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Fibroblast growth factors as growth suppressors for medulloblastoma: an in vitro study

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Abstract

Medulloblastoma (MB) is a malignant embryonal brain tumour that originates from neuronal precursors of the cerebellum. In the developing cerebellum, several members of the fibroblast growth factor (FGF) family have been shown to play important roles in controlling aspects of cerebellar neuronal maturation. In view of this, it is possible that these growth factors may prove useful in controlling the aggressive nature of this cerebellar tumour. To test this, I have characterized two new MB cell lines, namely HSJ and SYR, that serve as additional models for this tumour. Using these two cell lines and a previously characterized MB line, UM-MB1, I first conducted an extensive comparative analysis of the properties of these three cell lines and then proceeded to determine if members of the FGF family which localize to the developing cerebellum, namely: aFGF/FGF-1, bFGF/FGF-2, FGF-5, FGF-6 and FGF-9 could be used to control the growth of MB.

Although all three cell lines originate from human cerebellar tumours, two, UM-MB1 and SYR, are derived from the most common variant of MB, the classic, while HSJ originates from a desmoplastic tumour. The doubling time for SYR is 49 hours, almost twice that of HSJ (29 hours). All three MB cell lines, like their original tumours, express antigens as determined immunocytochemically and by Western blot analyses, consistent with a neuronal etiology. However, differences were noted amongst the three cell lines in the intensity of staining and expression of some of these neuron-

specific antigens. Like cells of the classic MB tumours, UM-MB1 and SYR exhibit immature phenotypes as evident from their high nuclear to cytoplasmic ratios. Cells of the desmoplastic variant are usually more mature and consistent with this, so are the cells in HSJ.

Amongst all three cell lines, only UM-MB1 and SYR, the two derived from classic variants, respond to FGF-2 and FGF-9 with advanced differentiation, growth suppression and apoptotic death. These same growth factors as well as FGF-5 and FGF-6 are mitogenic for HSJ. When UM-MB1 and SYR are treated with FGF-2 or FGF-9 and begin to undergo apoptosis, their expression of Bax, a pro-apoptotic effector of the Bcl-2 family, increases. Of interest and most probably related to its antimitotic function, Bcl-2 expression is also upregulated in UM-MB1 treated with FGF-2 or FGF-9. Finally, while UM-MB1 and SYR exhibit immunoreactive sites for all four FGF receptors, HSJ, whose growth is not suppressed by the FGFs only expresses two, FGFR1 and FGFR4.

In conclusion, it appears that the type(s) of effect that FGF exerts upon MB may depend upon the type(s) of FGFR expressed on the tumour cell as well as the histopathological variant from which the cells originate.

Résumé

Durant le développement du cervelet, plusieurs membres de la famille des facteurs de croissance fibroblastique (FGF) semblent jouer un rôle important dans le contrôle de la maturation des cellules neuronales. Une telle propriété pourrait être exploitée en tant qu'approche thérapeutique éventuelle pour le traitement du médulloblastome (MB), une tumeur embryonnaire maligne du système nerveux central (SNC), dont l'origine est attribuée à la transformation néoplasique des cellules souches neuronales du cervelet. Dans ce contexte, nous avons voulu tester la possibilité de contrôler la nature aggressive de cette tumeur à l'aide de ces facteurs de croissance. A cette fin, deux nouvelles lignées cellulaires, HSJ et SYR, ont été établies à titre de modèles additionnels de cette tumeur. Ces deux nouvelles lignées, ainsi que la lignée cellulaire UM-MB1, établie et caractérisée lors d'une étude antérieure, ont, dans un premier temps, fait l'objet d'une analyse comparative détaillée de leurs propriétés. Dans un deuxième temps, j'ai déterminé si les membres de la famille des FGFs, aFGF/FGF-1, bFGF/FGF-2, FGF-5, FGF-6 et FGF-9, présents dans le cervelet en cours de développement, exerçaient des effets sur la croissance de ces lignées cellulaires médulloblastomales.

Même si ces trois lignées cellulaires sont issues d'un même type tumoral, le médulloblastome, deux d'entre elles, UM-MB1 et SYR, provenaient de tumeurs classiques, et HSJ de la variante histopathologique

desmoplasique. L'analyse comparative des propriétés des trois lignées à démontré que le temps de dédoublement des cellules SYR était de 49 heures, ce qui équivaut à une vitesse de croissance presque deux fois plus lente que celle des cellules HSJ (29 heures). Par immunocytochimie et immunobuvardage, nous avons constaté que ces trois lignées exprimaient, tout comme leurs tumeurs d'origine, un phénotype antigénique neuronal. Par contre, des différences d'expression ou d'intensité d'expression de certains de ces marqueurs neuronaux ont été notées. Finalement, tout comme les cellules d'une tumeur de MB classique, les lignées cellulaires UM-MB1 et SYR sont apparues constituées de cellules immatures montrant un ratio noyau/cytoplasme très élevé. Les cellules de la variante histopathologique desmoplasique présentaient un phénotype plus mature, en accord avec la morphologie cellulaire des cellules HSJ.

Parmi les divers FGFs examinés, seulement bFGF/FGF-2 et FGF-9 ont paru favoriser la différenciation neuronale, le ralentissement de la vitesse de croissance et l'induction de la mort cellulaire par un mécanisme apoptotique, tant chez UM-MB1 que SYR, nos deux lignées provenant de médulloblastomes classiques. Par contre, ces mêmes facteurs de croissance, de même que le FGF-5 et FGF-6, ont montré un effet mitotique sur les cellules HSJ. Lorsque les cellules UM-MB1 et SYR ont été exposées au bFGF/FGF-2 ou au FGF-9 et qu'elles ont atteint le stade de la mort cellulaire, l'expression de la molécule pro-apoptotic Bax est apparue

augmentée. De plus, nous avons constaté une augmentation de l'expression de la molécule anti-apoptotique Bcl-2 dans les cellules UM-MB1 traitées avec le bFGF/FGF-2 ou le FGF-9. Finalement, il s'est avéré que HSJ, la lignée cellulaire dont aucun FGF n'inhibe la vitesse de croissance, exprime seulement les récepteurs FGFR1 et FGFR4, tandis que les lignées cellulaires UM-MB1 et SYR expriment les quatre types de récepteurs fibroblastiques (FGFR1-FGFR4).

Nous en avons conclu que les effets produits par les FGFs sur les lignées cellulaires médulloblastomales dépendent du (des) type(s) de récepteur(s) qu'elles expriment, de même que de la variante histopathologique des tumeurs dont elles sont issues.

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Abbreviations

aa	amino acid
BDNF	brain-derived-neurotrophic factor
CNS	central nervous system
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGL	external granular layer
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GT	germinal trigone
GFAP	glial fibrillary acidic protein
HSPGs	heparin sulfate proteoglycans
IGL	internal granular layer
MB	medulloblastoma et/ou médulloblastome
MAPs	microtubule-associated-proteins
NGF	nerve growth factor
NRG	neuregulin
NEP	neuroepithelial
NF	neurofilament
NSE	neuron specific enolase
NT-3	neurotrophin-3
NT-4/5	neurotrophin-4/5
PTCH	patched
PNETs	primitive neuroectodermal tumours
smo	smoothened
SNC	système nerveux central
SHH	sonic hedgehog
TNF	tumour necrosis factor

Trk	tropomyosine-receptor-kinase and/or tyrosine kinase receptor
VM	ventricular matrix
WHO	World Health Organization

A...

Dieu, ma force, mon courage et mon guide spirituel

Ma famille, présente et dans les cieux

Et tous ceux qui croient en moi...

CHAPTER ONE:

GENERAL INTRODUCTION

INTRODUCTION

After perinatal complications, congenital anomalies and motor vehicle accidents, cancer is the most common cause of death in the pediatric population in Canada. In fact, more than 800 new cases of cancer are diagnosed annually and approximately a quarter of these children die. Amongst all childhood neoplasms, tumours of the central nervous system (CNS) are the second most common. Medulloblastoma (MB), which accounts for 20% of all brain malignancies in children, is second only to astrocytomas in order of frequency (Canadien Cancer society, 1999). The peak incidence for MB is 7 years of age and it tends to have a higher rate of occurrence in males than in females. The principal location of this tumour is the posterior fossa with a tendency to project into the fourth ventricle (Giangaspero, 1999). Medulloblastoma is a highly aggressive brain tumour which tends to re-occur and metastasize via cerebral spinal fluid pathways. Although refinements have been made on conventional therapies (surgery, irradiation, and conventional chemotherapy), MB still continues to represent a frustrating therapeutic challenge (Choux, 1982; Oberfield et al., 1986; Jenkin et al., 1996; Maureen et al., 1996). Therefore, there is a need for new, less toxic ways to treat this brain malignancy that develops primarily in an immature and extremely vulnerable nervous system. To design such alternatives, it is imperative that we attain a better

understanding of the biology and etiology of MB.

1. Historical overview of medulloblastoma nomenclature

The formal introduction of medulloblastoma dates from 1925 when Cushing and Bailey first coined the name "*medulloblastoma cerebelli*" to define a densely cellular, midline cerebellar tumour that arises in the vermis or over the roof of the fourth ventricle and occurs mainly in children. This choice was in fact their second, as initially, Cushing and Bailey regarded this tumour as a primitive form of an astrocytic glioma and called it "*spongioblastoma cerebelli*" (Bailey and Cushing, 1925). However, Globus' and Strauss' simultaneous description of the "*spongioblastoma multiform*", a clearly different glial tumour entity, led them to disregard a spongioblastic origin for these tumours of the posterior fossa (Rubinstein , 1975). Therefore, Cushing and Bailey proposed that these childhood cerebellar tumours originate from the medulloblast, one of the five different bi-potential neuroepithelial stem cells that they equated to the indifferent cells of Schaper and hence they named it "*Medulloblastoma*" (MB) (Schaper. 1897; Bailey and Cushing, 1925).

2. Medulloblastoma classification

Although the name MB has been used throughout the world from one

generation of neuropathologists and neurosurgeons to the next, the appropriate nomenclature for this embryonal CNS tumour has been the subject of much debate. The current controversy actually dates back to the original description of this tumour and reflects the continued uncertainty regarding its histogenesis.

One of the fervent defenders of Cushing and Bailey's hypothesis concerning MB's nature and origin is Lucien J. Rubinstein. He argued, for approximately a two-decade period, that: 1) MB is a unique tumour which exists only in the cerebellum; 2) although some MB tumours may arise from the internal granular cells or embryonal cell nests located in the cerebellar vermis, most originate from a germinative cell derived from the external granular layer of the cerebellum, capable of bi-potential differentiation into both glial cells and neurons (Rubinstein, 1975). In conclusion, Lucien J. Rubinstein has maintained that MB is a unique type of embryonal CNS tumour.

On the other hand, Lucy B. Rorke proposed a new simplified classification for CNS neoplasms like MB that occur primarily in infancy and childhood. She classified these tumours as primitive neuroectodermal tumours (PNETs) (Hart and Earle, 1973), which suggests that neoplastic transformation of primitive neuroepithelial cells in the subependymal zones at all levels of the CNS can lead to the development of tumours with similar histological appearances. This classification scheme does not, however,

take into consideration the site of origin of the tumour, nor whether it is composed of undifferentiated or differentiated cells or cells with one or more lines of differentiation. As a result, PNET tumours have been further sub-classified into five subtypes to reflect at least differences in their inherent differentiation potentials. These subtypes are: 1) PNET, not otherwise specified; 2) PNET with glial differentiation; 3) PNET with ependymal differentiation; 4) PNET with neuronal differentiation; 5) PNET with multi- or bi-potential differentiation (Rorke, 1983). However, Lucien J. Rubinstein continued to argue against Rorke's classification scheme pointing out its failure to consider the possibility of restrictions in the differentiating potential of specific tumours at different anatomic sites, as well as the inherent differentiation potential of the stem cell from which they may have arisen (Rubinstein, 1985). In conclusion, to obviate these inconsistencies, in 1993, the World Health Organization (WHO) international classification of CNS tumours retained the classification of "embryonal childhood tumours" and defined all the primitive neuroectodermal tumours of the CNS as PNET, regardless of whether they are localized inside or outside the cerebellum (Kleihues, 1993). However, due to a long tradition, a tumour in the cerebellum with specific histopathological characteristics will probably always be called MB rather than a PNET of the cerebellum.

3. Medulloblastoma histogenesis

As mentioned previously, although it is generally accepted that MBs are usually restricted to the cerebellum, the precise histogenesis of these tumours has been, and still remains, to a lesser extent, an issue of controversy. The origin of MB is attributed to three main sources: (i) the cerebellar neuroepithelium/ventricular matrix(VM); (ii) the external granular layer (EGL), and (iii) the internal granular layer (IGL) of the cerebellum. The first two correspond to the two germinal zones of the cerebellum which give rise to the cellular elements that form the cerebellar cortex (Altman and Bayer, 1978; 1997). These two possibilities are not mutually exclusive as they may explain the differences noted in the two most commonly documented histopathological variants of MB, i.e. the classic and the desmoplastic (Giangaspero et al., 1999). Properties of these histopathological variants correlate well with those of the cells found in these two germinal zones (Katsetos et al., 1993; Katsetos and Burger, 1994; Katsetos et al., 1995). The third site of origin for MB, the IGL, was proposed based upon experimentally induced tumours in mice (Zimmerman, 1967) or hamsters (Zu Rhein and Varakis, 1979) following the intracerebral administration of carcinogenic hydrocarbons or JC papovavirus, respectively. Interestingly, however, although the tumours were detected in the IGL of adult animals, this layer of the cerebellum did not exist at the time of the initial injections. Consequently, the transforming events most likely

targeted the precursors of the IGL, the EGL.

3.1 The ventricular matrix (VM)

Although less attention has been given to the VM as a potential site of origin for MB, this hypothesis has become more and more popular recently. In fact, this etiology, although not yet exploited for the design of novel therapeutic modalities for this embryonal tumour, can explain the vermal location of some MB tumours. In this regard, Raaf and Kernohan in 1944, followed by Ringertz and Tola in 1950, suggested that several of the midline examples of MB may originate from small foci of embryonal cell nests located in the germinal zone of the posterior and anterior VM, respectively (Raaf and Kernohan, 1944; Ringertz and Tola, 1950). This germinal bud, situated at the hind end of the posterior VM, gives rise to the EGL and the choroid plexus (Altman and Bayer, 1997), and normally disappears before the first month of extra-uterine life in humans (Raaf and Kernohan, 1944). However, it is thought that some MB tumours, namely the classic variant, arise from the abnormal persistence of these primitive cells beyond this time. More recently, studies using specific neuronal differentiation-markers that co-localize to certain sub-populations of cerebellar neurons with distinct ontogenic profiles support this concept further (Katsetos et al., 1993; Katsetos and Burger, 1994; Katsetos et al., 1995). For example, Katsetos et al., have shown that calbindin-D_{28k}, a ventricular matrix-associated

neuronal calcium binding protein which is only expressed in neuronal cell populations of this matrix zone, not in the internal granular layer (IGL) of the cerebellum nor its precursors (EGL), is also found in classical MB, a histopathological variant of this tumour that is predominantly located to the cerebellar vermis (Katsetos et al., 1993; Katsetos and Burger, 1994; Katsetos et al., 1995).

3.2 The external granular layer (EGL)

An hypothetical origin of MB from portions of the EGL (the fetal granular layer of Obersteiner) was originally suggested by Stevenson and Echlin, in 1934, and subsequently supported by Kershmann, in 1938 (Stevenson and Echlin, 1934; Kershman, 1938). Apart from being the first postulated site of origin for MB, it is also the most accepted one. In fact, many of the new prognostic indices and diagnostic venues taken to treat this embryonal neoplasm have focused upon understanding the biology of these cerebellar precursors (Segal et al, 1992; Segal et al., 1994). In the past, investigators supported this etiology mostly because of the morphological similarity of MB tumour cells to the small primitive cells of the fetal EGL. For example, the architecture of many MBs, composed of subpial aggregations of tumour cells in the superficial part of the molecular layer, resemble cells in the layer of Obersteiner (Kadin et al., 1970; Rubinstein, 1975). Although there was general agreement on the EGL as the most likely site of origin for

MB, the differentiating potential of these germinal cells has been a subject of controversy. On one hand, Stevenson and Echlin, in agreement with Ramon y Cajal (1911), believed that the fetal granular layer could only give rise to neurons (Ramon y Cajal, 1911; Stevenson and Echlin, 1934). In contrast, Kershman supported Schaper's idea of a bi-potential differentiating capacity for the EGL cells (Kershman, 1938).

Consistent with Ramon y Cajal, and in support of a neuronal differentiating potential for the EGL, is that nearly 90% of the original tumour samples and MB cell lines generated express markers such as: the neurofilament triplets (NF-L, NF-M, NF-H), neuron specific enolase (NSE), microtubule-associated-proteins (MAPs) and synaptophysin, which are consistent with a neuronal etiology (Friedman et al., 1985; 1988; He et al., 1991; Keles et al., 1995; Kenigsberg et al., 1997; Pomeroy et al., 1997). Furthermore, studies have demonstrated genotypic and phenotypic similarities between the neurons of the EGL and neoplastic cells of this tumour. In this regard, genes coding for transcription factors present and probably involved in the genesis of the granule neurons of the internal granular layer (IGL) of the cerebellum such as: PAX6, a member of the paired box-containing (PAX) gene family, and EN1 and EN2, two homeobox-containing genes, are also expressed in MB tumours samples (Kozmik Zbynek et al., 1995). In addition, the human zic, a zinc finger protein with a restricted expression pattern in the cerebellar granule cell lineage, and the

class III β-tubulin isotype (βIII), one of the earliest appearing markers of neuronal differentiation in the pre-migratory/post-mitotic neurons of the EGL, have also been found to be present in MB (Katsetos et al., 1993; Aruga et al., 1994; Katsetos and Burger, 1994; Yokota et al., 1996).

Although only seen in rare cases, other histopathological variants of MB, such as the glial, melanotic and mesenchymal, have also been reported (Choux, 1982; Tomlinson et al., 1992; Burger and Scheithauer, 1993). Divergent differentiation toward melanotic and mesenchymal elements have been observed on very rare occasions. Glial differentiation is the most controversial, as it is only in the primary tumours that glial differentiation was noted. In fact, none of the cell lines derived from these tumours express glial fibrillary acidic protein (GFAP), a marker for astrocytic glial cells. Furthermore, GFAP is found in a very low percentage of the cells within the tumours, suggesting that positive staining for this intermediate filament may not be a manifestation of intrinsic glial differentiation but represent normal entrapped or reactive astrocytes that develop in the presence of a lesion, like a tumour (Friedman et al., 1985; 1988; He et al., 1991; Keles et al., 1995).

4. Histogenesis of the cerebellar cortex

The rhombencephalon is the portion of the developing nervous system that surrounds the fourth ventricle. This region is further subdivided

into the myelencephalon, which gives rise to the medulla, and the metencephalon, which is considered to be the primordium of the cerebellum and the pons (Nolte, 1993).

Unlike the other brain structures, the cellular elements of the cerebellar cortex are derived from two distinct germinal zones: i) the cerebellar neuroepithelium/VM, and ii) a later forming secondary germinal matrix, the EGL. The former consists of the typical neuroepithelial sheet and is responsible for the sequential generation of the deep nuclear neurons, the Purkinje cells, the interneurons-Golgi type II, stellate and basket cells- and the neuroglia (Zhang and Goldman; 1996; Altman and Bayer, 1978; 1997). The second germinal matrix, the EGL, arises from the caudal segment of the cerebellar neuroepithelium known as the germinal trigone (GT) or the rhombic lip, at a time when division of the nuclear and Purkinje cells ceases (Altman and Bayer, 1978; 1997).

The GT or rhombic lip is an intermediary proliferative matrix composed of three zones: 1) the neuroepithelium, lining the roof of the fourth ventricle; 2) the EGL, and 3) the choroid plexus. The former constitutes the core of the GT/rhombic lip, while the latter two represent its expansions (Altman and Bayer, 1978; 1997). The core, the neuroepithelium lining, contains two parts, one internal and one external. The internal lining is in contact with the fourth ventricle and provides stem cells to the formative choroid plexus, while the external occupies a subpial position and is

continuous with the EGL. Cytological studies have shown an association between the expansion of the EGL and increasing mitotic activity in this external lining (Altman and Bayer, 1978; 1997). Therefore, proliferating precursors within this lining are thought to be the direct source of the EGL neuronal cells. This is further supported by the finding of Alder et al. (1996) which show that neurons of the EGL and IGL like the GT cells (or rhombic lip cells) express a zinc finger protein, RU49, which is a granule cell marker (Alder et al., 1996; Yang et al., 1996). As the EGL keeps on proliferating, the bulk of the GT gradually declines to finally disappear around the end of the first extra-uterine month in humans (Raaf and Kernohan, 1944). Thereafter, the EGL disperses in two directions. The first takes place from posteroventral to anterodorsal over the future vermis in order to unify the developing cerebellum, and the second, from lateroventral to mediodorsal over the future cerebellar hemispheres to form the EGL, the secondary germinal zone. While cells in the former zone cease to multiply a few days after birth, those in the latter has been found to persist up to one year of life in humans (Stevenson and Echlin, 1934; Altman and Bayer, 1997).

4.1 Contribution of the EGL to the cerebellar structure

It is now well established that the EGL gives rise to only one class of neurons, the granule neurons of the IGL. Evidence supporting this conclusion comes from implantation, retroviral labelling, chimeric and

retrospective clonal analysis studies done in rat (Zhang and Goldman, 1996), mouse (Gao and Hatten, 1994; Jankovski et al., 1996), chick/quail (Martinez and Alvarado-Mallart, 1989; Hallonet et al., 1990; Hallonet and Le Douarin, 1993; Hallonet and Alvarado-Mallart, 1997) and other mammals (Mathis et al., 1997).

Before reaching their final destination, i.e. the IGL, the granule neurons first proliferate in the proliferating zone of the EGL and subsequently migrate inwards in the pre-migratory zone just underneath the proliferative area. At that stage, they become post-mitotic and start differentiating, a process that will be finalized once they have settled in the IGL. Guided by the Bergmann radial glial fibers, the granule neurons migrate further inward to finally settle and form the IGL of the cerebellum (Ramon y Cajal, 1911; Miale and Sidman, 1961; Sidman and Rakic, 1973).

4.2 Cerebellar granule neuronal development

4.2.1 Cellular interactions

Granule neuronal development is dependent on epigenetic influences from neighbouring granule cell precursors as well as other cerebellar cell types. In this regard, in vitro experiments have shown that homotypic interactions of the granule cell progenitors in the EGL stimulate cell DNA synthesis, whereas heterotypic neuron-glial interactions inhibit granule cell precursor proliferation and induce morphological differentiation, i.e. process

outgrowth (Gao et al., 1991). Moreover, mutual interactions between Purkinje cells and their future presynaptic regulatory partners, the granule neurons, are essential for the normal development of both cell types (Hatten and Heintz, 1995; Smeyne et al., 1995). Finally, elimination of the Purkinje cells produces a concomitant reduction in the size of the granule cell population most likely by cell death or apoptosis (Herrup and Kuemerle, 1997).

4.2.2 Molecular factors

Like any other cell types, the normal and complete maturation of the granule neurons of the cerebellum depends on the presence of various factors. These factors are biologically active, naturally occurring multi-functional peptides that upon interaction with their appropriate receptors induce a variety of changes in cellular function such as: proliferation, differentiation and survival. These same growth factors, and receptors, may, when expressed aberrantly, be implicated in a number of pathologies including cancer.

Most studies designed to identify factors involved in the development of the granule neurons have focussed upon factors affecting the maturation of the neurons of the EGL up to their migration and final settlement in the IGL. Table 1 lists the members of four growth factor families, their cognate receptors and the roles that they are currently believed to play during

cerebellar granule cells maturation.

4.2.2.1 Sonic hedgehog (SHH)

***Sonic hedgehog and granule neuronal development*:**

Sonic hedgehog peptide is one of three mammalian hedgehog (hh) gene product that is expressed and secreted by the Purkinje cells of the cerebellum (Traiffort et al., 1998; Wallace et al., 1999). It mediates its effects via two integral membrane proteins encoded by the patched (ptc) and smoothened (smo) genes which are expressed by dividing neurons of the EGL. It is believed that both receptors are needed to transduce SHH signals. PTCH is presumably the primary receptor while smo is essential for the actual signalling (Chen et al., 1996; Traiffort et al., 1998). During cerebellum histogenesis, a mutual antagonism between PTCH and SHH is believed to control the proliferation of the external granule neurons. While SHH would promote the proliferation of these neuronal precursors, PTCH function as an antagonist of shh signalling, thus, inducing their differentiation (Chen et al., 1996; Wallace et al., 1998; Dahmane and i Altaba, 1999; Wechsler-Reya and Scott, 1999).

***Sonic hedgehog and MB*:**

Evidence for the implication of SHH or/and PTCH in MB etiology comes from studies in both human and mouse. Goodrich et al. (1997) have shown that about 30% of mice heterozygous for mutations in the ptc gene

develop tumour masses resembling MB (Goodrich et al., 1997). This is consistent with the above showing PTCH to promote cellular maturation rather than division. In humans, mutations in ptc tumour suppressor gene is noted in nevoid basal cell carcinoma syndrome (Johnson et al., 1996), an autosomal dominant disorder in which there is a predisposition to develop abnormalities and various neoplasms including MB (Lacombe et al., 1990). And finally, a number of mutations in the human ptc gene have been found in both sporadic and some desmoplastic variants of MB (Pietch et al., 1997; Raffel et al., 1997; Vorechovsky et al., 1997; Vortmeyer et al., 1999), the variant that has been thought to originate from neuronal precursors of the EGL (Katsetos and Burger, 1994).

4.2.2.2 Neuregulins (NRG)

***Neuregulins and granule neuronal development*:**

The NRG constitute a group of related peptides that are members of the epidermal growth factor (EGF) family. At least thirteen members of the NRG have been identified to date, which all arise from alternative splicing of a single gene mapped to the short arm of the human chromosome 8 at position 8p12-p21 (Orr-Urtreger et al., 1993). All NRG family members share a common EGF-like domain, which is a critical part of the molecule, essential for receptor binding, activation and autophosphorylation (Chen et al., 1994; Carraway III, 1996; Pinkas-Kramarski et al., 1997). NRG signal

through a family of protein tyrosine kinases of the class I EGF receptor (EGFR) family, namely erbB2/HER2, erbB3/HER3 and erbB4/HER4. Two of these, erbB3/HER3 and erbB4/HER4, function as the binding receptors for the various NRG, while erbB2/HER2 does not directly bind any. However, upon ligand binding, erbB3/HER3 and erbB4/HER4 receptors can form homo- or heterodimers, which may include erbB2/HER2 (Carraway and Cantley, 1994; Carraway III, 1996; Erickson et al., 1997). In the rodent, all four erbB receptor types and all the NRG molecules are expressed in the developing cerebellum (Ozaki et al., 1998). In the human, however, HER2 is not detected during cerebellar development (Gilbertson et al., 1998). During rat cerebellar development, the NRG-erbB/HER system has been recently postulated to play a role in the migration of the granule neurons from the EGL to their final destination, the IGL. The interactions between NRG which are expressed by the granule cells, and the erbB4 receptor, which localize to the Bergmann glia, may induce the latter to acquires a radial glial morphology (fibre network) and facilitate the movement of the granule neurons along these glial fibres (Rio et al., 1997).

***Neuregulins and MB*:**

As mentioned previously, during human cerebellar granule cell development, HER2 is not detected at any developmental stage (Gilbertson et al., 1998). However, in MB primary tumour samples, HER2 has been shown to be co-expressed with HER4 and NRG. It has been proposed that

this aberrant expression of HER2 may be involved in the progression of this disease. As a consequence, co-expression of HER2 and HER4 has been thought to be a negative prognostic indicator for MB (Gilbertson et al., 1995; 1997; Herms et al., 1997).

4.2.2.3 Neurotrophins

***Neurotrophins and granule neuronal development*:**

The neurotrophin family includes: nerve growth factor (NGF), brain-derived-neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). These growth factors exert their biological activities by interacting with members of two families of receptors, namely the p75 neurotrophin receptor, a member of the tumour necrosis factor (TNF) receptor family, and the tyrosine kinase receptors (Trk) family. Although p75 neurotrophin receptor binds all neurotrophins with similar affinity, the Trk receptors binds them selectively. In this regard, the first member of this trk receptor family, TrkA/Trk, preferentially binds NGF, while TrkB binds BDNF and NT-4/5 with highest affinity. The preferred ligand for TrkC is NT-3 (Lamballe, 1991; Bothwell, 1995; Muller and Clos, 1997).

During rodent cerebellar granule cell development, BDNF, NT-3 and NT-4/5 have been shown to be expressed and involved in the maturation of these neurons (Gao et al., 1995; Segal et al., 1992; 1995; 1997). As reported by Segal and colleagues (1992), BDNF and NT-3 affect developing

granule cells at distinct stages of their maturation. For example, early granule cells in the EGL express TrkB and respond to endogenous BDNF, whereas later in their maturational process, once they have migrated to the inner portion of the cerebellum, these postmitotic granule neurons begin to express TrkC and become responsive to NT-3 (Segal et al., 1992; 1995; Gao et al., 1995). Thus, TrkB is expressed in less mature granule cells, while TrkC is present in the more mature ones. Although known to mediate a wide variety of effects on responsive neurons during granule maturation, BDNF and NT-4/5, acting through TrkB, enhance and stabilize neurite outgrowth and neuronal survival. Subsequently, NT-3, which appears later developmentally, induces axonal fasciculation in these same neurons (Segal et al., 1992;1995; Gao et al., 1995).

***Neurotrophins and MB*:**

Neurotrophins and their cognate receptors have been found to be variably expressed in MB (Keles et al., 1993; Segal et al., 1994; Washiyama et al., 1996). For example, Washiyama et al. (1996), in their study of 29 PNET biopsy samples (27 of which were MBs), reported that neoplastic cells immunoreactie to TrkA, TrkB and TrkC was present in 27%, 62% and 48% of these tumours, respectively. Additionally, the corresponding ligands, i.e. BDNF, NT-3 and NT-4/5, were also expressed in 22%, 9% and 19% of these biopsies respectively. However, this study did not explore the possible relationship between neurotrophin expression and patient outcome.

Segal et al. (1994), were the first to provide evidence of a possible correlation between neurotrophin expression and MB patient survival. They noted that patients with MB tumours expressing high levels of TrkC mRNA had significantly longer disease-free intervals and better overall survival rates than those with low levels of this receptor. Moreover, they observed that tumour cell lines that can be maintained in vitro had no detectable full-length TrkC (Segal et al., 1994). Paradoxically however, when two commercially available MB cell lines, namely D283 and Daoy were transfected with either TrkA or TrkC, only the TrkA not the TrkC transfectants died by apoptosis when exposed to their appropriate ligands (Muragaki et al., 1997). This early finding was recently challenged in a study published by Kim and colleagues (1999), who showed that Daoy cells transfected with TrkC did indeed undergo apoptosis when exposed to NT-3 (Kim et al., 1999). Furthermore, freshly isolated MB tumour cells expressing TrkC were found to respond similarly. Lastly, TrkC-transfected Daoy, when grown as intracerebral xenografts, responded to NT-3 in a manner similar to that seen in vitro (Kim et al., 1999).

4.2.2.4 Fibroblast growth factors (FGF)

FGFs : general properties

The fibroblast growth factors (FGF) constitute one of the largest growth factor family. Although seventeen members of this family have been

identified to date, all transcribed from distinct genes, in humans, only thirteen genes have yet been cloned (Szebenyi and Fallon, 1999). These factors, which share 30-70% amino acid (aa) sequence homology, are designated as FGF-1 through FGF-17. However, alternative names are often used to identify certain members. Table 2 lists other names for the FGFs encountered in the literature, as well as the human chromosomal localisation of the gene that encodes for each of them (Basilico and Moscatelli, 1992; Szebenyi and Fallon, 1999).

FGF receptors :

Members of the FGF family exert their biological effects by binding to and activating specific cell surface receptors. At least four distinct receptor types for the FGFs, namely FGF receptor 1 (FGFR1), FGFR2, FGFR3 and FGFR4, arising from four distinct genes located on chromosomes 8p11.2-p11.1, 10q26, 4p16.3 and 5q35.1-qter, respectively, have been identified to date (Mattei et al., 1991; Thompson et al., 1991 Dionne et al., 1992; Warrington et al., 1992; Wood et al., 1995). The basic structure of these receptors is an extracellular region composed of three immunoglobulin-like domains containing a continuous stretch of 4-8 acidic aa, called the acidic box, between Ig domains I and II. Contiguous with the extracellular region is a single transmembrane domain followed by a cytosolic tyrosine kinase domain split by a 14 aa insert and a short carboxy-terminal tail. With the exception of FGFR4, FGFR1 through 3 can, by alternative splicing of the

genes, give rise to additional structural variants that are either membrane bound or soluble, thus increasing the complexity of the FGFR family (Givol et Yayon, 1992; Johnson and Williams, 1993; Green et al., 1996). In addition to their ability to bind to specific cell surface signalling receptors, all the FGFs have a high affinity for heparin sulfate proteoglycans (HSPGs) and related extracellular matrix components. These components are thought to act as either a reservoir for the FGFs or to be essential components in the FGF signalling pathway itself. Although the exact sequence of binding events is unresolved, the signal transduction complex seems to implicate the FGF polypeptide, HSPGs and the FGFR (Baird, 1994; Galzie et al., 1997; Szebenyi and Fallon, 1999).

FGFs during development :

The FGF family is one of the largest family of growth factors that is expressed very early during development. In this regard, multiple studies have demonstrated the involvement of certain FGFs in gastrulation, neurulation as well as anteroposterior specification of body segments and neuronal structures in vertebrates (Crossley and Martin, 1995; Yamaguchi and Rossant, 1995; Goldfarb, 1997; Lewandosky et al., 1997). FGFs are known to act as growth and differentiation regulators for cells of endo-, meso- and ectodermal origin. In addition, they have been shown to play important roles in wound healing, tissue repair and maintenance (Cuevas et al., 1988; Burgess and Marciag, 1989; Basilico and Moscatelli, 1992; Clarke

et al., 1993; Galzie et al., 1997).

Like their specific ligands, the FGFRs have been found to be expressed very early in embryogenesis where they are thought to regulate key steps in neuroepithelial stem cell differentiation (Kalyani et al., 1999). The early appearance of both growth factor and receptor renders this family particularly interesting when looking at the etiology of embryonal tumours like MB, which originate from cerebellar precursors deriving from the neuroepithelium. Although not yet implicated in the pathogenesis of developmental abnormalities in the nervous system, the FGFs, when aberrrantly expressed, have been shown to be involved in some neurodegenerative diseases and tumours of non-neural origin (Logan, 1990; Baird, 1994; Weiner, 1995).

With respect to the maturation of cerebellar precursors, cells from which MB has been proposed to arise, only certain members of the FGF family have thus far been documented to play a role. Table 1 lists the members of this family, i.e. aFGF/FGF-1 (Wilcox and Unnerstall, 1991; McAndrew et al., 1998), bFGF/FGF-2 (Hatten et al., 1988; El-Husseini et al., 1994; Matsuda et al., 1994; Tao et al., 1997), FGF-5 (Hattori et al., 1997), FGF-6 (Ozawa et al., 1996) and FGF-9 (Tagashira et al., 1995; Todo et al., 1998), and their cognate receptors (Yazaki et al., 1994; Goldfarb, 1996; Green et al., 1996; Szebenyi and Fallon, 1999) that are expressed in the cerebellum during neuronal development. Unlike the neurotrophins, the

exact temporal pattern of expression and role that these factors and receptors may play in cerebellar maturation are still uncertain. However, bFGF/FGF-2, the most documented member of this family has been found to induce granule neurons proliferation (Tao et al., 1997) and to promote neurite outgrowth (Hatten et al., 1988) in vitro and in vivo.

***FGF and MB*:**

Consistent with a differentiating role for bFGF/FGF-2 in cerebellar neuronal maturation and the neuronal etiology of MB, we have reported that bFGF/FGF-2 can indeed induce cellular differentiation, slow the growth and promote the death by apoptosis of a newly established and characterized MB cell line, UM-MB1 (Kenigsberg et al., 1997). These MB cells responded similarily to bFGF/FGF-2 when grown in vivo as intracerebral xenografts (Vachon et al., 1998). These findings were corroborated by the observations of Wechsler-Reya and Scott (1999), who reported that bFGF/FGF-2 can inhibit the mitogenic effects of shh on granule cell precursors (Wechsler-Reya and Scott, 1999).

5. Therapeutic modalities

5.1 Surgery

Surgical intervention is the initial treatment for patients diagnosed with MB. It is the quickest and only way to reduce the bulky tumour and consequently remove the intracranial pressure. Although a mortality rate of

32% following surgery was reported by Cushing in the 1930's, improvements in general anaesthesia and neurosurgical techniques have resulted in an operative mortality rate of less than 5% (Choux, 1982; Friedman, 1991).

The goal of brain tumour surgery is to perform as complete a resection as possible without causing neurological harm. In this regard, cognitive deficits are usually not attributed to the surgery for MB as the cerebellum is not usually associated with intellectual functions. However, perioperative factors such as bacterial meningitis and cerebrospinal fluid (CSF) leaks do appear to be associated with neurocognitive deficits particularly in younger children (Johnson et al., 1994; Sutton et al., 1996). The real question about surgery is whether or not a gross total resection offers a survival advantage over a near-total resection. The answer remains controversial as studies both support (Norris et al., 1981; Park et al., 1983) and refute (Allen et al., 1986) a survival advantage for those children receiving a gross-total resection compared to a partial resection. Nevertheless, performing either total or partial resection allows acquisition of the brain tissue for histopathological examination and definitive diagnosis. Lastly, but not least, tumour resection relieves hydrocephalus, an obstruction of the fourth ventricle associated with 60% of MB cases, which allows neurosurgeons to obviate the need for preresection CSF shunting (DeVita et al., 1997).

5.2 Radiation therapy

Although Cushing and Bailey knew since 1919 that MB was a radiosensitive tumour, it was not before the late 1940's that Patterson introduced radiation therapy as an integral part of MB treatment. Because MB tumours have a propensity to disseminate throughout the CSF early in the course of the disease, Patterson tried irradiation of the entire neural axis in patients following surgery and/or biopsy to target disseminated malignant tissue. This approach resulted in a dramatic impact on long-term survival rates (Patterson, 1953). Since then, major efforts have been placed on improving radiotherapeutic techniques in order to optimize radiation dose for the brain and spinal cord. The effective doses used are: 54-55 Gy to the posterior fossa (primary location of the tumour) and 35-36 Gy to the craniospinal axis (Choux , 1982; DeVita et al., 1997). Although these doses are required to improve the survival rate, they often produce severe neurological, neuropsychological and neuroendocrinological damage (Choux, 1982; Oberfield et al., 1986; Maureen et al., 1996). These sequelae are even more pronounced in children under the age of three. Therefore, radiation therapy is often delayed until that age in most centres (Jenkin, 1996). However, if no other treatment modality is possible, these aforementioned doses are often reduced by approximately 10% for these younger patients (Choux, 1982; DeVita et al., 1997). Although MB is one of the most radiosensitive tumours of the CNS, it seems that the maximum

benefits that can be achieved with conventional radiation have been reached. In this regard, although recent studies demonstrate an increase of 10-20% in the number of survivors, the number of survival years do not extended beyond 5 years (Jenkin et al., 1990).

5.3 Chemotherapy

Adjunctive chemotherapy has become part of the standard treatment for infants with MB since 1980 (Cohen and Parker, 1996). Chemotherapy is a way to try to delay or even replace post-irradiation in infants under the age of three, and to reduce the dose of craniospinal irradiation in low-risk patients in order to spare the detrimental cognitive side-effects as well as improve overall survival rates (Kühl, 1998). However, although not yet definitive, chemotherapy may potentiate the negative effects of radiotherapy on growth velocity (Olshan et al., 1992), and may also act as a negative factor in the development of secondary neoplasms, i.e. resistance (Parker et al., 1994). Some of the most commonly used chemotherapeutic agents for recurrent or progressive MB and their mechanisms of action are summarized in Table 3. Although these agents may be used individually, the best outcomes are obtained when they are used in multi-drug chemotherapy regimes and in combination with conventional doses of craniospinal radiation (Krischer et al., 1991; Parker et al., 1994). Studies investigating the use of chemotherapy prior to radiation give comparable results to those where both

treatments were given simultaneously (Cohen and Parker, 1996). Furthermore, reducing the dose of radiation, although potentially less damaging to the CNS, has been reported by Prados et al. (1989) to reduce the 5 year survival rates by 30% (Prados et al., 1989).

6. Project

6.1 Hypothesis and objectives

Although the clinical picture of MB has improved with the refinement of conventional therapeutic modalities, these treatments often result in a high incidence of neurotoxicity. It is therefore imperative that less toxic, more specific and natural therapeutic interventions be designed to reduce the incidence of these sequellae, as well as to improve survival rates for individuals diagnosed with MB.

Based on MB's presumed origin from portions of the EGL of the cerebellum or the VM, wehave begun to investigate whether "differentiation-induction" with growth factors shown to advance cerebellar maturation might control MB cell growth. In the past, we have demonstrated, using our novel MB cell line UM-MB1, that one such factor, bFGF/FGF-2, could advance MB maturation, slow its growth and ultimately kill it by apoptosis (Kenigsberg et al., 1997). These exciting results on this novel cell line and model for MB provided the impetus for the present research project and its two aims:

i) To establish and characterize two new human MB cell lines, to serve as

additional experimental models for this complex cerebellar tumour. The properties of these models will be compared with those of the original tumours from which they were derived as well as amongst MB cell lines. This comparative analysis could be instrumental in identifying prognostic or diagnostic indicators which may be important for future studies.

ii) To determine if bFGF/FGF-2 can reduce the proliferative potential of the aforementioned novel MB cell lines in a manner similar to that documented for UM-MB1. In addition, other members of the FGF family, namely FGF-1, FGF-5, FGF-6 and FGF-9, that have been more recently shown to be expressed in the developing cerebellum, will be investigated to determine if they induce responses similar to those noted with bFGF/FGF-2 in UM-MB1. According to the results of the above, it will also be attempted to correlate responsiveness of the MB cell lines to the FGFs with their selective expression of certain types of FGFRs.

Table 1: Molecular factors and cognate receptors involved in cerebellar neuronal development

Molecular factors	Receptors	Roles
Neuregulins (NRG)	**erbB3/HER3** **erbB4/HER4**	**Migration** (Rio et al., 1997)
Sonic hedgehog (shh)	**PTCH/smo**	**Proliferation** (Wechsler-Reya & Scott, 1997 Wallace, 1999)
Neurotrophins (NT)		
BDNF	**TrKB** (Segal et al., 1992)	**Outgrowth/Stabilization of neurites** **Promote survival/Prevent apoptosis** (Segal et al., 1995; 1997)
NT-3	**TrkC** (Segal et al., 1992)	**Fasciculation** (Segal et al., 1995)
NT-4/5	**TrkB**	**Outgrowth/Stabilization of neurites** **Promote survival** (Gao et al., 1995)

Table 1: Molecular factors and cognate receptors involved in cerebellar neuronal development cont'd

Molecular factors	Receptors	Roles
Fibroblast growth factors (FGF)		
aFGF/FGF-1 (Wilcox & Unnerstall, 1991; McAndrew et al., 1998)	**FGFR1-FGFR4 (all variants)***	
bFGF/FGF-2 (El-Husseini et al., 1994; Matsuda et al., 1994)	**FGFR1, FGFR3-FGFR4IIIc> FGFR1IIIb, FGFR2IIIc***	**Proliferation** (Tao et al.,1997) **Neurite outgrowth/differentiation** (Hatten et al., 1988)
FGF-5 (Hattori et al., 1997)	**FGFR1IIIc>FGFR2, FGFR3IIIc***	
FGF-6 (Ozawa et al., 1996)	**FGFR4IIIc>FGFR1, FGFR2IIIc***	
FGF-9 (Tagashira et al., 1995; Todo et al., 1998)	**FGFR2-FGFR4IIIc>FGFR3IIIb> FGFR1IIIc***	

(* in Ornitz et al., 1996)

Table 2: Nomenclature of FGF and human chromosomal location of the genes

Name	Alternative names	Chromosomal location
FGF-1	Acidic FGF (aFGF)	5q31.3-33.2
FGF-2	Basic FGF (bFGF)	4q26-27
FGF-3	INT-2	11q13
FGF-4	HST-1, k-FGF (kaposi)	11q13.3
FGF-5		4q21
FGF-6	HST-2	12p13
FGF-7	KGF (keratinocyte GF)	15
FGF-8	AIGF (androgen induced)	10q24
FGF-9	GGF (glial)	13q12-q13
FGF-10		5p12-13
FGF-11	FHF-3	17p12
FGF-12	FHF-1	3q28
FGF-13	FHF-2	Xq21
FGF-14	FHF-4	
FGF-15		
EGL-17		
BNL	Branchless	

(Adapted from: Szebenyi & Fallon, 1999)

Table 3: Drugs used for recurrent or progressive MB

General categories	Drugs	Mechanism of action
Alkylating agents and related compound	-Nitrosoureas	
	*lomustine (CCNU)	
	*carmustine (BCNU)	
	-Platinum compounds	Forms covalent bonds with DNA and thus impedes DNA replication
	*cisplastin	
	*carboplatin	
	-Nitrogen mustards	
	*cyclophosphamide	
Antimetabolites	-Folate antagonists	
	*methotrexate	Inhibits purine nucleotide synthesis
Plant derivatives	-Vinca alkaloids	
	*vincristine	Inhibits microtubule function, hence the formation of mitotic spindle
	*etoposide	Inhibits DNA synthesis by an action on topoisomerase II
		Inhibits mitochondrial function
Miscellaneous agents	*Procarbazine	Inhibits DNA and RNA synthesis
		Interferes with mitosis at interphase
		Monoamine oxidase (MOA) inhibitor

(Adapted from: Rang et al., 1995 and DeVita et al., 1997)

CHAPTER TWO:

ARTICLE

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INDUCTION OF DIFFERENTIATION AND APOPTOSIS IN HUMAN MEDULLOLASTOMA BY FIBROBLAST GROWTH FACTORS: A COMPARATIVE STUDY USING THREE NEW CELL LINES[1]

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Running title: *Fibroblast growth factors affect medulloblastomas*

Key words: brain tumour, medulloblastoma, fibroblast growth factor, fibroblast growth factor receptors, apoptosis, differentiation.

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[3] The abbreviations used are: FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GFAP, glial fibrillary acidic protein; EGL, external granular layer; MB, medulloblastoma; MAP, microtubule associated protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NF, neurofilament; NGF, nerve growth factor; NSE, neuron specific enolase; NT-3, neurotrophin-3; PTCH, patched; PCD, programmed cell death; Shh, sonic hedgehog; TGFβ, transforming growth factor beta; VM, ventricular matrix.

ABSTRACT

Members of the fibroblast growth factor (FGF) family play important roles in controlling mammalian brain development. In the cerebellum, FGFs promote neurogenesis as well as activate the differentiation of postmitotic neurons. This latter activity may prove invaluable in the management of medulloblastoma (MB), an embryonal brain tumour which is thought to arise from cerebellar neuronal precursors. In this regard, we previously found FGF-2 could slow the growth, advance the differentiation and ultimately trigger apoptosis of a MB cell line we developed and characterized, UM-MB1[1]. In the present study, we extend this investigation to include two new MB cell lines, HSJ and SYR, and test, in addition to FGF-2, other FGFs that localize to the developing cerebellum (i.e. FGF-1, -5, -6 and -9) for biological activity. We presently report that although all three lines, like their original tumours, exhibit immature neuronal phenotypes, differences in their doubling times, morphologies, states of differentiation, as well as their antigenic profiles are evident. Furthermore, only UM-MB1 and SYR, the two cell lines derived from classic variants of MB, respond selectively to FGF-2 and -9 with advanced differentiation, increases in doubling times, followed by apoptosis. Although all three cell lines express the pro-apoptotic effector Bax, anti-apoptotic Bcl-2 is only detected in UM-MB1 and SYR. When UM-MB1 and SYR are treated with FGF-2 or -9 and undergo apoptosis, expression of Bax

increases. Finally, while UM-MB1 and SYR exhibit immunoreactive sites for all four FGF receptor (FGFR) types, HSJ, whose growth is not suppressed by the FGFs only expresses two, FGFR1 and FGFR4. In conclusion, these findings suggest that members of the FGF family may act as growth suppressors for certain histopathological variants of MB that express specific FGFRs.

INTRODUCTION

Medulloblastoma (MB), a highly aggressive embryonal tumour of the cerebellum that occurs most frequently in childhood, accounts for close to 20% of all intra-cranial tumours in the pediatric population[2]. Despite marked improvements in surgical, imaging, and radiation techniques, as well as the introduction of adjuvant chemotherapy in its management, MB's high incidence for recurrence and proclivity for dissemination render its long-term clinical picture consistently poor[3].

Although little is known about the transforming events that underlie the development of this neoplasm, MB is thought to originate from two distinct germinal zones of the cerebellar cortex namely: **(1)** the earlier forming cerebellar neuroepithelium or ventricular matrix (VM)[4] which gives rise to the deep cerebellar neurons, Purkinje cells, neurons of the molecular layer as well as glia and ependyma[5] or, **(2)** a later forming matrix located to the outer surface of the cerebellum, the external granule layer (EGL) of Obersteiner[6,7] that gives rise exclusively to internal granule neurons[8]. The differential localization of calbindin-D_{28k} and class III β-tubulin which distinguish neuronal progeny of these two germinal zones[9] to the two most common variants of MB, suggest that the classic variant originates from the VM while the desmoplastic arises from cells of the EGL[10].

Regardless of MB's precise etiology, its derivation from the developing cerebellum is amply supported in studies which show this tumour

to express distinct differentiation and lineage-related antigens found in cerebellar neuronal progenitors[11-18]. Expression of these markers in MB suggests that this tumour can, if appropriately stimulated, reinstate a normal differentiation program to generate more mature and less aggressive progeny. To initiate this event, growth factors normally found in the developing cerebellum may be the most appropriate stimuli.

Unlike select neurotrophins which specifically impact the survival and differentiation of internal granule neurons, progeny of the EGL[19,20], the FGFs could, in view of their more widespread distribution in the developing cerebellum[21-23], influence cells of both germinal zones. In this regard, at least five members of the FGF family namely, FGF-1/aFGF[24], FGF-2/bFGF[25,26], FGF-5[27], FGF-6[23]and FGF-9[28-30] are found in the developing cerebellum. However, physiological roles for only one, FGF-2, in promoting cerebellar neurogenesis[31,32] and aspects of neuronal differentiation[33,34] have been evidenced to date. Since levels of FGF-2 in the developing cerebellum peak before the generation of the internal granule neurons[25], it is likely that FGF-2 is more important to the maturation of neurons that originate from the VM, the matrix from which the classic, and most common variant of MB is thought to arise [10,35].

In view of the above, we began testing the differentiating activity of FGF-2 for MB. Results from our study show that FGF-2 can promote the maturation of a new MB cell line that we developed and characterized, UM-

MB1[1]. This cell line, which originates from a classic MB tumour, responds to FGF-2 with advanced neuronal-like differentiation, growth suppression followed by apoptotic death[1]. In addition, these responses to FGF-2 are maintained in vivo for UM-MB1 grown as intracerebral xenografts[36]. However, UM-MB1 is only one cell line and representative of one variant of this tumour, and FGF-2, only one member of this growth factor family that localizes to the developing cerebellum. Consequently, in the present study, we first developed and characterized two new MB cell lines to include in our panel. Using all of our three cell lines, two derived from classic tumours, one from a desmoplastic one, we proceeded to study the effects of all of the aforementioned FGFs for differentiating, cytostatic and apoptotic activities. This comparative study was designed to help identify markers shared by responsive tumour cells that could have prognostic and/or diagnostic value. Furthermore, it may provide clues for elucidating the underlying mechanism(s) by which the FGFs control the aggressive behaviour of this tumour.

MATERIALS AND METHODS

Characterization of original cerebellar tumours

Surgical specimens were obtained at craniectomy from two intrinsic MB tumours located to the posterior fossi of two- and three-year-old males. Tumoural samples were either frozen, or formalin-fixed and paraffin embedded for the immmunohistochemical and histochemical analyses, or placed immediately in saline for preparing the two continuous MB cell lines, HSJ and SYR.

Establishment, maintenance and growth factor treatment of MB cell lines

The tumour specimens were minced and mechanically dissociated and the continuous lines selected by subsequently passing proliferating cells. These initial cultures, and the two new adherent cell lines generated from them, HSJ and SYR, as well as our previously characterized MB cell line, UM-MB1[1], were all maintained in logarithmic phase growth in DMEM, (Gibco, Burlington, Ont.) supplemented with 10 mM HEPES (pH 7.4) (Gibco), 10% FBS (Gibco) and penicillin/streptomycin (Sigma, Oakville, Ont.) and subcultured weekly by trypsinization[1]. To study the effects of the FGFs, equimolar (6.25 pM - 6.25 nM) concentrations of human recombinant acidic FGF (FGF-1), basic FGF (FGF-2), FGF-5, FGF-6 and FGF-9, (R& D Systems Inc, Minneapolis, MN) were added to all three MB cell lines immediately after seeding and replenished every two days thereafter.

Viability and proliferation assays

Changes in MB mitochondrial dehydrogenase activity, viable cell number and rates of proliferation following FGF treatment were assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) reduction colorimetric assay[37], trypan blue exclusion and ^{3}H-thymidine incorporation, respectively. For thymidine incorporation, cells seeded on 24 multiwell dishes were incubated with 0.5 µCi methyl-^{3}H-thymidine /well (25 Ci/mmol, Amersham Pharmacia Biotech, Baie d'Urfé, Qc) for 12 h[38] on various culture days. Wells were subsequently washed extensively with cold PBS, and TCA precipitable radioactivity solubilized in 0.1 N NaOH/1% SDS measured by liquid (Aquasol-2, Mississauga, Ont.) scintillation spectrometry.

Immunocytochemistry:

(a) Lineage analysis

The presence of specific lineage-related antigens was assessed immunocytochemically on formalin (3.7%) fixed cultures as detailed previously[1]. Primary antibodies used at the indicated working dilutions include rabbit primaries such as: anti-bovine neuron specific enolase (NSE) (1:300) (Dako, Dimension Labs, Mississauga, Ont.), anti- neurofilament (NF) 68 kd (1:200), anti-NF 150 kd (1:400), anti-NF 200 kd (1;400; Chemicon, Temecula, CA), anti-bovine glial fibrillary acidic protein (GFAP) (1:350; Dako) and the following mouse monoclonals: anti-bovine

synaptophysin clone Sy35 (1:20; Dako), anti-vimentin clone V9 (1:50, Dako), anti-microtubule associated protein (MAP) 1 clone HN-1, anti-MAP2 clone HN-2 and anti-MAP1b (MAP5) clone AA6 (all at 1:400; Sigma). Immunoreactive sites were revealed using the Cy3-conjugated goat antibodies raised against rabbit or mouse IgG (1:1000; Jackson Research Labs, West Grove, PA). Fluorescent observations were recorded on T-MAX 400 ASA film (Kodak, Rochester, NY). Specificity of the immunoreactions was determined routinely with omission of the primaries and inclusion of appropriate pre-immune sera.

(b) Fibroblast growth factor receptor (FGFR) expression

Immunoreactivities for FGFR1 through FGFR4 were examined on similar preparations using the following rabbit polyclonal antibodies raised against peptides corresponding to amino acid sequences mapping to the carboxy termini of the precursor forms of the following FGFRs of human origin: Flg (C-15) anti-(FGFR1) (1:300), Bek (C-17) anti-Bek (FGFR2) (1:400), FGFR3 (C-15) anti- (FGFR3) (1:300) and FGFR4 (C-16) anti-(FGFR4) (1:300) (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). Immunoreactive sites were revealed and recorded as detailed above. Specificity of the immunoreactions was determined routinely by omission of appropriate primaries, inclusion of the appropriate pre-immune sera and by using the rat L6 myoblast cell line which lacks any endogenous FGFR[39] as negative control, and the human MCF7 breast cell line (ATCC, Manassas,

VA) which expresses FGFR1, 2 and 4[40,41] and the human Caco-2 intestinal epithelial cell line (ATCC) which expresses FGFR3[42] as positives.

(c) In situ detection of DNA fragmentation

The presence of apoptotic cell death was assessed by the direct immunofluorescent detection of digoxigenin-labeled genomic DNA using the Oncor Apoptag in situ apoptosis detection kit (Oncor, Gaithersburg, MD) in control and FGF-treated cultures. Samples were simultaneously stained with Hoechst (Ho) 33342 (5 µg/ml, Sigma) to monitor for changes in nuclear morphology. Immunoreactive nuclei were only scored as apoptotic when evidence of necrotic cell death was ruled out as detailed[1].

Western blots

Exponentially growing MB cells maintained in the absence (control) or presence of 0.625 nM FGF-2 or FGF-9 for 3 to 4 (for NF expression) or 7 (for Bax and Bcl-2 expression) culture days were collected by trypsinization, washed several times in PBS by centrifugation and homogenized in either SDS sample buffer (for NF) or Tris-buffered saline lysis buffer (137 mM NaCl and 20 mM Tris pH 8.0)[43] containing 1% NP-40, 10% (V/V) glycerol and 0.1% SDS (for Bax and Bcl-2) and, in both instances, the following protease inhibitors; leupeptin (0.2 µg/ml), aprotonin (5 µg/ml) and phenyl methyl sulfonyl fluoride (PMSF) (1 mM). Equal amounts of cell homogenate protein (70 µg per well) were separated by SDS PAGE (7.5% for NF and 12% for Bax and Bcl-2), transferred electrophoretically onto

Immobilon P membranes (Millipore, Nepean, Ont.). Equal protein loading was controlled by Ponceau red staining of membranes. After washing out the stain, membranes were blocked for 1 h at room temperature in 5% skim milk /0.2% Tween in Tris-buffered saline (TBST), and probed with either of the three anti-NF antibodies (1:500, Chemicon) or mouse monoclonal anti-Bcl-2 (1:500; Transduction Labs, Mississauga, Ont.) or purified mouse anti-Bax (2 µg/ml; Pharmingen, Mississauga, Ont.). Membranes were then extensively washed and incubated for 20 mins with either HRP-conjugated donkey anti-rabbit IgG (1:5000) or sheep anti-mouse IgG (1:5000) (both from Amersham Pharmacia Biotech). Immunopositive bands were visualized using enhanced chemiluminescence (Amersham Pharmacia Biotech) and X-ray film (Kodak).

Ultrastructure

All three cell lines (control and FGF-treated) were washed with PBS, fixed in cold 2% glutaraldehyde in 0.2M sodium cacodylate buffer, dehydrated in alcohol, Epon embedded directly in their culture flasks and further processed for electron microscopy as previously described[1].

RESULTS

Characteristics of the original tumours

The cerebellar tumours from which HSJ and SYR cell lines originate were both comprised primarily of small, undifferentiated cells, with extremely high nuclear to cytoplasmic ratios, and hyperchromatic nuclei, all indices of their immaturity (Fig. 1). While the mitotic index was extremely high for HSJ, it was low to moderate for SYR. Nevertheless, numerous Homer-Wright rosettes were evident in both. Generalized cytoplasmic immunoreactivity for synaptophysin was noted in the majority of the neoplastic cells from HSJ, and approximately 30% from SYR. In contrast, GFAP immunoreactive sites colocalized only to the vascular tissue in both tumours. Furthermore, the majority of the neoplastic cells in both tumour specimens were intensely NSE immunopositive. Histological analyses of HSJ indicate that this densely cellular and reticulin fibre-rich tumour has the cytological and morphological characteristics of the desmoplastic variant of MB. Although low to moderate desmoplasia was seen in SYR, it was mainly localized to the spinal canal, and like UM-MB1[1], it represents a classic variant of MB.

Properties of the MB Cell Lines

Immunological

The lineage and differentiation-related antigenic profiles of the two new cell lines, HSJ and SYR, were analysed and compared to those of their

original tumours. An extensive immunocytochemical comparison was done between all of our three MB cell lines. As shown in Table 1, the immunophenotypes of the new MB lines are consistent with those of their original tumour specimens (both positive for synaptophysin and NSE). Although, all three MB lines express antigens consistent with a neuronal etiology, differences were noted in the intensity of staining for some antigens, and the expression of others. In this regard, UM-MB1 is unique in that it does not express any MAP2 and SYR is the only cell line that is immunonegative for NF-H. While vimentin and MAP5 immunoreactive sites are noted in all three lines, their staining intensity is greatest in UM-MB1 (Fig. 2A) and HSJ (Fig. 2B), respectively.

Morphology and Growth

The three cell lines we developed and characterized to date represent predominantly adherent cultures. They are however, distinct, as is evident from their antigenic profiles (Table 1), sizes, shapes (Fig. 2), states of differentiation and rates of division. As previously reported, UM-MB1 are poorly differentiated round to oval-shaped cells which measure 15-20 μm in diameter and extend short neurite-like processes[1]. Our new MB cell line SYR, like UM-MB1, is also extremely immature as evidenced ultrastructurally (data not shown). SYR cells are small (mean cell diameter = 10-12 μm), round, often multinucleated and do not, at the light microscopic level appear to have any process outgrowth. The other newly characterized MB cell line,

HSJ, contains larger (mean soma diameter = 20-25 μm) cells which are more mature as evident by their lower nuclear to cytoplasmic ratios (data not shown). These larger cells extend extremely long bipolar fibrous-like processes which measure on average 75-100 μm and can reach lengths up to ten times those of their cell bodies. Doubling times, determined from exponentially growing HSJ and SYR cultures by linear regression are, 29 (r=0.98) and 48.4 h (r=0.99), respectively.

Specific FGFs differentially affect MB cell mitochondrial dehydrogenase activity, proliferation and survival

In order to quickly screen the FGFs for growth suppressing activity on our new MB cell lines as well as extend this study with UM-MB1, we proceeded first to use the MTT "viability" assay. This assay rapidly monitors for changes in functional mitochondrial dehydrogenase activity which should reflect alterations in viable cell number[37]. Based on our previous findings with UM-MB1, the MTT assays were done after 7 days of growth factor treatment, a time when FGF-2 was shown to significantly decrease UM-MB1 number as determined manually by counting viable cells that exclude trypan[1]. Viable cell counts were done similarly in parallel with the MTT assays.

Values obtained from both these determinations are presented on Table 2 and clearly indicate that changes in mitochondrial dehydrogenase activities do not accurately reflect changes in MB viable cell number. This

was particularly evident for HSJ, where FGF -5, -6 and -9 are clearly mitogenic yet they significantly decrease, rather than increase mitochondrial dehydrogenase activities. Although the changes in viable cell number and OD from the MTT assay were directionally similar for UM-MB1 and SYR treated with FGF-2 and -9, they were magnitudinally discrepant. FGF-2 produces a much greater decrease in cell number than in ODs when compared to appropriate control values.

For UM-MB1, the results of the MTT assay were at least directionally consistent with the viable cell counts (Table 2) and thymidine incorporation values. In this regard, FGF-2 is the most potent growth suppressor for UM-MB1 (18 ± 2 vs 25 ± 4.8% of control for thymidine uptake on day 5 for 0.625 nM FGF-2 or -9 respectively).

Although the MTT assay shows FGF-2 and -9 to be equipotent growth inhibitors for SYR (Table 2), by trypan blue exclusion, and thymidine incorporation, FGF-2 is more potent. For example, thymidine incorporation in SYR after 4 days of growth factor treatment is suppressed to 50 or 71% of control with equimolar (0.625 nM) amounts FGF-2 or FGF-9, respectively. Inhibition of the growth of SYR with either FGF is maximal at 0.625 nM (equivalent to 10 ng/ml FGF-2) (data not shown). Furthermore, when tested in combination at their maximal doses (0.625 nM) on day 4, inhibition of thymidine uptake was not additive nor significantly different from FGF-2 alone (51% of control).

In order to fully characterize the growth inhibitory response of our new MB cell line SYR to FGF-2 we proceeded to compare, in parallel cultures, time-dependent changes in viable cell number, thymidine incorporation, and cell death. As shown in figure 3, significant decreases in thymidine uptake are first evident in SYR by 3 days of FGF-2 treatment (Fig. 3B), concomitant with significant changes in viable cell number (Fig. 3A). Furthermore, although FGF-2 promotes death of SYR (Fig. 3C), in view of the magnitude of this phenomena (12.6% of total cells dead on day 7), FGF-2 most likely exerts its greatest effects on tumour cell number (26% of control on day 7) by slowing cell proliferation. This is reflected by a marked increase in SYR's doubling time which goes from 48.4h (r=0.99) in control, to 61.9h (r=0.97) following FGF-2 (10 ng/ml) treatment.

FGF-2 and FGF-9 advance differentiation and trigger apoptosis of two MB cell lines

We previously showed that FGF-2 could induce three responses in UM-MB1, which apparently occur sequentially[1]. In this regard, FGF-2 first suppresses the UM-MB1 cell growth while it also advances maturation by increasing neurite-like outgrowth, NF expression and ultrastructural cytodifferentiation. Finally, this growth factor triggers death in these cells by apoptosis[1]. We presently report that UM-MB1 responds to FGF-9 in an identical fashion (Fig 4). As seen in the photomicrographs in figure 4 taken at two time points, FGF-9, like FGF-2, first increases process outgrowth and

slows cell doubling before it impacts UM-MB1 viability.

Growth of SYR, like UM-MB1, is only inhibited by FGF-2 and FGF-9 (Table 2). Although these FGFs advance SYR ultrastructural maturation (data not shown), they do not enhance neurite-like process elongation nor do they upregulate NF expression (data not shown).

At the light microscopic level, the only notable change in SYR following FGF treatment is that in cell density (see Fig. 5 for example) due to both the inhibition of proliferation (see Fig. 3) and the triggering of apoptosis, as illustrated in figure 5 by the in situ detection of fragmented DNA.

FGF-2 and FGF-9 affect expression of Bcl-2 and Bax in MB cells

The expression of Bcl-2 and Bax, anti- and pro-apoptotic products of the bcl-2 family of cell death-regulating genes were examined in our MB cell lines. While both apoptotic effectors are detected by Western blot analyses in UM-MB1 and SYR, HSJ only expresses Bax , albeit at higher levels (Fig. 6). By day 7, levels of Bax are found to be significantly greater in FGF-treated UM-MB1 and SYR cultures when compared to same day controls (Fig. 6). Interestingly, although increases in Bax were most pronounced in SYR following FGF-treatment, the apoptotic responses of UM-MB1 to these FGFs are greater. In addition, while the upregulation of Bax in UM-MB1 is greater following FGF-9 treatment, it is FGF-2 that triggers the larger apoptotic response in these cells. The levels of Bcl-2, which are

barely detectable in untreated control UM-MB1 cells, also increase following FGF application (Fig. 6). In contrast, these growth factors do not modulate expression of Bcl-2 in SYR nor do they induce it in HSJ (Fig.6).

MB cell lines express different FGFR types

It is well known that the FGFs mediate their biological effects through membrane-bound receptors. In this regard, four tyrosine kinase receptor types coded by four distinct genes, FGFR1-4, have been identified and shown to be involved in the FGF signalling network. However, alternative splicing of the genes encoding for FGFR1, FGFR2 and FGFR3, further increases the number and complexity of this family by generating variants with distinct binding and signalling capacities[44]. To determine which FGFR types can be detected inmmunocytochemically in our cell lines, we used commercially available defined epitope specific antibodies which should recognize all known membrane bound variants of each receptor type. Results from these immunocytochemical analyses are shown in figure 7. We find both UM-MB1 and SYR, the two MB lines derived from classic tumours that respond to FGF-2 or -9 with differentiation, growth suppression and death, display immunoreactive sites for all four FGFR types (Fig. 7). In contrast, HSJ, which originates from the desmoplastic tumour, and is not growth suppressed by any of the FGFs, only exhibits immunoreactivity for FGFR1 and FGFR4 (Fig. 7).

Although receptor immunostaining associates primarily with the

plasmalemma in all three cell lines, the staining pattern is somewhat different between lines and receptors. In this regard, FGFR2 more frequently associates with the neurite-like processes and growth cone-like structures in UM-MB1. In contrast, FGFR1-like immunoreactivity is consistently diffuse. This diffuse pattern for FGFR1 was also seen in the human MCF7 breast cells which serve as our positive controls. In HSJ, FGFR1 is found to associate with both soma and processes while FGFR4 localizes more to the body. In both cell lines that were immunopositive for FGFR3 (UM-MB1 and SYR), FGFR3 is seen on the cell surface as well as with intracellular perinuclear vesicles. This latter type of pattern was not noted in the human Caco-2 intestinal epithelial cell line which serves are our positive control for FGFR3. And finally, treatment of these MB cells with FGF-2 or -9, did not change the pattern or relative intensity of the immunoreactions for these receptors nor did it induce expression of any in instances when they were not normally detected (i.e. untreated HSJ).

DISCUSSION

In the present report we demonstrate, that physiological concentrations of certain members of the FGF family, can advance the maturation, suppress the growth, and trigger apoptosis of two MB cell lines that we developed and characterized. These two cell lines , UM-MB1 and SYR, originate from classic MB tumours and express immunoreactive sites for all four FGFR types. In contrast, our third cell line, HSJ, derived from a desmoplastic tumour, responds to the same FGFs with accelerated growth and only expresses FGFR1 and FGFR4. Although the pro-apoptotic regulatory protein Bax can be detected in all three lines, Bcl-2 is only noted in UM-MB1 and SYR. Furthermore, when UM-MB1 and SYR are treated with FGF and undergo apoptosis, expression of Bax increases. Interestingly however, Bcl-2 levels also increase in UM-MB1 treated with these same growth factors.

Consistent with a neuronal etiology for MB, all three tumours, and the cell lines generated from them, express neuronal lineage-related antigens. Although these lines represent the clonal expansion of select tumoural cells, they are representative models for MB since they do retain many of the properties of most of the transformed cells in the original tumour specimens. For example, the state of differentiation of UM-MB1[1] and SYR, as evidenced ultrastructurally with their high nuclear to cytoplasmic ratios, sparsity of cytoplasm, rough endoplasmic reticulum and Golgi (data not shown) is

consistent with the immature neoplastic cells found in classic variants of this tumour[35]. In contrast, the pale islands of desmoplastic MB represent areas of more advanced cellular maturity[11,45] and HSJ, the cell line derived from this variant, exhibits a more advanced state of cytodifferentiation. Cells of these pale islands however, have been documented to have low Ki-67 labelling indices[11], while the doubling time for HSJ is the shortest of our three cell lines. Although the proliferation rates of the cell lines may appear inconsistent with their states of maturity, they are consistent with the mitotic indices of the original tumour specimens which are highest for HSJ, and lowest for SYR.

When looking at the immunocytochemical profiles of our three MB cell lines, the predominant immunophenotype is neuronal-like, similar to that noted for the majority of MB lines generated to date[1,46-52]. Although it is difficult to speculate on the biological and behavioural significance of the subtle differences in the antigenic profiles of our cells, the higher expression of vimentin in UM-MB1 and SYR, cells derived from classic MB, is consistent with that documented in the literature for this tumour variant[53]. This high level of expression for vimentin, an intermediate filament found in neuroepithelial progenitors and neural precursors that appears prior to that of the NFs in cells derived from classic MBs is noteworthy, as it corroborates the proposed etiology of this variant from a more primitive germinal layer of the cerebellum, the VM[4]. On the other hand, the complete absence of NF-H in

SYR may simply hold structural, rather that developmental significance for a cell that is round and devoid of processes.

The effects of the FGFs on the MB cells were screened by comparing results from a simple commonly employed "survival" or "proliferation" MTT assay which uses the activity of mitochondrial dehydrogenases as an index of cell number, to that determined by manual counting of viable cells that exclude trypan. The conflicting results we obtain from these assays for HSJ in particular should flag a cautionary note as they clearly suggest that many variables, other than loss of cell viability may alter mitochondrial enzymatic activity. The marked decreases in mitochondrial activities in HSJ treated with FGFs noted concomitantly with proliferative rather that cytostatic or cytotoxic responses has also been reported for human fibroblasts treated with transforming factor β_1 (TGFβ_1)[54]. Inconsistencies between viable cell number and MTT values in cells induced to proliferate has be suggested to arise from the increase in S phase duration and decrease in G_1, the phase in which the synthesis of mitochondrial dehydrogenases takes place[54]. The differentiating activities of the FGFs should be taken into consideration as well. When FGF-2 and -9 advance cytodifferentiation, as is the case with UM-MB1[1] and SYR (data not shown), intracellular organelles, such as mitochondria increase and so should their enzymes. Therefore, a more mature MB cell, treated with FGF, would have higher mitochondrial dehydrogenase activity per cell than its less differentiated untreated

counterpart.

Of all the FGFs tested in our study, only two, FGF-2 and -9, advance differentiation, slow growth and trigger apoptosis of our two cell lines derived from classic MB tumours. In contrast, HSJ, the cell line derived from a desmoplastic tumour responds to these same growth factors as well as FGF-5 and -6 with increased proliferation. On an equimolar basis, all these FGFs are equipotent mitogens for HSJ while FGF-2 is the most effective growth inhibitor for UM-MB1 and SYR. Nevertheless, as the effects of FGF-2 and -9 on UM-MB1 and SYR are not additive, they likely act via common mechanism(s), possibly via common receptor(s) and signalling pathway(s).

From our immunocytochemical data it appears that the differential expression of the four FGFR types in our MB cell lines may underlie their distinct responses. Both UM-MB1 and SYR display intense immunoreactivity for all four FGFR types while HSJ is only immunoreactive for FGFR1 and FGFR4. Therefore, the differentiating, cytostatic and cytotoxic effects of FGF-2 and -9 are likely mediated by FGFR2 or FGFR3. However, based on the ligand specificities of the FGFRs, the IIIc variants of these receptors which preferentially bind FGF-2 and-9, in particular FGF-2[55] are the isoforms that likely mediate the effects of these FGFs on UM-MB1 and SYR. In support of this, and in agreement with MB's histogenesis from the developing cerebellum, within the CNS, FGFR1, FGFR2 and FGFR3 are expressed predominantly in their IIIc forms[56]. The expression of the specific splice

variants of these receptors in MB is currently under investigation in our laboratory using RT-PCR and splice-specific restriction digests.

When comparing responses between cell lines, it is evident that the FGFs promote the greatest changes in differentiation and the largest apoptotic response in UM-MB1. In this regard, while FGFs advance ultrastructural maturation in both UM-MB1 and SYR, they only induce neurite-like outgrowth and increase NF expression in UM-MB1[1]. Furthermore, after 7 days of FGF-2 treatment, more than 50% of UM-MB1 cells undergo apoptosis while only a small percentage of SYR die. Therefore, a correlation may exist between the extent of cellular differentiation and apoptosis in MB.

The apoptotic response to FGF, which appears coupled to or dependent upon differentiation in MB, is consistent with this tumour's etiology from immature neuronal precursors. Furthermore, it suggests that FGF may be stimulating MB to reinstate a more normal differentiation program along a neuronal-like lineage pathway. In this regard, during embryonic and early postnatal periods, more than half of all neuronal precursors are eliminated by an active process called "programmed cell death" (PCD)[57]. MB, which is thought to originate from such precursors, may have arisen as a result of failed PCD possibly due to their inability to respond to differentiating agents that prime them to exit the cell cycle, or, undergo apoptosis if growth factor deprived. Fibroblast growth factors may

reinstate that response in this tumour by any one, or combinations of the following mechanisms:

(1) FGF may act to initiate differentiation, a process which not only primes cell cycle exit in neuronal progenitors but increases their expression of apoptotic effectors and mediators (reviewed in [58]). In support of this, we find FGF-2 or -9 to increase the levels of the pro-apoptotic modulator Bax in both SYR and UM-MB1 cells committed to die. Furthermore, Bax has been shown to play essential roles in a number of different neuronal death paradigms[59-62] including those that take place in developing cerebellar neurons[63]. However, since Bax promotes cell death when it homodimerizes and is inactive when it heterodimerizes with anti-apoptotic family members like Bcl-2, (reviewed in [64]) an increase in Bax does not automatically imply more apoptosis. In this regard, we find FGF-2 and -9 to increase Bax more in SYR than UM-MB1 although UM-MB1 undergoes the most death. This may be due to the fact that Bcl-2, which antagonizes Bax's apoptotic activity, is constitutively expressed more abundantly in SYR. Furthermore, separate from its role as an anti-apoptotic effector, Bcl-2 has recently been shown to prevent cell cycle progression (reviewed in [64]). Therefore, it is possible that FGFs increase Bcl-2 in UM-MB1 to slow its growth and, the absence of Bcl-2 in HSJ may underlie its rapid doubling rate.

(2) FGF may prime differentiation of MB yet alone, be insufficient to maintain a viable fully differentiated state. In support of this, FGF has been

shown to prime a number of neuronal progenitors for responsiveness and dependence upon other neurotrophic and differentiating factors like NGF for survival. In primary chromaffin cell cultures[65] and MAH, a sympathoadrenal progenitor cell line[66], FGF has been found to initiate neuronal-like differentiation which creates a responsiveness and dependence in these cells upon other neuroactive substances for their long-term survival. In the absence of these other substances, these cells, like our MB lines treated only with FGF, die.

(3) FGF may trigger apoptosis in MB tumours by promoting their differentiation while they continue to divide. Such a conflict has been thought to induce inappropriate cell cycle genes that initiate the signalling pathways leading to apoptosis[67]. Fibroblast growth factor-2 has been shown to act similarly in differentiated oligodendroglia which make an attempt to re-enter the cell cycle but, instead, undergo apoptosis[68].

FGF-2's antimitotic activity for UM-MB1 and SYR may be mediated by a number of different mechanisms as well. FGFs could antagonize mitogenic signals, or, induce the expression of cytostatic ones. In the case of the former, the activity of Sonic hedgehog (Shh), which has been found to increase the proliferation and prevent the differentiation of cerebellar granule cell precursors[69-70] can be antagonized by FGF-2[69]. On the other hand, the FGFs may slow MB growth by upregulating TGFβ expression and release from these cells. It may reinstate a growth inhibitory autocrine loop that is

normally mediated by this cytokine yet found to be lost in MBs that are hyperdipoloid[71] like our own[1,72]. Preliminary findings from immunoneutralization studies suggest that FGF-2 may suppress UM-MB1 cell growth by increasing availability of bioactive TGFβ (unpublished personal observations).

Finally, while several agents like phenylacetate[73], human T-lymphotrophic retrovirus-1[74] and cholera toxin β[75] have been shown to promote MB cell differentiation, none trigger cell death. On the other hand, betulinic acid[76], manumycin A[77], lovastatin[78] or CD95 receptor ligands[79] induce apoptosis of human MB cells without affecting their differentiation. Furthermore, neurotrophins like NGF and NT-3, known for their differentiating activity in the nervous system and the developing cerebellum, promote apoptosis without advancing the maturation of TrkA- or TrkC-transfected MB cells, respectively[80,81]. Therefore, our results are the first to show that one substance, FGF, can modify all three parameters in MB, their state of differentiation, rate of division, and survival. These findings are both novel and physiologically relevant as they corroborate this tumour's etiology from the developing cerebellum and suggest that MB can, if appropriately stimulated, reinstate a more normal type of differentiation program. Although ineffective in completely eliminating MB cells and possibly useful for only one variant of this tumour that expresses specific FGFRs, FGF's potential as an anti-tumoural agent for MB certainly warrants further

investigation.

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Table 1. Immunocytochemical Profiles of the New MB Cell Lines: A Comparative Analysis Using Anti-Lineage and Differentiation-Specific Antibodies

Antibody specificity	UM-MB1	HSJ	SYR
NSE	++	++	++
Synaptophysin	+	+	+
Vimentin	++++	++	+++
GFAP	-	-	-
Map1	+++	+	++
Map2	-	+	+/-
Map5	++	++++	+
NF-L*	++	+++	+
NF-M*	++++	+++	++
NF-H*	++	+/-	-

The results presented in this table summarizes the antigenic profiles of our three human MB cell lines, UM-MB1, HSJ and SYR as determined on formalin-fixed cultures by immunocytochemistry and, when indicated by *, on cell homogenates by Western blotting as well (see Materials and Methods for details). Intensity of the immunoreaction is graded as follows;: -, negative; +/- to ++++, weakly to intensely positive.

Table 2. FGFs May Differentially Affect MB Cell Viabilty and Mitochondrial Dehydrogenase Activities

CELL LINE	UM-MB1		HSJ		SYR	
	Viable Cells (% of control)	OD MTT assay (% of control)	Viable Cells (% of control)	OD MTT assay (% of control)	Viable Cells (% of control)	OD MTTassay (% of control)
CONDITION						
Control	100±1.42	100±1.18	100±1.89	100±2.15	100±0.92	100±2.47
FGF-1	112±6.68	93.2±1.45	113±2.45	95±2.44	107±2.64	98.3±2.75
FGF-2	41.8±2.92**	63.7±1.78**	128±2.65**	91.5±4.28	35.9±.41**	78.8±3.63*
FGF-5	92.4±4.99	98.8±2.63	123±6.95*	72.8±1.11**	87.6±5.13	102±5.09
FGF-6	103±1.00	96.3±1.52	131±2.13**	71.8±3.09**	87.7±1.47	105±5.44
FGF-9	68.1±3.59**‡a	79.5±2.32**‡b	121±2.74*	65.2±1.83**	63.6±3.06**‡c	77.3±1.48*

MB cells were seeded at the same initial densities and maintained in the absence (control) or continuous presence of equimolar (0.625nM) amounts of the rhFGFs indicated above for 7 days. Viable cell number and mitochondrial dehydrogenase activity were determined by trypan blue exclusion or the MTT colorimetric assay respectively as detailed in Materials and Methods. For comparative purposes, values are expressed as percentage of control ± S.E.M., n= 4 or 6 for viable counts or MTT assay respectively. Values significantly different from their appropriate controls indicated with * for $P<0.01$ and ** for $P<0.001$. Significantly different among FGFs-treatments indicated with ‡ for $P<0.001$ (One way ANOVA, $P<0.0001$, $F=55.54;57.56$ or 90.63 for a, b and c respectively, post hoc Tukey Kramer multiple comparison test).

FIGURE LEGENDS

Figure 1. Photomicrographs of the resected MB tumours HSJ (**A**) and SYR (**B**). Both lesions are composed primarily of small immature cells with high nucleocytoplasmic ratios. Note the clustered architecture in **A** consistent with the desmoplastic variant of MB. Arrows indicate aggregates of Homer Wright (neuroblastic) rosettes. (H & E). Scale bar represents 20 μm.

Figure 2. Fluorescent micrographs show immunoreactive sites for vimentin in UM-MB1 (**A**), MAP5 in HSJ (**B**) and NF 150KD in SYR cultures (**C**). Note the marked difference in the general morphology and size of these three MB cell lines. Scale bar represents 20μm.

Figure 3. FGF-2 affects the proliferation and viability of the SYR cell line. Cells were seeded in 24 MW dishes and maintained in the absence (open circles) or continuous presence of 0.625 nM FGF-2 (closed circles) for the days indicated on the abscissae. Changes in viable cell number (**A**), ^{3}H-thymidine incorporation (**B**), and cell morbidity (**C**) were determined as detailed in Materials and Methods. Values represent means ± S.D. from quadruplicate or triplicate wells for **A** and **B** or **C** respectively. Values in **A** are significantly different from same day controls from day 2 onwards at $P<0.0001$ for days 2,5,6 and 7 and $P<0.001$ for days 3 and 4. In **B**, values from day 3 onwards are significantly different from their appropriate controls

at P<0.01 for day 3, P<0.001 for day 4 and P<0.0001 for days 5 and 6. In **C**, values are significantly different from controls at P<0.0001 for days 1, 3, 5,6 and 7 and at P<0.001 for day 4 (Student's t-test).

Figure 4. Fibroblast growth factor-2 and FGF-9 slow the growth, induce neurite extension and promote the death of UM-MB1 cells in culture. Cells were seeded at identical densities in 24 MW dishes and maintained up to 8 days in vitro in the absence (control) or presence of 0.625 nM FGF-2 or FGF-9. Photomicrographs taken with phase contrast optics of unfixed cultures show that both FGFs slow the growth and promote the extension of neurites from these cells by day 4 of treatment. Although untreated control cells are still viable by day 8, FGF-treated cells are predominantly dead. Scale bar represents 40μm.

Figure 5. Morphological evidence for FGF-2-induced apoptosis in SYR cells. Cells were seeded in the absence (**A** and **B**) or continuous presence of 0.625 nM FGF-2 (**C** and **D**) for 7 days. Identical fields (**A** and **B** for control or **C** and **D** for FGF-2-treated) were processed for DNA stainability with Hoechst 33342 (**A** and **C**) as well as for the immunofluorescent detection of digoxigenin-labeled DNA (**B** and **D**) as detailed in Materials and Methods. Note the presence of immunofluorescence in FGF-2 treated cultures (**D**) which is associated with cells in which the nuclei are

fragmented and chromatin condensed (**C**). Normal chromatin (**A**) that is not immunopositive (**B**) is seen in control. Scale bar represents 20μm.

Figure 6. Effects of FGF-2 and FGF-9 on Bcl–2 and Bax expression in MB cell lines as determined by Western blot analysis. Cell lysates prepared from cultures maintained in the absence (Ctrl) or presence of equimolar (0.625 nM) concentrations of FGF-2 or FGF-9 for 7 days were separated by SDS-PAGE, transferred electrophoretically and probed with specific antibodies as detailed in Material & Methods. These data are representative of at least 3 separate determinations.

Figure 7. MB cell lines differentially express FGF receptors. Fluorescent photomicrographs of the immunocytochemical localization of FGFR1 through FGFR4 (indicated as R1-R4) in UM-MB1, SYR and HSJ cell lines. Cells were seeded on glass coverslips in 24 MW dishes, maintained in culture for several days before formalin fixing and processing for the immunocytochemical detection of FGFR-like immunoreactivity as detailed in Materials and Methods. Note that intense immunoreactivity for all four receptor types is evident in UM-MB1 and SYR while HSJ only exhibits FGFR1- and FGFR4-like immunoreactive sites. Scale bar represents 20μm.

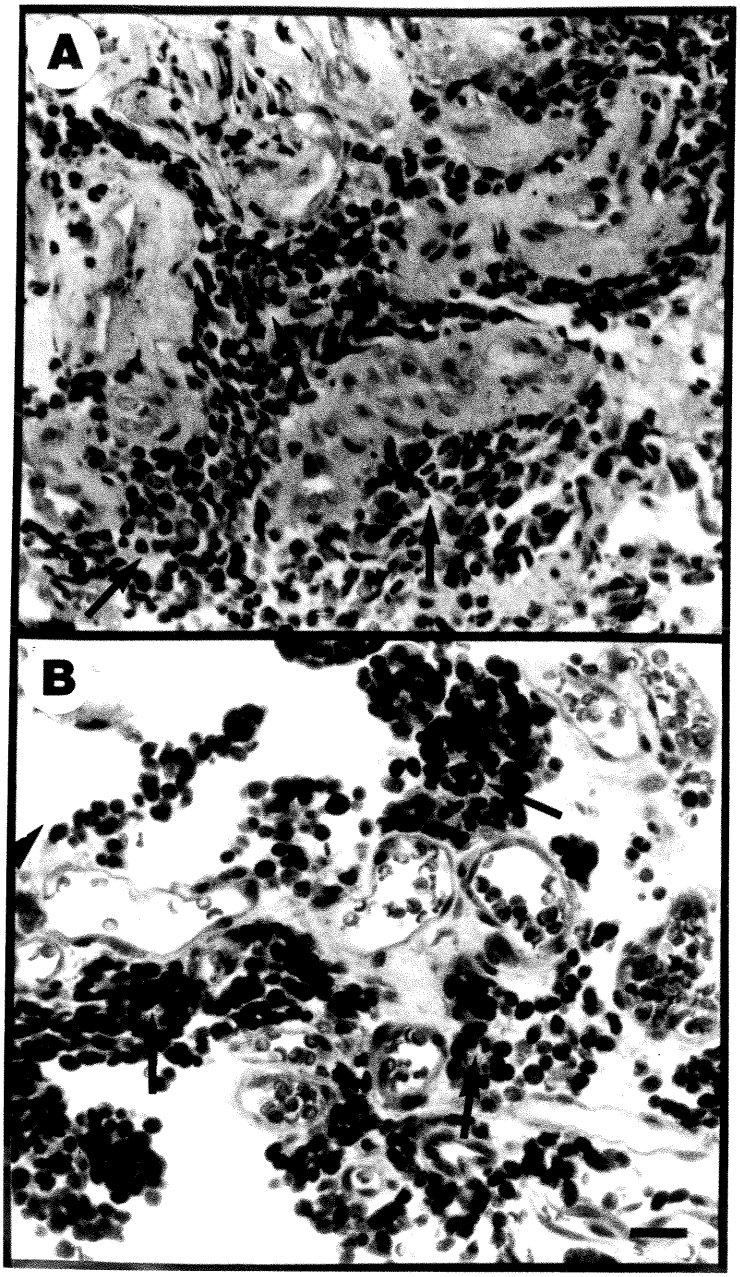

Figure 1. Photomicrographs of the resected MB tumours HSJ (**A**) and SYR (**B**).

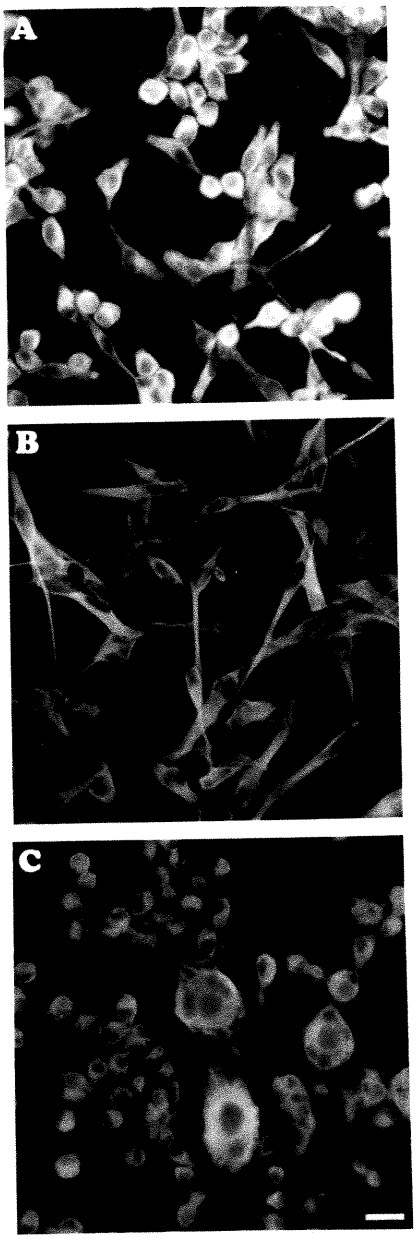

Figure 2. Fluorescent micrographs show immunoreactive sites for vimentin in UM-MB1 (**A**), MAP5 in HSJ (**B**) and NF 150KD in SYR (**C**).

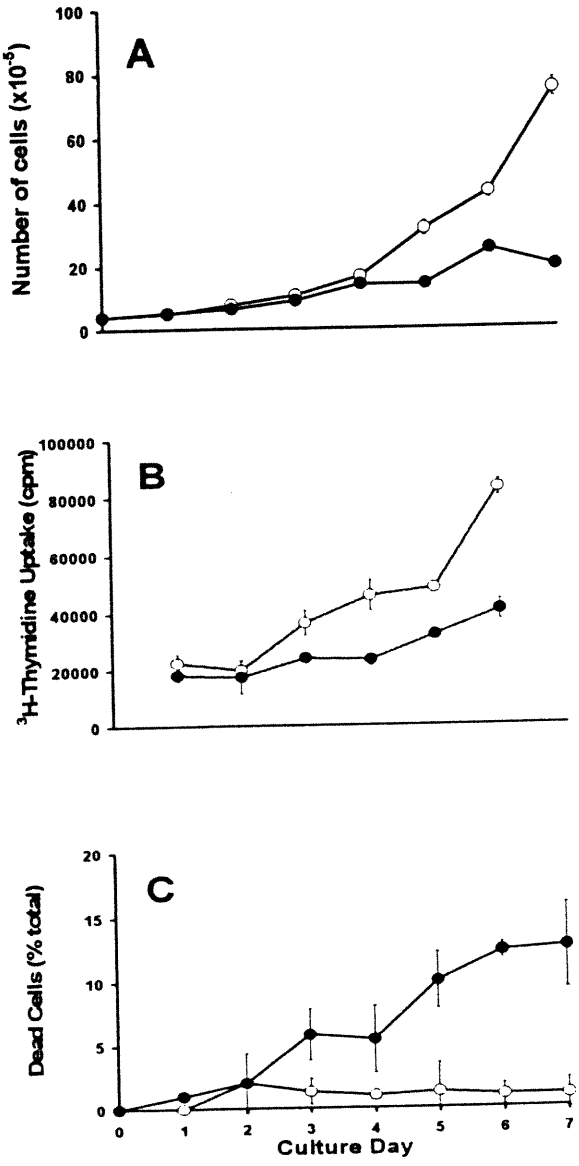

Figure 3. FGF-2 affects the proliferation and viability of SYR cell line.

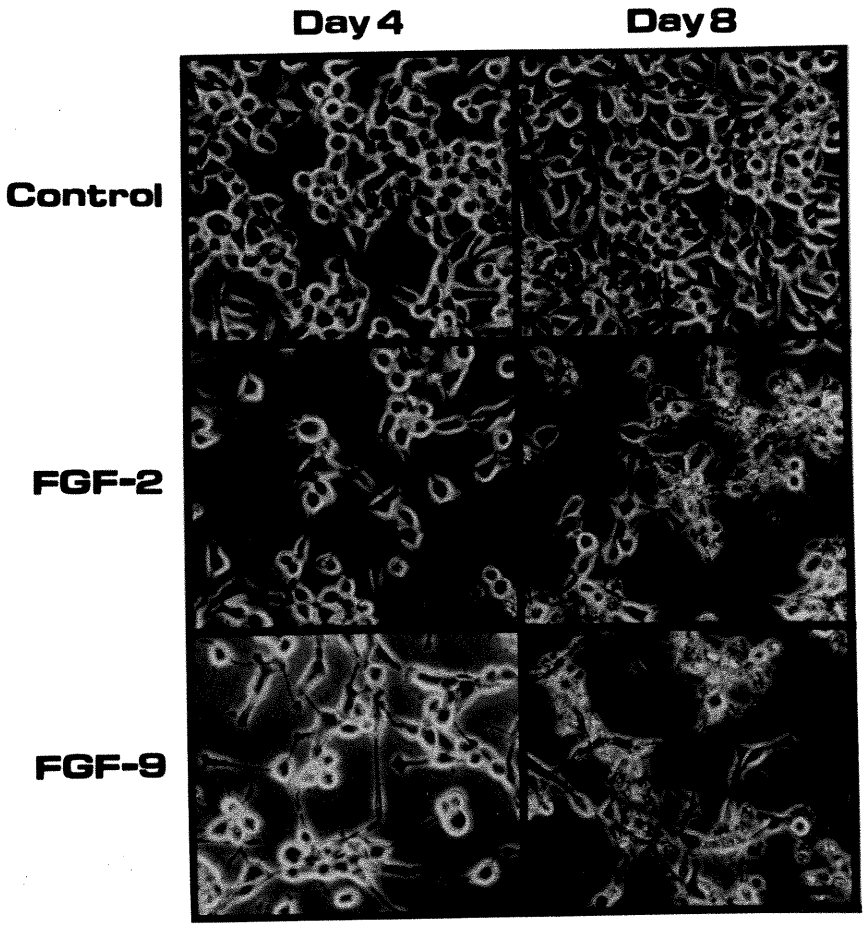

Figure 4. Fibroblast growth factor-2 and FGF-9 slow the growth, induce neurite extention and promote the death of UM-MB1 cells.

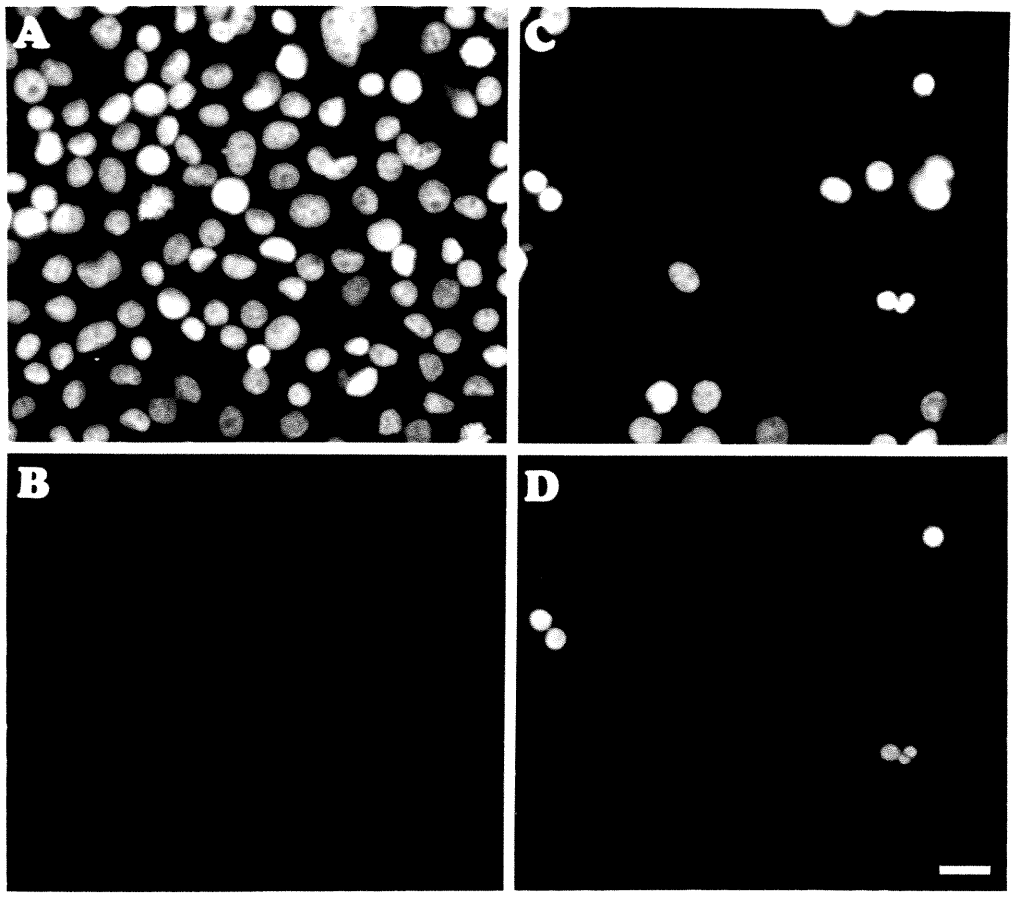

Figure 5. Morphological evidence for FGF-2-induced apoptosis in SYR cells.

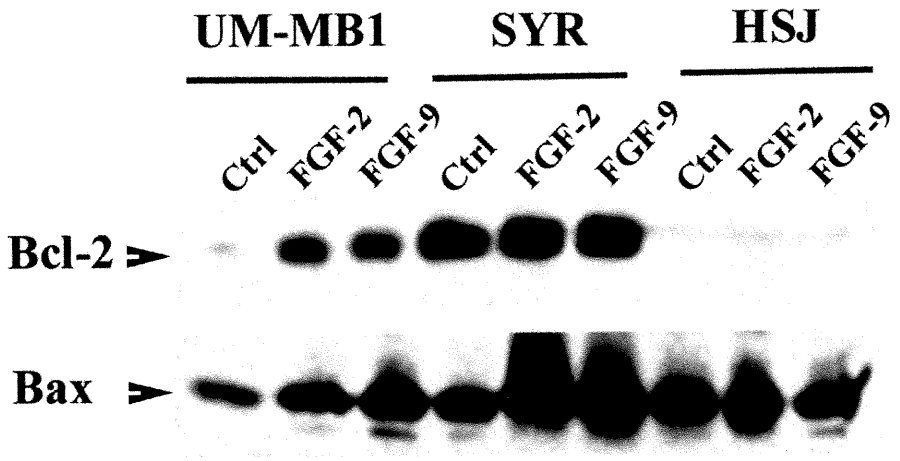

Figure 6. Effects of FGF-2 and FGF-9 on Bcl-2 and Bax expression in MB cell lines as determined by Western blot analysis.

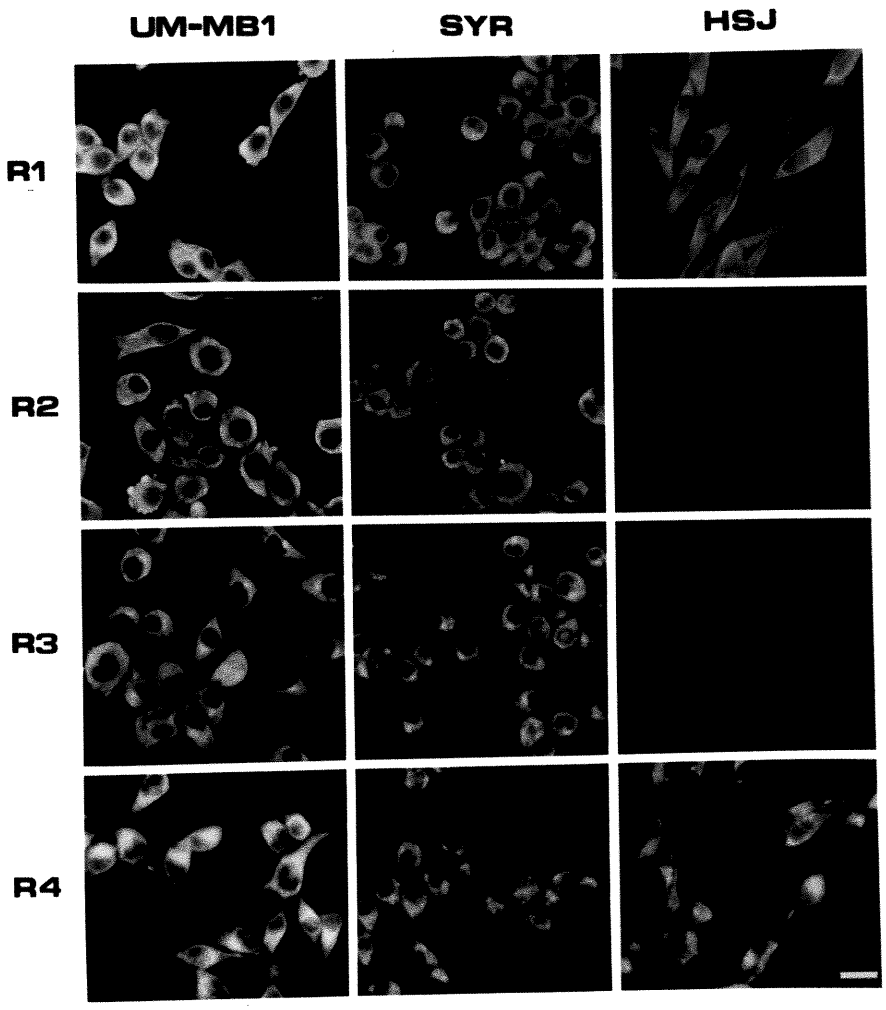

Figure 7. MB cell lines differentially express FGF receptors.

CHAPTER THREE:

GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

Basic fibroblast growth factor/FGF-2 is the first member of the FGF family which has been shown to control cerebellar neuronal maturation. Using a novel MB cell line, UM-MB1 derived from a human cerebellar tumour, Kenigsberg and colleagues (1997) demonstrated that this growth factor could advance maturation, slow the growth and promote the death by apoptosis of this cell line (Kenigsberg et al., 1997). In order to determine if similar changes could be induced in other MB cell lines, my Masters' project was to: *first*, establish and characterize two new human MB cell lines from the two principal histopathological variants of MB that will serve as additional experimental models for this tumour, *second*, to determine if bFGF/FGF-2 and other members of this growth factor family such as: aFGF/FGF-1, FGF-5, FGF-6 and FGF-9 which are also present in the developing cerebellum, could induce similar responses in our three cell lines, and *third*, to identify which FGFR types are expressed in our MB cell lines.

In the present study, I report that one of the newly established and characterized cell line, SYR, like UM-MB1, responds to bFGF/FGF-2 with advanced maturation, growth retardation and apoptotic cell death. In addition, FGF-9 induces similar changes in UM-MB1 and SYR that are, however, less potent as compared to FGF-2. Both cell lines are derived from the classic variant of MB and express all four FGFR types. In contrast, the third cell line, HSJ which shows a more differentiated phenotype

consistent with the desmoplastic variant of MB from which it originates, responds to the same FGFs but with growth acceleration. Interestingly, this cell line exhibits immunoreactive sites for FGFR1 and FGFR4 only.

These results suggest that responsiveness to certain members of the FGF family by MB may depend upon: (1) the histopathological variant of the tumour and/or (2) the expression of certain types of FGFR which may mediate distinct cellular responses. These two possibilities are the major topics of the following discussion.

1. FGFR expression during cerebellar granule cell development may distinguish the two histopathological variants of MB and thus predict responsiveness

MB has been sub-classified into two main histopathological variants, the classic and desmoplastic (Rubinstein and Northfield, 1964; Rubinstein, 1975). This classification scheme was introduced not only to describe the differences in architecture and gross appearance noted in this tumour, but also to describe distinct subsets of MB which may have evolved from cerebellar neuroblasts of either of the two germinal matrices of the cerebellum: (i) the cerebellar neuroepithelium/VM, and (ii) the later forming EGL (Altman and Bayer, 1978; 1997). In this regard, the classic variant of MB is thought to originate from transformed cells of the cerebellar

neuroepithelium/VM, a primary germinal matrix located in the midline cerebellum which disappears early after birth (Raaf and Kenoham, 1944; Katsetos and Burger, 1994). The desmoplastic variant is thought to derive from remnant cells of the EGL, the germinal zone that covers the cerebellar hemispheres and persists up to one year postnatally in human (Stevenson and Echlin, 1934; Katsetos and Burger, 1994). This idea was first put forward by Kadin's group (1970) who tried to reconcile the different locations of these tumours variants based on the normal migration of cells in the developing mouse and human cerebellum. They suggested that since the EGL is an expansion of the cerebellar neuroepithelium/VM that migrates upward and laterally to form this secondary germinal layer that covers the cerebellar hemispheres, it is logical that the classic variant of MB, which has a midline location, occurs early during this migratory process. In contrast, the desmoplastic variant which tends to have a hemispheric location originates from cells that form the EGL (Kadin et al., 1970). This idea has been supported by the numerous observations listed below.

First, the maturational state of the cells in each histopathological variant is similar to that described for cells of each germinal layer of the cerebellum. In this regard, the cells of the classic variant are more undifferentiated. They are usually round-to-oval or carrot-shaped with a high nuclear to cytoplasmic ratio and a scanty cytoplasm containing very few cytoplasmic organelles (Katsetos et al., 1988; 1989). This is consistent with

their origin from a more primitive germinal zone, like the cerebellar neuroepithelium/VM. In contrast, cells in the desmoplastic variant, like cells of the inner pre-migratory/post-mitotic zone of the EGL display features of maturation such as: enlargement of cytoplasm, increase in mitochondria and Golgi and endoplasmic reticulum complexes, and elaboration of neurite-like processes (Ramon y Cajal, 1911; Katsetos et al., 1988; 1989; Altman and Bayer, 1997; Giangaspero et al., 1997). These differences in cellular maturation are also observed in our MB cell lines derived from these two different histopathological variants. Both of our MB cell lines that originate from classic tumours show a more immature phenotype. For example, they both exhibit a high nuclear to cytoplasmic ratio and a strong immunostaining for vimentin, a type III intermediate filament protein expressed by cerebellar neuroepithelial precursor cells (Trojanowsky et al., 1994). Moreover, the cell line derived from a desmoplastic tumour exhibit neurite-like processes consistent with its high expression of MAP5, a microtubule-associated protein found during neurites formation, stabilization and maintenance (Tucker et al., 1988; Maccioni and Cambiazo, 1995), and an increase in cytoplasmic volume and organelles, all consistent with a more mature phenotype.

Second, each histopathological variant has been shown to express proteins that are exclusively present in cells of either the cerebellar neuroepithelium/VM or the EGL, the two germinal matrices of the

cerebellum. For example, calbindin-D_{28k}, a cytosolic calcium binding protein, expressed exclusively in immature cerebellar neuroepithelial/VM cells, is also seen in a subset of classic MB tumours and in the commercially available D283 cell line. In addition, an early neuronal differentiating marker, the class III β-tubulin present in the EGL and its progeny, i.e the IGL, is also detected in desmoplastic MBs (Katsetos et al., 1993; 1995). Finally, mutations in the PTCH receptor which is expressed by dividing neurons of the EGL have been found in some desmoplastic MBs as well (Pietch et al., 1997; Wallace et al., 1998; Wechsler-Reya, 1999). Nevertheless, expression pattern of these antigens on our MB models still await elucidation and could perhaps help strengthen this proposal.

Lastly, clinicopathological studies demonstrate that the desmoplastic variant tends to occurs in an older group of patients, which is consistent with their etiology from remnant cells of a later forming germinal matrix, the EGL. The classic variant, which originates from the earlier forming neuroepithelium/VM is in turn seen more often in children (Spitz et al., 1947; Ringertz and Tola, 1950; Rubinstein and Northfield, 1964; Katsetos et al., 1994).

Thus, each histopathological variant of MB probably represents neoplastic transformed cells originating from different germinal matrices that have different developmental profiles and different growth factors dependencies as well. If that is the case, perhaps the different responses

we have observed in our studies reflect developmental changes in the expression or levels of the FGFR types by cells of the two germinal zones of the cerebellum. In agreement with this, Heuer and colleagues (1990) have noted temporal changes in the level of FGFR mRNA transcripts during the chick cerebellar development. They have reported a high level of FGFR mRNA transcripts in the primary cerebellar germinal matrix as compared to the EGL (Heuer et al., 1990). Although no indications about which FGFR types were present and/or absent in either cerebellar germinal zone, studies on rat neuroepithelial (NEP) stem cells demonstrate that while all four receptor types are expressed in these undifferentiated cells, only FGFR2 is down-regulated during neuronal differentiation (Kalyani et al., 1999). Interestingly, our cell line that originates from a desmoplastic MB tumour, the variant thought to arise from the later forming germinal matrix of the cerebellum, does not exhibit immunoreactive sites for FGFR2.

In addition, differences in the regional expression of the FGFRs have also been noted. In the rat cerebellum, it has been shown that FGFR1 (Wanada et al., 1990; Yazaki et al., 1994) and FGFR4 (Miyaki et al., 1995) mRNAs are strongly expressed in both the proliferative and pre-migratory/post-mitotic zones of the EGL. Consistent with this, FGFR1 and FGFR4 are both found in HSJ, the cell line that comes from the desmoplastic MB tumour and thus the EGL. The choroid plexus, one prong of the germinal trigone (GT) which also includes the cerebellar neuroepithelium/VM

(Altman and Bayer, 1978; 1997), preferentially expresses FGFR2 mRNA (Yazaki et al., 1994). This is consistent with our finding of FGFR2 expression in our cells derived from classic tumours.

Therefore, it seems like the expression pattern of the FGFRs in the two histopathological variants of MB is consistent with its etiology from transformed cells of either germinal zone of the cerebellum and is, to a certain extent, preserved in these cells although they are transformed. Maybe, like calbindin-D_{28k} and class III β-tubulin proteins, the differential pattern of expression of these receptors could serve as markers that would distinguish these two most commonly encountered variants of MB, i.e. the classic and desmoplastic.

2. FGFR2 and FGFR3, possible FGFRs in mediating bFGF/FGF-2 and FGF-9 responses.

In the present study, we found that amongst the five FGFs tested, only bFGF/FGF-2 and FGF-9 could induce differentiation, slow growth and promote death by apoptosis in the two cell lines derived from the classic tumours. In contrast, these same FGFs as well as FGF-5 and FGF-6, induced mitogenic responses in the third cell line that originates from a desmoplastic MB. Interestingly, both cell lines derived from the classic MB tumours exhibited immunoreactive sites for FGFR1 through FGFR4, while the one which originates from the desmoplastic tumour did not express

FGFR2 and FGFR3. This observation raises the possibility that these two FGFRs, i.e. FGFR2 and FGFR3, may be involved in the growth suppression and/or apoptotic cell death responses induced by bFGF/FGF-2 and FGF-9. This possibility is supported by the following observations.

First, when looking at the FGFR family, it was found that four distinct receptors, i.e. FGFR1-FGFR4, are involved in the FGF signalling network. However, multiple variants of FGFR1, FGFR2 and FGFR3, but not FGFR4, can be generated by alternative splicing to give rise to receptors with different ligand specificities, affinities and signalling capacities thus, making this family of receptors even more complex (Johnson and Williams, 1993; Goldfarb, 1996; Szebenyi and Fallon, 1999). Interestingly, and in agreement with our finding, is that FGFR2 and FGFR3 of the IIIc isoform exhibit higher binding affinities for both bFGF/FGF-2 and FGF-9 (Ornitz et al., 1996; Santos-Ocampo, 1996). Unfortunately, due to limitations in our methods of detection, we were not able to determine which isoforms of FGFR2 or FGFR3 were present in our MB cell lines which express these two receptors.

Second, the loss of FGFR2 or FGFR3 has been shown to induce uncontrolled cell proliferation of prostate tumours (Yang et al., 1993; Feng et al., 1997; Matsubara et al., 1998) and astrocytomas (Morrison et al., 1994) or immature chondrocytes (Li et al., 1999), respectively. In addition, mutations in several different domains of FGFR3 induce a gain of function

which potentiates its negative effect on growth and leads to several forms of dwarfism (Legeai-Mallet et al., 1998; Li et al., 1999). These findings may explain the mitogenic response of HSJ to these FGFs, since both FGFR2 and FGFR3 are not expressed in this cell line.

In conclusion, our study supports the proposal that the two most common histopathological variants of MB do indeed originate from different cerebellar neuronal precursors which are not necessarily unrelated. A further understanding of the different developmental stages of cerebellar cells could shed light on the biological similarities and differences between these histopathological variants, and may hopefully help identify markers which can serve as prognostic and /or diagnostic indicators for this childhood brain tumour.

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