCG can down-regulate cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), two important enzymes that mediate inflammatory processes, through the suppression of NFkB activation [240]. However, the anti-inflammatory properties of EGCG have not been directly tested *in vivo* in gliomas given the relative protection of

the CNS from the immune system throughout the blood brain barrier. EGCG has also been recognized for antioxidant properties. It has been demonstrate that at low concentrations of EGCG, activation of MAPK leads to antioxydant-response element-mediated gene expression including detoxifying enzymes. At higher concentrations EGCG may induce a sustained activation of MAPKs such as JNK leading to apoptosis [241]. Others have demonstrated that at low concentrations, reactive oxygen species (ROS) scavenging activity of EGCG might predominate over its reducing power and lead to its protective effect on DNA. However, at higher concentrations, the relatively higher reductive power of EGCG may gradually predominate over its ROS scavenging activity and result in a pro-oxidant effect of EGCG on DNA [242]. The numerous anticancer and antiangiogenic effects of EGCG occur at variable doses, depending on the period of treatment with EGCG, concurrent treatments, and cells studied. In our experiences EGCG seemed to potentiate the cytotoxic effect of IR on malignant glioma and brain endothelial cells, maybe through its pro-oxidant effect or through other anticancer properties such as cell death.

Multiple signalling pathways have been found to be regulated by EGCG including: expression of VEGF, MMPs, uPA, EGFR, cell cycle regulatory proteins, NFkB, PI3-K/Akt, and Ras/Raf/MAPK. However, the exact molecular mechanism of EGCG is not completely understood. Studies have shown that the inhibitory effect of EGCG on tumor cell proliferation might be transduced through its binding to the 67-kDa laminin receptor (67LR) [160], a protein whose expression is strongly correlated with tumor aggressiveness [243]. Interestingly, studies have shown that through its

binding to the 67LR, EGCG may also reduce the phosphorylation of myosin II regulatory light chain, resulting in an increase in cells in the G_2/M phase of cell cycle and ultimately in an inhibition of cell growth [244]. More studies are required however.

Until now, the best way of assuring a regular intake of green tea polyphenols, especially EGCG, is through its daily consummation in tea. Although good habits are worth working for, it is recognized that chronic habits are difficult to change. Also, the quantity of EGCG extracted after infusion is dependent on numerous and difficult to control variables. A Phase 1 pharmacokynetic study has been conducted to determine the systemic availability of green tea catechins after single oral dose administration of EGCG and Polyphenon E, a decaffeinated green tea catechin mixture. Authors found that the two catechin formulations resulted in similar plasma EGCG levels and that the systemic availability of EGCG increased at higher doses, possibly due to saturable presystemic elimination of orally administered green tea polyphenols [233]. Importantly, there is evidence in animal models that EGCG is capable of penetrating through the blood brain barrier, although its brain distribution is less than the plasmatic distribution [245]. Another step towards human studies was the need for biomarkers representing green tea consumption. Wang et al analyzed green tea polyphenol components in plasma and urine samples collected from a phase II intervention trial carried out in 124 healthy adults receiving 500- or 1000-mg green tea polyphenols or placebo for 3 months [246]. A significant dose-dependent elevation was found for ECG and EGCG concentrations in plasma at both 1-month and 3-months after intervention with green tea polyphenols, rendering them reliable

biomarkers for green tea consumption at the population level [246]. Results of the interventional trial are not yet published however.

Another promising avenue is the possible combination of nutrients to optimize their individual anticancer properties. Since EGCG pretreatment potentiates IR effects' on glioblastoma cells, could adding nutrients with anticancerous properties optimize the effectiveness of this combined treatment? U-87 human glioblastoma cells were treated with EGCg (10μM) and/or Curcumin (1μM) for 6 hours. Irradiated cells were exposed to a single 3 Gy treatment with a linear accelerator. After irradiation, MEM containing 20% FBS was added and cells were left to recuperate for 18 hours. Non-treated control cells were handled similarly to the cells subjected to EGCG and/or Curcumin and/or IR. Cell death was assessed 18 hours after treatments and cell growth was assessed after 48 hours as described in the methodology section of chapters II, III and IV. EGCG and Curcumin individually induced U-87 cell death (Figure 2) [246]. Furthermore, the combined pretreatment with EGCG and Curcumin followed by IR significantly decreased growth of cancerous cells. Therefore, pretreatment with a combination of nutrients might enable lowering IR doses without compromising the anticancerous effect of treatment. There is promise that by integrating nutritherapy in the global management of cancer that we might succeed in decreasing treatment regiments that are less tolerated by patients, improve their quality of life by decreasing adverse effects, and above all, increase their survival.

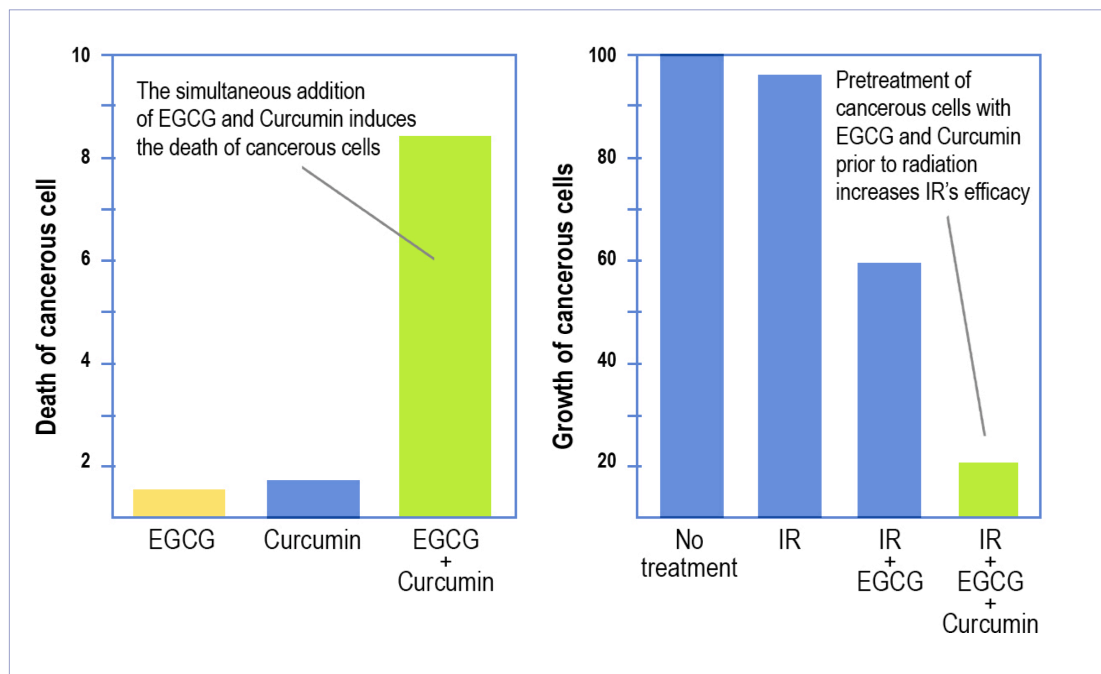

Figure 2. Reproduced from [247].

5.3 Conclusion

The first goal of this doctoral project was to determine if EGCG, in synergy with radiotherapy, can sensitize GBM's response to radiation and whether specific molecular markers are involved. We documented that U-87 cells were relatively radioresistant and that Survivin, in conjunction with RhoA signaling, may be implicated in GBM's radioresistance. Also we found that pre-treatment of U-87 cells with EGCG could overcome the cytoprotective effect of Survivin overexpression and potentiate the cytoreductive effect of IR.

The second goal was to characterize the impact of IR on HBMEC survival and to determine whether EGCG, also recognized for its anti-angiogenic properties, could optimize this effect. We found that although EGCG treatment and IR individually decreased HBMEC survival, the combined treatment synergistically reduced survival. We documented that the combined treatment increased cell death, more specifically necrosis.

The third goal was to investigate impact of IR exposure on the angiogenic functions i.e. cell proliferation, migration in response to brain tumor-derived growth factors, and capacity for tubulogenesis of surviving human brain tumor-derived ECs. The Rho signalling pathway was also investigated in relation to the functional properties of radioresistant HBMEC. Our data suggest that IR significantly alters crucial steps of angiogenesis, namely cell proliferation, migration and tubulogenesis in radioresistant HBMEC. Response to growth factors important for tumor expansion and angiogenesis is significantly attenuated in radioresistant HBMEC.

In conclusion, this doctoral project confirmed IR's cytoreductive properties on malignant gliomas and proposed a novel mechanism to explain GBMs' radioresistance. The thesis documented for the first time IR's cytotoxic effect in human brain endothelial cells. It also described the existence of radioresistant HBMEC and characterized their altered angiogenic functions. The thesis documented that the combination of natural anti-cancerous and antiangiogenic molecules such as EGCG with radiotherapy could improve IR's effect on human malignant glioma cells and tumor-derived ECs, especially through increased necrosis for HBMEC. The present thesis supports integrating nutrients with anticancerous and antiangiogenic properties, such as EGCG, in the management of gliomas to sensitize tumor cells and tumor-associated endothelial cells to conventional therapies.

CHAPTER VI BIBLIOGRAPHY

1. Martin, J.H., ed. *Neuroanatomy - Text and Atlas*. Second edition ed. 1996, Appleton & Lange: Stamford. 578.

2. Winn, H.R., ed. *Youmans Neurological Surgery*. Fifth edition ed. Oncology, ed. H.a.S.R. Brem. Vol. 1. 2004, Saunders: Philadelphia. 1457.

3. Oldendorf, W.H. and E.M. Cornford, *A comparison of total body and local spinal cord irradiation in experimental allergic encephalomyelitis.* J Neuropathol Exp Neurol, 1977. **36**(1): p. 50-61.

4. Surawicz, T.S., et al., *Descriptive epidemiology of primary brain and CNS tumors: results from the Central Brain Tumor Registry of the United States, 1990-1994.* Neuro Oncol, 1999. **1**(1): p. 14-25.

5. Yates, A.J., *An overview of principles for classifying brain tumors.* Mol Chem Neuropathol, 1992. **17**(2): p. 103-20.

6. Louis, D.N., et al., *The 2007 WHO classification of tumours of the central nervous system.* Acta Neuropathol, 2007. **114**(2): p. 97-109.

7. Collins, V.P., *Brain tumours: classification and genes.* J Neurol Neurosurg Psychiatry, 2004. **75 Suppl 2**: p. ii2-11.

8. Daumas-Duport, C., et al., *Grading of astrocytomas. A simple and reproducible method.* Cancer, 1988. **62**(10): p. 2152-65.

9. Ellison D, L.S., ed. *Neuropathology - A reference text of CNS pathology*. First edition ed. 1998, Mosby: Barcelona.

10. Norden, A.D. and P.Y. Wen, *Glioma therapy in adults.* Neurologist, 2006. **12**(6): p. 279-92.

11. Kraus, J.A., et al., *Shared allelic losses on chromosomes 1p and 19q suggest a common origin of oligodendroglioma and oligoastrocytoma.* J Neuropathol Exp Neurol, 1995. **54**(1): p. 91-5.

12. Biernat, W., et al., *Identical mutations of the p53 tumor suppressor gene in the gliomatous and the sarcomatous components of gliosarcomas suggest a common origin from glial cells.* J Neuropathol Exp Neurol, 1995. **54**(5): p. 651-6.

13. Li, L. and T. Xie, *Stem cell niche: structure and function.* Annu Rev Cell Dev Biol, 2005. **21**: p. 605-31.

14. Li, L. and W.B. Neaves, *Normal stem cells and cancer stem cells: the niche matters.* Cancer Res, 2006. **66**(9): p. 4553-7.

15. Singh, S. and P.B. Dirks, *Brain tumor stem cells: identification and concepts.* Neurosurg Clin N Am, 2007. **18**(1): p. 31-8, viii.

16. Yuan, X., et al., *Isolation of cancer stem cells from adult glioblastoma multiforme.* Oncogene, 2004. **23**(58): p. 9392-400.

17. Beier, D., et al., *CD133(+) and CD133(-) glioblastoma-derived cancer stem cells show differential growth characteristics and molecular profiles.* Cancer Res, 2007. **67**(9): p. 4010-5.

18. Hunter, K.E. and M.E. Hatten, *Radial glial cell transformation to astrocytes is bidirectional: regulation by a diffusible factor in embryonic forebrain.* Proc Natl Acad Sci U S A, 1995. **92**(6): p. 2061-5.

19. Jemal, A., et al., *Cancer statistics, 2002.* CA Cancer J Clin, 2002. **52**(1): p. 23-47.

20. Wrensch, M., et al., *Epidemiology of primary brain tumors: current concepts and review of the literature.* Neuro Oncol, 2002. **4**(4): p. 278-99.

21. Grossman, S.A., et al., *Central nervous system cancers in first-degree relatives and spouses.* Cancer Invest, 1999. **17**(5): p. 299-308.

22. Sanson M, T.S., *Données générales de neuro-oncogenèse.* Encyclopédie Médico-Chirurgicale, 2004. **17**(205): p. 1-10.

23. Hodges, L.C., et al., *Prevalence of glioblastoma multiforme in subjects with prior therapeutic radiation.* J Neurosci Nurs, 1992. **24**(2): p. 79-83.

24. Brat, D.J., et al., *Molecular genetic alterations in radiation-induced astrocytomas.* Am J Pathol, 1999. **154**(5): p. 1431-8.

25. Simmons, N.E. and E.R. Laws, Jr., *Glioma occurrence after sellar irradiation: case report and review.* Neurosurgery, 1998. **42**(1): p. 172-8.

26. Sankaranarayanan, K. and R. Chakraborty, *Impact of cancer predisposition and radiosensitivity on the population risk of radiation-induced cancers.* Radiat Res, 2001. **156**(5 Pt 2): p. 648-56.

27. Wrensch, M., et al., *Are prior head injuries or diagnostic X-rays associated with glioma in adults? The effects of control selection bias.* Neuroepidemiology, 2000. **19**(5): p. 234-44.

28. Tortosa, A., et al., *Prognostic implication of clinical, radiologic, and pathologic features in patients with anaplastic gliomas.* Cancer, 2003. **97**(4): p. 1063-71.

29. Price, A.C., et al., *Primary glioma: diagnosis with magnetic resonance imaging.* J Comput Tomogr, 1986. **10**(4): p. 325-34.

30. Kelly, P.J., et al., *Imaging-based stereotaxic serial biopsies in untreated intracranial glial neoplasms.* J Neurosurg, 1987. **66**(6): p. 865-74.

31. Osborn, A., ed. *Diagnostic Imaging - Brain.* ed. S.K. Blaser SI, Katzman GL, Provenzale J, Castillo M, Hedlund GL, Illner A, Harnsberger HR, Cooper JA, Jones BV, Hamilton BE 2004, Amirsys: Altona.

32. Bagley, L.J., et al., *Gliomas: correlation of magnetic susceptibility artifact with histologic grade.* Radiology, 1997. **202**(2): p. 511-6.

33. Watanabe, M., R. Tanaka, and N. Takeda, *Magnetic resonance imaging and histopathology of cerebral gliomas.* Neuroradiology, 1992. **34**(6): p. 463-9.

34. Earnest, F.t., et al., *Cerebral astrocytomas: histopathologic correlation of MR and CT contrast enhancement with stereotactic biopsy.* Radiology, 1988. **166**(3): p. 823-7.

35. Kleihues, P. and H. Ohgaki, *Primary and secondary glioblastomas: from concept to clinical diagnosis.* Neuro Oncol, 1999. **1**(1): p. 44-51.

36. Watanabe, K., et al., *Overexpression of the EGF receptor and p53 mutations are mutually exclusive in the evolution of primary and secondary glioblastomas.* Brain Pathol, 1996. **6**(3): p. 217-23; discussion 23-4.

37. Ohgaki, H. and P. Kleihues, *Genetic pathways to primary and secondary glioblastoma.* Am J Pathol, 2007. **170**(5): p. 1445-53.

38. Reardon, D.A. and P.Y. Wen, *Therapeutic advances in the treatment of glioblastoma: rationale and potential role of targeted agents.* Oncologist, 2006. **11**(2): p. 152-64.

39. Lassman, A.B., *Molecular biology of gliomas.* Curr Neurol Neurosci Rep, 2004. **4**(3): p. 228-33.

40. Hlobilkova, A., et al., *Could changes in the regulation of the PI3K/PKB/Akt signaling pathway and cell cycle be involved in astrocytic tumor pathogenesis and progression?* Neoplasma, 2007. **54**(4): p. 334-41.

41. Yahanda, A.M., et al., *Astrocytes derived from p53-deficient mice provide a multistep in vitro model for development of malignant gliomas.* Mol Cell Biol, 1995. **15**(8): p. 4249-59.

42. Altieri, D.C., *Survivin, versatile modulation of cell division and apoptosis in cancer.* Oncogene, 2003. **22**(53): p. 8581-9.

43. Shin, S., et al., *An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and -7.* Biochemistry, 2001. **40**(4): p. 1117-23.

44. Conway, E.M., et al., *Three differentially expressed survivin cDNA variants encode proteins with distinct antiapoptotic functions.* Blood, 2000. **95**(4): p. 1435-42.

45. Tamm, I., et al., *IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs.* Cancer Res, 1998. **58**(23): p. 5315-20.

46. Grossman, D., et al., *Transgenic expression of survivin in keratinocytes counteracts UVB-induced apoptosis and cooperates with loss of p53.* J Clin Invest, 2001. **108**(7): p. 991-9.

47. Croci, D.O., et al., *Silencing survivin gene expression promotes apoptosis of human breast cancer cells through a caspase-independent pathway.* J Cell Biochem, 2008.

48. Kang, D.W., et al., *Ciglitazone induces caspase-independent apoptosis through down-regulation of XIAP and survivin in human glioma cells.* Neurochem Res, 2008. **33**(3): p. 551-61.

49. Li, F., et al., *Control of apoptosis and mitotic spindle checkpoint by survivin.* Nature, 1998. **396**(6711): p. 580-4.

50. Temme, A., et al., *Localization, dynamics, and function of survivin revealed by expression of functional survivinDsRed fusion proteins in the living cell.* Mol Biol Cell, 2003. **14**(1): p. 78-92.

51. Wheatley, S.P., et al., *INCENP is required for proper targeting of Survivin to the centromeres and the anaphase spindle during mitosis.* Curr Biol, 2001. **11**(11): p. 886-90.

52. Blanc-Brude, O.P., et al., *Therapeutic targeting of the survivin pathway in cancer: initiation of mitochondrial apoptosis and suppression of tumor-associated angiogenesis.* Clin Cancer Res, 2003. **9**(7): p. 2683-92.

53. O'Connor, D.S., et al., *Control of apoptosis during angiogenesis by survivin expression in endothelial cells.* Am J Pathol, 2000. **156**(2): p. 393-8.

54. Ricci, M.S. and W.X. Zong, *Chemotherapeutic approaches for targeting cell death pathways.* Oncologist, 2006. **11**(4): p. 342-57.

55. Chaudhry, I.H., et al., *Vascular endothelial growth factor expression correlates with tumour grade and vascularity in gliomas.* Histopathology, 2001. **39**(4): p. 409-15.

56. Zadeh, G. and A. Guha, *Angiogenesis in nervous system disorders.* Neurosurgery, 2003. **53**(6): p. 1362-74; discussion 1374-6.

57. Bergers, G. and L.E. Benjamin, *Tumorigenesis and the angiogenic switch.* Nat Rev Cancer, 2003. **3**(6): p. 401-10.

58. Hristov, M. and C. Weber, *Endothelial progenitor cells in vascular repair and remodeling.* Pharmacol Res, 2008.

59. Asahara, T. and A. Kawamoto, *Endothelial progenitor cells for postnatal vasculogenesis.* Am J Physiol Cell Physiol, 2004. **287**(3): p. C572-9.

60. Holash, J., et al., *Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF.* Science, 1999. **284**(5422): p. 1994-8.

61. Kaur, B., et al., *Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis.* Neuro Oncol, 2005. **7**(2): p. 134-53.

62. Sun, L., et al., *Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain.* Cancer Cell, 2006. **9**(4): p. 287-300.

63. Folkman, J., *The role of angiogenesis in tumor growth.* Semin Cancer Biol, 1992. **3**(2): p. 65-71.

64. Waeber, C., N. Blondeau, and S. Salomone, *Vascular sphingosine-1-phosphate S1P1 and S1P3 receptors.* Drug News Perspect, 2004. **17**(6): p. 365-82.

65. Auguste, P., et al., *Molecular mechanisms of tumor vascularization.* Crit Rev Oncol Hematol, 2005. **54**(1): p. 53-61.

66. Dome, B., et al., *Alternative vascularization mechanisms in cancer: Pathology and therapeutic implications.* Am J Pathol, 2007. **170**(1): p. 1-15.

67. Hendrix, M.J., et al., *Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma.* Nat Rev Cancer, 2003. **3**(6): p. 411-21.

68. Maniotis, A.J., et al., *Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry.* Am J Pathol, 1999. **155**(3): p. 739-52.

69. Rojiani, A.M. and K. Dorovini-Zis, *Glomeruloid vascular structures in glioblastoma multiforme: an immunohistochemical and ultrastructural study.* J Neurosurg, 1996. **85**(6): p. 1078-84.

70. Brat, D.J. and E.G. Van Meir, *Glomeruloid microvascular proliferation orchestrated by VPF/VEGF: a new world of angiogenesis research.* Am J Pathol, 2001. **158**(3): p. 789-96.

71. Preusser, M., P. Birner, and J.A. Hainfellner, *Algorithm for the standardized assessment of vascular patterns in glioblastoma specimens.* Clin Neuropathol, 2004. **23**(5): p. 238-40.

72. Sharma, S., et al., *Angiogenic patterns and their quantitation in high grade astrocytic tumors.* J Neurooncol, 2006. **79**(1): p. 19-30.

73. Less, J.R., et al., *Microvascular architecture in a mammary carcinoma: branching patterns and vessel dimensions.* Cancer Res, 1991. **51**(1): p. 265-73.

74. Papadopoulos, M.C., et al., *Emerging molecular mechanisms of brain tumour oedema.* Br J Neurosurg, 2001. **15**(2): p. 101-8.

75. Charalambous, C., T.C. Chen, and F.M. Hofman, *Characteristics of tumor-associated endothelial cells derived from glioblastoma multiforme.* Neurosurg Focus, 2006. **20**(4): p. E22.

76. Charalambous, C., F.M. Hofman, and T.C. Chen, *Functional and phenotypic differences between glioblastoma multiforme-derived and normal human brain endothelial cells.* J Neurosurg, 2005. **102**(4): p. 699-705.

77. Birner, P., et al., *Vascular patterns in glioblastoma influence clinical outcome and associate with variable expression of angiogenic proteins: evidence for distinct angiogenic subtypes.* Brain Pathol, 2003. **13**(2): p. 133-43.

78. Preusser, M., et al., *Histopathologic assessment of hot-spot microvessel density and vascular patterns in glioblastoma: Poor observer agreement limits clinical utility as prognostic factors: a translational research project of the European Organization for Research and Treatment of Cancer Brain Tumor Group.* Cancer, 2006. **107**(1): p. 162-70.

79. Leon, S.P., R.D. Folkerth, and P.M. Black, *Microvessel density is a prognostic indicator for patients with astroglial brain tumors.* Cancer, 1996. **77**(2): p. 362-72.

80. Miebach, S., et al., *Isolation and culture of microvascular endothelial cells from gliomas of different WHO grades.* J Neurooncol, 2006. **76**(1): p. 39-48.

81. Papadopoulos, M.C., et al., *Occludin expression in microvessels of neoplastic and non-neoplastic human brain.* Neuropathol Appl Neurobiol, 2001. **27**(5): p. 384-95.

82. Giese, A., et al., *Dichotomy of astrocytoma migration and proliferation.* Int J Cancer, 1996. **67**(2): p. 275-82.

83. Charalambous, C., et al., *Interleukin-8 differentially regulates migration of tumor-associated and normal human brain endothelial cells.* Cancer Res, 2005. **65**(22): p. 10347-54.

84. Alessandri, G., et al., *Phenotypic and functional characteristics of tumour-derived microvascular endothelial cells.* Clin Exp Metastasis, 1999. **17**(8): p. 655-62.

85. Tozer, G.M., C. Kanthou, and B.C. Baguley, *Disrupting tumour blood vessels.* Nat Rev Cancer, 2005. **5**(6): p. 423-35.

86. Tuettenberg, J., C. Friedel, and P. Vajkoczy, *Angiogenesis in malignant glioma--a target for antitumor therapy?* Crit Rev Oncol Hematol, 2006. **59**(3): p. 181-93.

87. Scott, J.N., et al., *How often are nonenhancing supratentorial gliomas malignant? A population study.* Neurology, 2002. **59**(6): p. 947-9.

88. Brandes, A.A., *State-of-the-art treatment of high-grade brain tumors.* Semin Oncol, 2003. **30**(6 Suppl 19): p. 4-9.

89. Willems, P.W., et al., *Effectiveness of neuronavigation in resecting solitary intracerebral contrast-enhancing tumors: a randomized controlled trial.* J Neurosurg, 2006. **104**(3): p. 360-8.

90. Wood, J.R., S.B. Green, and W.R. Shapiro, *The prognostic importance of tumor size in malignant gliomas: a computed tomographic scan study by the Brain Tumor Cooperative Group.* J Clin Oncol, 1988. **6**(2): p. 338-43.

91. Keles, G.E., et al., *Volume of residual disease as a predictor of outcome in adult patients with recurrent supratentorial glioblastomas multiforme who are undergoing chemotherapy.* J Neurosurg, 2004. **100**(1): p. 41-6.

92. Crocker, I.R. and Y. Popowski, *Basic physics and biology of radiation therapy.* Semin Interv Cardiol, 1997. **2**(2): p. 89-94.

93. Bernard, A. and J. Bourhis, *[Radiotherapy. Radiobiological aspects, principal secondary effects].* Rev Prat, 2001. **51**(11): p. 1265-71.

94. Dunne-Daly, C.F., *Principles of radiotherapy and radiobiology.* Semin Oncol Nurs, 1999. **15**(4): p. 250-9.

95. Bolus, N.E., *Basic review of radiation biology and terminology.* J Nucl Med Technol, 2001. **29**(2): p. 67-73; test 76-7.

96. Hill, M.A., *Radiation damage to DNA: the importance of track structure.* Radiat Meas, 1999. **31**(1-6): p. 15-23.

97. Fine, H.A., et al., *Meta-analysis of radiation therapy with and without adjuvant chemotherapy for malignant gliomas in adults.* Cancer, 1993. **71**(8): p. 2585-97.

98. Gupta, T. and K. Dinshaw, *Modified optimal fractionation for poor prognosis malignant gliomas: an elusive search.* Acta Oncol, 2005. **44**(2): p. 105-13.

99. Souhami, L., et al., *Randomized comparison of stereotactic radiosurgery followed by conventional radiotherapy with carmustine to conventional radiotherapy with carmustine for patients with glioblastoma multiforme: report of Radiation Therapy Oncology Group 93-05 protocol.* Int J Radiat Oncol Biol Phys, 2004. **60**(3): p. 853-60.

100. Akabani, G., et al., *Dosimetry and radiographic analysis of 131I-labeled anti-tenascin 81C6 murine monoclonal antibody in newly diagnosed patients with malignant gliomas: a phase II study.* J Nucl Med, 2005. **46**(6): p. 1042-51.

101. Wild-Bode, C., et al., *Sublethal irradiation promotes migration and invasiveness of glioma cells: implications for radiotherapy of human glioblastoma.* Cancer Res, 2001. **61**(6): p. 2744-50.

102. Cordes, N., et al., *Irradiation differentially affects substratum-dependent survival, adhesion, and invasion of glioblastoma cell lines.* Br J Cancer, 2003. **89**(11): p. 2122-32.

103. Little, J.B., *Genomic instability and bystander effects: a historical perspective.* Oncogene, 2003. **22**(45): p. 6978-87.

104. Chakravarti, A., et al., *Survivin enhances radiation resistance in primary human glioblastoma cells via caspase-independent mechanisms.* Oncogene, 2004. **23**(45): p. 7494-506.

105. Kajiwara, Y., et al., *Expression of survivin in astrocytic tumors: correlation with malignant grade and prognosis.* Cancer, 2003. **97**(4): p. 1077-83.

106. Chakravarti, A., et al., *Quantitatively determined survivin expression levels are of prognostic value in human gliomas.* J Clin Oncol, 2002. **20**(4): p. 1063-8.

107. Ader, I., et al., *RhoB controls the 24 kDa FGF-2-induced radioresistance in HeLa cells by preventing post-mitotic cell death.* Oncogene, 2002. **21**(39): p. 5998-6006.

108. Ader, I., et al., *Inhibition of Rho pathways induces radiosensitization and oxygenation in human glioblastoma xenografts.* Oncogene, 2003. **22**(55): p. 8861-9.

109. Forget, M.A., et al., *The expression of rho proteins decreases with human brain tumor progression: potential tumor markers.* Clin Exp Metastasis, 2002. **19**(1): p. 9-15.

110. Gingras, D., et al., *Localization of RhoA GTPase to endothelial caveolae-enriched membrane domains.* Biochem Biophys Res Commun, 1998. **247**(3): p. 888-93.

111. Regina, A., et al., *Down-regulation of caveolin-1 in glioma vasculature: modulation by radiotherapy.* J Neurosci Res, 2004. **75**(2): p. 291-9.

112. Chalmers, A.J., *Radioresistant glioma stem cells--therapeutic obstacle or promising target?* DNA Repair (Amst), 2007. **6**(9): p. 1391-4.

113. Fortin, D., et al., *Enhanced chemotherapy delivery by intraarterial infusion and blood-brain barrier disruption in malignant brain tumors: the Sherbrooke experience.* Cancer, 2005. **103**(12): p. 2606-15.

114. Toth, K., et al., *MDR1 P-glycoprotein is expressed by endothelial cells of newly formed capillaries in human gliomas but is not expressed in the neovasculature of other primary tumors.* Am J Pathol, 1996. **149**(3): p. 853-8.

115. Demeule, M., et al., *Brain endothelial cells as pharmacological targets in brain tumors.* Mol Neurobiol, 2004. **30**(2): p. 157-83.

116. Hegi, M.E., et al., *MGMT gene silencing and benefit from temozolomide in glioblastoma.* N Engl J Med, 2005. **352**(10): p. 997-1003.

117. Hegi, M.E., et al., *Clinical trial substantiates the predictive value of O-6-methylguanine-DNA methyltransferase promoter methylation in glioblastoma patients treated with temozolomide.* Clin Cancer Res, 2004. **10**(6): p. 1871-4.

118. Esteller, M., et al., *Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents.* N Engl J Med, 2000. **343**(19): p. 1350-4.

119. Lanzetta, G., et al., *Temozolomide in radio-chemotherapy combined treatment for newly-diagnosed glioblastoma multiforme: phase II clinical trial.* Anticancer Res, 2003. **23**(6D): p. 5159-64.

120. Scott, J.N., et al., *Which glioblastoma multiforme patient will become a long-term survivor? A population-based study.* Ann Neurol, 1999. **46**(2): p. 183-8.

121. Tentori, L. and G. Graziani, *Pharmacological strategies to increase the antitumor activity of methylating agents.* Curr Med Chem, 2002. **9**(13): p. 1285-301.

122. Bodell, W.J., et al., *Levels of N7-(2-hydroxyethyl)guanine as a molecular dosimeter of drug delivery to human brain tumors.* Neuro Oncol, 2001. **3**(4): p. 241-5.

123. Mathieu, D. and D. Fortin, *The role of chemotherapy in the treatment of malignant astrocytomas.* Can J Neurol Sci, 2006. **33**(2): p. 127-40.

124. Stupp, R., et al., *Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma.* N Engl J Med, 2005. **352**(10): p. 987-96.

125. *Randomized trial of procarbazine, lomustine, and vincristine in the adjuvant treatment of high-grade astrocytoma: a Medical Research Council trial.* J Clin Oncol, 2001. **19**(2): p. 509-18.

126. Boyle, F.M., S.L. Eller, and S.A. Grossman, *Penetration of intra-arterially administered vincristine in experimental brain tumor.* Neuro Oncol, 2004. **6**(4): p. 300-5.

127. Bello, L., et al., *Low-dose chemotherapy combined with an antiangiogenic drug reduces human glioma growth in vivo.* Cancer Res, 2001. **61**(20): p. 7501-6.

128. Doll, R. and R. Peto, *The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today.* J Natl Cancer Inst, 1981. **66**(6): p. 1191-308.

129. Key, T.J., et al., *The effect of diet on risk of cancer.* Lancet, 2002. **360**(9336): p. 861-8.

130. Anand, P., et al., *Cancer is a preventable disease that requires major lifestyle changes.* Pharm Res, 2008. **25**(9): p. 2097-116.

131. Khan, N., et al., *Targeting multiple signaling pathways by green tea polyphenol (-)-epigallocatechin-3-gallate.* Cancer Res, 2006. **66**(5): p. 2500-5.

132. Milner, J.A., *Molecular targets for bioactive food components.* J Nutr, 2004. **134**(9): p. 2492S-2498S.

133. Block, G., B. Patterson, and A. Subar, *Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence.* Nutr Cancer, 1992. **18**(1): p. 1-29.

134. Schweigert, F.J., *Nutritional proteomics: methods and concepts for research in nutritional science.* Ann Nutr Metab, 2007. **51**(2): p. 99-107.

135. Afman, L. and M. Muller, *Nutrigenomics: from molecular nutrition to prevention of disease.* J Am Diet Assoc, 2006. **106**(4): p. 569-76.

136. Nichenametla, S.N., et al., *A review of the effects and mechanisms of polyphenolics in cancer.* Crit Rev Food Sci Nutr, 2006. **46**(2): p. 161-83.

137. Zaveri, N.T., *Green tea and its polyphenolic catechins: medicinal uses in cancer and noncancer applications.* Life Sci, 2006. **78**(18): p. 2073-80.

138. Mandel, S. and M.B. Youdim, *Catechin polyphenols: neurodegeneration and neuroprotection in neurodegenerative diseases.* Free Radic Biol Med, 2004. **37**(3): p. 304-17.

139. Sano, J., et al., *Effects of green tea intake on the development of coronary artery disease.* Circ J, 2004. **68**(7): p. 665-70.

140. Carlson, J.R., et al., *Reading the tea leaves: anticarcinogenic properties of (-)-epigallocatechin-3-gallate.* Mayo Clin Proc, 2007. **82**(6): p. 725-32.

141. Demeule, M., et al., *Green tea catechins as novel antitumor and antiangiogenic compounds.* Curr Med Chem Anticancer Agents, 2002. **2**(4): p. 441-63.

142. McLaughlin, N., et al., *The Survivin-mediated radioresistant phenotype of glioblastomas is regulated by RhoA and inhibited by the green tea polyphenol (-)-epigallocatechin-3-gallate.* Brain Res, 2006. **1071**(1): p. 1-9.

143. Leone, M., et al., *Cancer prevention by tea polyphenols is linked to their direct inhibition of antiapoptotic Bcl-2-family proteins.* Cancer Res, 2003. **63**(23): p. 8118-21.

144. Yokoyama, S., et al., *Inhibitory effect of epigallocatechin-gallate on brain tumor cell lines in vitro.* Neuro Oncol, 2001. **3**(1): p. 22-8.

145. Shankar, S., G. Suthakar, and R.K. Srivastava, *Epigallocatechin-3-gallate inhibits cell cycle and induces apoptosis in pancreatic cancer.* Front Biosci, 2007. **12**: p. 5039-51.

146. Gupta, S., T. Hussain, and H. Mukhtar, *Molecular pathway for (-)-epigallocatechin-3-gallate-induced cell cycle arrest and apoptosis of human prostate carcinoma cells.* Arch Biochem Biophys, 2003. **410**(1): p. 177-85.

147. Annabi, B., et al., *Probing the infiltrating character of brain tumors: inhibition of RhoA/ROK-mediated CD44 cell surface shedding from glioma cells by the green tea catechin EGCg.* J Neurochem, 2005. **94**(4): p. 906-16.

148. Ho, Y.C., et al., *Epigallocatechin-3-gallate inhibits the invasion of human oral cancer cells and decreases the productions of matrix metalloproteinases and urokinase-plasminogen activator.* J Oral Pathol Med, 2007. **36**(10): p. 588-93.

149. Tosetti, F., et al., *Angioprevention': angiogenesis is a common and key target for cancer chemopreventive agents.* FASEB J, 2002. **16**(1): p. 2-14.

150. Masuda, M., et al., *Epigallocatechin-3-gallate decreases VEGF production in head and neck and breast carcinoma cells by inhibiting EGFR-related pathways of signal transduction.* J Exp Ther Oncol, 2002. **2**(6): p. 350-9.

151. Kojima-Yuasa, A., et al., *Green tea extract inhibits angiogenesis of human umbilical vein endothelial cells through reduction of expression of VEGF receptors.* Life Sci, 2003. **73**(10): p. 1299-313.

152. Lamy, S., D. Gingras, and R. Beliveau, *Green tea catechins inhibit vascular endothelial growth factor receptor phosphorylation.* Cancer Res, 2002. **62**(2): p. 381-5.

153. Jung, Y.D., et al., *EGCG, a major component of green tea, inhibits tumour growth by inhibiting VEGF induction in human colon carcinoma cells.* Br J Cancer, 2001. **84**(6): p. 844-50.

154. Annabi, B., et al., *Radiation induced-tubulogenesis in endothelial cells is antagonized by the antiangiogenic properties of green tea polyphenol (-) epigallocatechin-3-gallate.* Cancer Biol Ther, 2003. **2**(6): p. 642-9.

155. Frei, B. and J.V. Higdon, *Antioxidant activity of tea polyphenols in vivo: evidence from animal studies.* J Nutr, 2003. **133**(10): p. 3275S-84S.

156. Azam, S., et al., *Prooxidant property of green tea polyphenols epicatechin and epigallocatechin-3-gallate: implications for anticancer properties.* Toxicol In Vitro, 2004. **18**(5): p. 555-61.

157. Kanadzu, M., Y. Lu, and K. Morimoto, *Dual function of (--)-epigallocatechin gallate (EGCG) in healthy human lymphocytes.* Cancer Lett, 2006. **241**(2): p. 250-5.

158. Yamamoto, T., et al., *Green tea polyphenol causes differential oxidative environments in tumor versus normal epithelial cells.* J Pharmacol Exp Ther, 2003. **307**(1): p. 230-6.

159. Liang, Y.C., et al., *Suppression of extracellular signals and cell proliferation through EGF receptor binding by (-)-epigallocatechin gallate in human A431 epidermoid carcinoma cells.* J Cell Biochem, 1997. **67**(1): p. 55-65.

160. Tachibana, H., et al., *A receptor for green tea polyphenol EGCG.* Nat Struct Mol Biol, 2004. **11**(4): p. 380-1.

161. Hess, C.F., et al., *Malignant glioma: patterns of failure following individually tailored limited volume irradiation.* Radiother Oncol, 1994. **30**(2): p. 146-9.

162. Leith, J.T., et al., *Intrinsic and extrinsic characteristics of human tumors relevant to radiosurgery: comparative cellular radiosensitivity and hypoxic percentages.* Acta Neurochir Suppl, 1994. **62**: p. 18-27.

163. Bian, X.W., et al., *Increased angiogenic capabilities of endothelial cells from microvessels of malignant human gliomas.* Int Immunopharmacol, 2006. **6**(1): p. 90-9.

164. Bian, C., et al., *Immortalization of human umbilical vein endothelial cells with telomerase reverse transcriptase and simian virus 40 large T antigen.* J Zhejiang Univ Sci B, 2005. **6**(7): p. 631-6.

165. Greiffenberg, L., et al., *Interaction of Listeria monocytogenes with human brain microvascular endothelial cells: InlB-dependent invasion, long-term intracellular growth, and spread from macrophages to endothelial cells.* Infect Immun, 1998. **66**(11): p. 5260-7.

166. Fritz, G., B. Kaina, and K. Aktories, *The ras-related small GTP-binding protein RhoB is immediate-early inducible by DNA damaging treatments.* J Biol Chem, 1995. **270**(42): p. 25172-7.

167. Gabrys, D., et al., *Radiation effects on the cytoskeleton of endothelial cells and endothelial monolayer permeability.* Int J Radiat Oncol Biol Phys, 2007. **69**(5): p. 1553-62.

168. Hovinga, K.E., et al., *Radiation-enhanced vascular endothelial growth factor (VEGF) secretion in glioblastoma multiforme cell lines--a clue to radioresistance?* J Neurooncol, 2005. **74**(2): p. 99-103.

169. Sheng-Hua, C., et al., *Radiation-enhanced hepatocyte growth factor secretion in malignant glioma cell lines.* Surg Neurol, 2007. **68**(6): p. 610-3; discussion 613-4.

170. Kumar, P., A.I. Miller, and P.J. Polverini, *p38 MAPK mediates gamma-irradiation-induced endothelial cell apoptosis, and vascular endothelial growth factor protects endothelial cells through the phosphoinositide 3-kinase-Akt-Bcl-2 pathway.* J Biol Chem, 2004. **279**(41): p. 43352-60.

171. Aznar, S. and J.C. Lacal, *Rho signals to cell growth and apoptosis.* Cancer Lett, 2001. **165**(1): p. 1-10.

172. Etienne-Manneville, S. and A. Hall, *Rho GTPases in cell biology.* Nature, 2002. **420**(6916): p. 629-35.

173. Knauer, S.K., W. Mann, and R.H. Stauber, *Survivin's dual role: an export's view.* Cell Cycle, 2007. **6**(5): p. 518-21.

174. Altieri, D.C., *New wirings in the survivin networks.* Oncogene, 2008. **27**(48): p. 6276-84.

175. Delmas, C., et al., *Farnesyltransferase inhibitor, R115777, reverses the resistance of human glioma cell lines to ionizing radiation.* Int J Cancer, 2002. **100**(1): p. 43-8.

176. Zhai, G.G., et al., *Radiation enhances the invasive potential of primary glioblastoma cells via activation of the Rho signaling pathway.* J Neurooncol, 2006. **76**(3): p. 227-37.

177. Saito, T., et al., *Centrosome amplification induced by survivin suppression enhances both chromosome instability and radiosensitivity in glioma cells.* Br J Cancer, 2008. **98**(2): p. 345-55.

178. Schmidt-Ullrich, R.K., et al., *ERBB receptor tyrosine kinases and cellular radiation responses.* Oncogene, 2003. **22**(37): p. 5855-65.

179. Chakravarti, A., A. Dicker, and M. Mehta, *The contribution of epidermal growth factor receptor (EGFR) signaling pathway to radioresistance in human gliomas: a review of preclinical and correlative clinical data.* Int J Radiat Oncol Biol Phys, 2004. **58**(3): p. 927-31.

180. Chakravarti, A., J.S. Loeffler, and N.J. Dyson, *Insulin-like growth factor receptor I mediates resistance to anti-epidermal growth factor receptor therapy in primary human glioblastoma cells through continued activation of phosphoinositide 3-kinase signaling.* Cancer Res, 2002. **62**(1): p. 200-7.

181. Kao, G.D., et al., *Inhibition of phosphatidylinositol-3-OH kinase/Akt signaling impairs DNA repair in glioblastoma cells following ionizing radiation.* J Biol Chem, 2007. **282**(29): p. 21206-12.

182. Shu, H.K., et al., *The intrinsic radioresistance of glioblastoma-derived cell lines is associated with a failure of p53 to induce p21(BAX) expression.* Proc Natl Acad Sci U S A, 1998. **95**(24): p. 14453-8.

183. Harrigan, M.R., *Angiogenic factors in the central nervous system.* Neurosurgery, 2003. **53**(3): p. 639-60; discussion 660-1.

184. Ferrara, N., *Vascular endothelial growth factor as a target for anticancer therapy.* Oncologist, 2004. **9 Suppl 1**: p. 2-10.

185. Aonuma, M., et al., *Tumorigenicity depends on angiogenic potential of tumor cells: dominant role of vascular endothelial growth factor and/or fibroblast growth factors produced by tumor cells.* Angiogenesis, 1998. **2**(1): p. 57-66.

186. Gorski, D.H., et al., *Blockage of the vascular endothelial growth factor stress response increases the antitumor effects of ionizing radiation.* Cancer Res, 1999. **59**(14): p. 3374-8.

187. Maj, J.G., et al., *Microvascular function regulates intestinal crypt response to radiation.* Cancer Res, 2003. **63**(15): p. 4338-41.

188. Fuks, Z., et al., *Basic fibroblast growth factor protects endothelial cells against radiation-induced programmed cell death in vitro and in vivo.* Cancer Res, 1994. **54**(10): p. 2582-90.

189. Pena, L.A., Z. Fuks, and R.N. Kolesnick, *Radiation-induced apoptosis of endothelial cells in the murine central nervous system: protection by fibroblast growth factor and sphingomyelinase deficiency.* Cancer Res, 2000. **60**(2): p. 321-7.

190. Paris, F., et al., *Endothelial apoptosis as the primary lesion initiating intestinal radiation damage in mice.* Science, 2001. **293**(5528): p. 293-7.

191. Browder, T., et al., *Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer.* Cancer Res, 2000. **60**(7): p. 1878-86.

192. Tan, J. and D.E. Hallahan, *Growth factor-independent activation of protein kinase B contributes to the inherent resistance of vascular endothelium to radiation-induced apoptotic response.* Cancer Res, 2003. **63**(22): p. 7663-7.

193. Kumar, P., et al., *Combination treatment significantly enhances the efficacy of antitumor therapy by preferentially targeting angiogenesis.* Lab Invest, 2005. **85**(6): p. 756-67.

194. Bao, S., et al., *Glioma stem cells promote radioresistance by preferential activation of the DNA damage response.* Nature, 2006. **444**(7120): p. 756-60.

195. Denekamp, J., *Endothelial cell proliferation as a novel approach to targeting tumour therapy.* Br J Cancer, 1982. **45**(1): p. 136-9.

196. Coleman, C.N., *Hypoxia in tumors: a paradigm for the approach to biochemical and physiologic heterogeneity.* J Natl Cancer Inst, 1988. **80**(5): p. 310-7.

197. Yu, J.L., B.L. Coomber, and R.S. Kerbel, *A paradigm for therapy-induced microenvironmental changes in solid tumors leading to drug resistance.* Differentiation, 2002. **70**(9-10): p. 599-609.

198. Schuuring, J., et al., *Irradiation combined with SU5416: microvascular changes and growth delay in a human xenograft glioblastoma tumor line.* Int J Radiat Oncol Biol Phys, 2005. **61**(2): p. 529-34.

199. Kirsch, M., G. Schackert, and P.M. Black, *Anti-angiogenic treatment strategies for malignant brain tumors.* J Neurooncol, 2000. **50**(1-2): p. 149-63.

200. Plate, K.H., et al., *Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo.* Nature, 1992. **359**(6398): p. 845-8.

201. Jain, R.K., *Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy.* Science, 2005. **307**(5706): p. 58-62.

202. Thorsen, F. and B.B. Tysnes, *Brain tumor cell invasion, anatomical and biological considerations.* Anticancer Res, 1997. **17**(6B): p. 4121-6.

203. Sutherland, R.M. and A.J. Franko, *On the nature of the radiobiologically hypoxic fraction in tumors.* Int J Radiat Oncol Biol Phys, 1980. **6**(1): p. 117-20.

204. Folkins, C., et al., *Anticancer therapies combining antiangiogenic and tumor cell cytotoxic effects reduce the tumor stem-like cell fraction in glioma xenograft tumors.* Cancer Res, 2007. **67**(8): p. 3560-4.

205. Tanaka, T., *Effect of adenoviral-mediated thymidine kinase transduction and ganciclovir therapy on tumor-associated endothelial cells.* Neurol Med Chir (Tokyo), 1997. **37**(10): p. 730-7; discussion 737-8.

206. Stins, M.F., F. Gilles, and K.S. Kim, *Selective expression of adhesion molecules on human brain microvascular endothelial cells.* J Neuroimmunol, 1997. **76**(1-2): p. 81-90.

207. Goldbrunner, R.H., et al., *Models for assessment of angiogenesis in gliomas.* J Neurooncol, 2000. **50**(1-2): p. 53-62.

208. Claes, A., et al., *Phenotypic and genotypic characterization of orthotopic human glioma models and its relevance for the study of anti-glioma therapy.* Brain Pathol, 2008. **18**(3): p. 423-33.

209. Fomchenko, E.I. and E.C. Holland, *Mouse models of brain tumors and their applications in preclinical trials.* Clin Cancer Res, 2006. **12**(18): p. 5288-97.

210. Catalano, A., et al., *Enhanced expression of vascular endothelial growth factor (VEGF) plays a critical role in the tumor progression potential induced by simian virus 40 large T antigen.* Oncogene, 2002. **21**(18): p. 2896-900.

211. Vanden Berghe, T., et al., *More than one way to die: methods to determine TNF-induced apoptosis and necrosis.* Methods Mol Med, 2004. **98**: p. 101-26.

212. Verheij, M., *Clinical biomarkers and imaging for radiotherapy-induced cell death.* Cancer Metastasis Rev, 2008. **27**(3): p. 471-80.

213. Tanida, I., T. Ueno, and E. Kominami, *LC3 and Autophagy.* Methods Mol Biol, 2008. **445**: p. 77-88.

214. Shvets, E., E. Fass, and Z. Elazar, *Utilizing flow cytometry to monitor autophagy in living mammalian cells.* Autophagy, 2008. **4**(5): p. 621-8.

215. Roninson, I.B., *Tumor cell senescence in cancer treatment.* Cancer Res, 2003. **63**(11): p. 2705-15.

216. Rainaldi, G., et al., *Induction of apoptosis or necrosis by ionizing radiation is dose-dependent in MG-63 osteosarcoma multicellular spheroids.* Anticancer Res, 2003. **23**(3B): p. 2505-18.

217. Shimamura, H., et al., *Irradiated pancreatic cancer cells undergo both apoptosis and necrosis, and could be phagocytized by dendritic cells.* Eur Surg Res, 2005. **37**(4): p. 228-34.

218. Kurita, H., et al., *Early effects of PRS-irradiation for 9L gliosarcoma: characterization of interphase cell death.* Minim Invasive Neurosurg, 2000. **43**(4): p. 197-200.

219. Baatout, S., et al., *Increased radiation sensitivity of an eosinophilic cell line following treatment with epigallocatechin-gallate, resveratrol and curcuma.* Int J Mol Med, 2005. **15**(2): p. 337-52.

220. Quto b, S.S. and C.E. Ng, *Comparison of apoptotic, necrotic and clonogenic cell death and inhibition of cell growth following camptothecin and X-radiation treatment in a human melanoma and a human fibroblast cell line.* Cancer Chemother Pharmacol, 2002. **49**(2): p. 167-75.

221. Lefranc, F., J. Brotchi, and R. Kiss, *Possible future issues in the treatment of glioblastomas: special emphasis on cell migration and the resistance of migrating glioblastoma cells to apoptosis.* J Clin Oncol, 2005. **23**(10): p. 2411-22.

222. Lefranc, F. and R. Kiss, *The sodium pump alpha1 subunit as a potential target to combat apoptosis-resistant glioblastomas.* Neoplasia, 2008. **10**(3): p. 198-206.

223. Ditsworth, D., W.X. Zong, and C.B. Thompson, *Activation of poly(ADP)-ribose polymerase (PARP-1) induces release of the pro-inflammatory mediator HMGB1 from the nucleus.* J Biol Chem, 2007. **282**(24): p. 17845-54.

224. Badie, B., et al., *Dexamethasone-induced abolition of the inflammatory response in an experimental glioma model: a flow cytometry study.* J Neurosurg, 2000. **93**(4): p. 634-9.

225. Wolff, J.E., J. Denecke, and H. Jurgens, *Dexamethasone induces partial resistance to cisplatinum in C6 glioma cells.* Anticancer Res, 1996. **16**(2): p. 805-9.

226. Wolff, J.E. and H. Jurgens, *Dexamethasone induced partial resistance to methotrexate in C6-glioma cells.* Anticancer Res, 1994. **14**(4A): p. 1585-8.

227. Das, A., et al., *Dexamethasone protected human glioblastoma U87MG cells from temozolomide induced apoptosis by maintaining Bax:Bcl-2 ratio and preventing proteolytic activities.* Mol Cancer, 2004. **3**(1): p. 36.

228. Satoh, M.S. and T. Lindahl, *Role of poly(ADP-ribose) formation in DNA repair.* Nature, 1992. **356**(6367): p. 356-8.

229. Berger, N.A., et al., *Poly(ADP-ribose) polymerase mediates the suicide response to massive DNA damage: studies in normal and DNA-repair defective cells.* Princess Takamatsu Symp, 1983. **13**: p. 219-26.

230. Ha, H.C. and S.H. Snyder, *Poly(ADP-ribose) polymerase is a mediator of necrotic cell death by ATP depletion.* Proc Natl Acad Sci U S A, 1999. **96**(24): p. 13978-82.

231. Huang, Q., et al., *A novel function of poly(ADP-ribose) polymerase-1 in modulation of autophagy and necrosis under oxidative stress.* Cell Death Differ, 2008.

232. Kim, K.W., et al., *Autophagy for cancer therapy through inhibition of pro-apoptotic proteins and mammalian target of rapamycin signaling.* J Biol Chem, 2006. **281**(48): p. 36883-90.

233. Chow, H.H., et al., *Phase I pharmacokinetic study of tea polyphenols following single-dose administration of epigallocatechin gallate and polyphenon E.* Cancer Epidemiol Biomarkers Prev, 2001. **10**(1): p. 53-8.

234. Baatout, S., et al., *Study of the combined effect of X-irradiation and epigallocatechin-gallate (a tea component) on the growth inhibition and induction of apoptosis in human cancer cell lines.* Oncol Rep, 2004. **12**(1): p. 159-67.

235. Ahmad, N., P. Cheng, and H. Mukhtar, *Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate.* Biochem Biophys Res Commun, 2000. **275**(2): p. 328-34.

236. Fassina, G., et al., *Mechanisms of inhibition of tumor angiogenesis and vascular tumor growth by epigallocatechin-3-gallate.* Clin Cancer Res, 2004. **10**(14): p. 4865-73.

237. Ahn, H.Y., Y. Xu, and S.T. Davidge, *Epigallocatechin-3-O-gallate inhibits TNFalpha-induced monocyte chemotactic protein-1 production from vascular endothelial cells.* Life Sci, 2008. **82**(17-18): p. 964-8.

238. Kim, S.H., et al., *Epigallocatechin-3-gallate protects toluene diisocyanate-induced airway inflammation in a murine model of asthma.* FEBS Lett, 2006. **580**(7): p. 1883-90.

239. Kawai, K., et al., *Epigallocatechin gallate attenuates adhesion and migration of CD8+ T cells by binding to CD11b.* J Allergy Clin Immunol, 2004. **113**(6): p. 1211-7.

240. Surh, Y.J., et al., *Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation.* Mutat Res, 2001. **480-481**: p. 243-68.

241. Chen, C., et al., *Activation of antioxidant-response element (ARE), mitogen-activated protein kinases (MAPKs) and caspases by major green tea polyphenol components during cell survival and death.* Arch Pharm Res, 2000. **23**(6): p. 605-12.

242. Tian, B., et al., *Chemiluminescence analysis of the prooxidant and antioxidant effects of epigallocatechin-3-gallate.* Asia Pac J Clin Nutr, 2007. **16 Suppl 1**: p. 153-7.

243. Berno, V., et al., *The 67 kDa laminin receptor increases tumor aggressiveness by remodeling laminin-1.* Endocr Relat Cancer, 2005. **12**(2): p. 393-406.

244. Umeda, D., H. Tachibana, and K. Yamada, *Epigallocatechin-3-O-gallate disrupts stress fibers and the contractile ring by reducing myosin regulatory light chain phosphorylation mediated through the target molecule 67 kDa laminin receptor.* Biochem Biophys Res Commun, 2005. **333**(2): p. 628-35.

245. Lin, L.C., et al., *Pharmacokinetics of (-)-epigallocatechin-3-gallate in conscious and freely moving rats and its brain regional distribution.* J Agric Food Chem, 2007. **55**(4): p. 1517-24.

246. Wang, J.S., et al., *Validation of green tea polyphenol biomarkers in a phase II human intervention trial.* Food Chem Toxicol, 2008. **46**(1): p. 232-40.

247. Béliveau, R., Gingras, Denis, ed. *Les aliments contre le cancer*. 2005, Edition du Trécarré: Montréal. pp.213.