Université de Montréal

Régulation des interactions entre les leucocytes humains et les cellules endothéliales: mécanismes pro- et anti-adhésifs.

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Ce mémoire intitulé:

Régulation des interactions entre les leucocytes humains et les cellules endothéliales: mécanismes pro- et anti-adhésifs

présenté par

Christine Al-Zouki

a été évalué par un jury composé des personnes suivantes:

John SD Chan, Ph.D.	**président du jury**
Janos G Filep, M.D.	**directeur de recherche**
Haroutioun M Hasséssian, Ph.D.	**membre du jury**

Mémoire accepté le.....

C'est avec fierté que je dédie ce mémoire à mes parents, ainsi qu'a mon cher ami Maher qui ont su me supporter tout au long de mes études. L'amour et l'attention qu'ils m'ont donnés m'ont permis de franchir cette étape de ma vie dans l'harmonie.

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Introduction

Des études récentes suggèrent que l'inflammation locale pourrait être impliquée dans la pathogénèse des maladies cardiovasculaires ischémiques. Les neutrophiles sont les cellules les plus nombreuses au site inflammatoire lors des premières étapes de l'inflammation, et on pense qu'ils contribueraient au dommage causé à l'endothélium. Explorer les mécanismes qui régulent et/ou inhibent le traffic des leucocytes est très important pour comprendre comment on pourrait limiter le recrutement des leucocytes, et conséquemment inhiber la réponse inflammatoire.

Nous hypothésons alors que l'endothéline-1 pourrait promouvoir l'adhésion des neutrophiles tandis que des agents endogènes tels la protéine C-réactive, les lipoxines induites par l'aspirine, et les antiflammines pourraient inhiber l'adhésion des leucocytes. Ces études ont été entreprises afin de vérifier ces hypothèses ainsi que les mécanismes qui régissent l'augmentation ou l'inhibition de l'adhésion des leucocytes.

Méthodes

Ces expériences ont été faites sur des leucocytes humains dans le sang total, sur des leucocytes isolés, et sur des cellules endothéliales d'artères coronariennes humaines en culture. L'expression des molécules d'adhésion à la surface des leucocytes et des cellules endothéliales fut étudiée par cytométrie en flux, en utilisant des anticorps monoclonaux marqués à la fluoroscéine ou à la phycoérithrine. Les essais d'adhésion ont été fait sous des conditions statiques. Nous avons utilisé des anticorps monoclonaux (pour bloquer la fonction) afin de caractériser les molécules d'adhésion impliquées dans l'adhésion. De plus, grâce à des inhibiteurs sélectifs d'enzymes et des antagonistes sélectifs pour certains récepteurs, nous avons explorer les mécanismes impliqués. Nous avons aussi fait des études de liaison pour identifier les récepteurs impliqués, ainsi que des ELISAs pour quantifier la L-sélectine soluble (clivée).

Résultats

Pour ce qui est de l'endothéline-1, notre étude indique qu'elle promouvoit l'adhésion des neutrophiles aux cellules endothéliales en activant le récepteur ET_A sur les neutrophiles, et subséquemment la production de PAF. La protéine C-réactive quant à elle, inhibe l'adhésion L-sélectine-dépendante des neutrophiles, et aux mêmes concentrations elle inhibent l'expression de la L-sélectine à la surface des neutrophiles sans induire d'activation cellulaire. Les analogues de la LXA_4 et la LXA_4 induite par l'aspirine modulent l'expression de la L-sélectine et de CD11/CD18 à la surface des leucocytes immunostimulés ou non et inhibent l'adhésion CD18-dépendante des neutrophiles aux cellules endothéliales. Puis finalement les antiflammines aussi inhibent l'adhésion CD18-dépendante des neutrophiles.

Conclusions

Ces études révèlent des mécanismes importants qui affectent l'adhésion des neutrophiles aux cellules endothéliales. Nous avons démontré que l'endothéline-1 promouvoit l'adhésion des neutrophiles, tandis que la protéine C-réactive, les peptides dérivés de la protéine C-réactive, la lipoxine native, la lipoxine induite par l'aspirine et les antiflammines peuvent inhiber de façon marquée les interactions entre les neutrophiles et les cellules endothéliales.

Donc, par différents mécanismes, en inhibant l'accumulation des leucocytes, nous pouvons limiter dans l'espace et dans le temps la réponse imflammatoire et donc limiter les dommages tissulaires, médiés par les neutrophiles, qui accompagnent les maladies cardiovasculaires ischémiques aigues ainsi que d'autres maladies inflammatoires (aigues ou chroniques) tel que l'arthrite.

Remerciements

J'aimerais remercier mon directeur de recherche, le Dr Janos G Filep, pour m'avoir donné l'opportunité de faire mes recherches dans son laboratoire. Non seulement il m'a fournit l'espace et le matériel nécessaire, mais il m'a aussi donner beaucoup de son temps, ce que j'ai beaucoup apprécié. J'ai eu l'occasion, grâce à lui, de travailler dans un environnement stimulant et de pratiquer de nombreuses techniques. Il a su me guider tout au long de mes recherches et ses connaissances dans le domaine de la recherche furent très utiles à mon cheminement.

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Sans oublier de remercier le Conseil de Recherche Médical du Canada pour m'avoir supporté avec une bourse d'étude.

Finalement, j'aimerais remercier les nombreux donneurs de sang, ainsi que les infirmières du centre de prélèvement de l'hôpital Maisonneuve-Rosemont.

CONTENU

Liste d'abréviations

Ac	Anticorps
ADNc	Acide desoxyribonucléique complémentaire
ADP	Adénosine diphosphate
AF	Antiflammine
AMPc	Adénosine monophosphate cyclique
AP-1	Activator protein-1
ATL	Aspirin-triggered lipoxin
bFGF	Basic fibroblast growth factor
COX II	Cyclooxygenase type II
CRP	C-reactive protein
ECE	Endothelin-converting enzyme
ELAM-1	Endothelial leukocyte adhesion molecule-1
15-epi-LXA_4	15-epi-lipoxin A_4
15-epi-LXB_4	15-epi-lipoxin B_4
Erk2	Extracellular signal-regulated kinase 2
ET	Endothéline
ET-1	Endothéline-1
FAK	Focal adhesion kinase
FMLP	Formyl methionine-leucine-phenylalanine
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HCAEC	Human coronary artery endothelial cells
15-*R*-HETE	15-R-hydroxy-eicosa-tetraenoic acid
ICAM-1,2,3	Intercellular adhesion molecule-1,2,3
IL-1β	Interleukin-1β
IL-6	Interleukin-6
IL-8	Interleukin-8
LDL	Low density lipoprotein
LFA-1	Lymphocyte function-associated antigen-1
5-LO	5-lipoxygenase
12-LO	12-lipoxygenase
15-LO	15-lipoxygenase
LPS	Lipopolysaccharide
LX	Lipoxin

LXA_4	Lipoxin A_4
LXB_4	Lipoxin B_4
mAbs	Monoclonal antibodies
Mac-1	CD11b/CD18
MadCAM-1	Mucosal address in cell adhesion molecule-1
MAPK	Mitogen activated protein kinase
MCP	Monocyte-chemotactic protein
M-CSF	Monocyte-colony-stimulating factor
MEK	MAPK kinase
MHC I	Major histocompatibility complex type I
MHC II	Major histocompatibility complex type II
MoAbs	Monoclonal antibodies
NF-κB	Nuclear factor kappa B
NK	Natural killer
NO	Nitric oxide
PAF	Platelet-activating factor
PAI-1	Plasminogen activator inhibitor-1
PDGF	Platelet-derived growth factor
PECAM	Platelet endothelial cell adhesion molecule
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin
PHGS-II	Prostaglandin H synthesis II (cyclooxygenase II)
PI-3	Phisphatidylinositol-3
PIPP	Polyisoprenyl phosphate
PKC	Protein kinase C
PMN	Polymorphonucléaire (polymorphonuclear leukocytes (PMNL))
PSGL	P-selectin glycoprotein ligand-1
PSL	P-selectin ligands
SRC	Short concensus repeat
TGF	Transforming growth factor
TNF	Tumor necrosis factor
tPA	Tissue type plasminogen activator
uPA	urokinase type plasminogen factor
VCAM	Vascular cell adhesion molecule
VLA-4	Very late activation antigen-4

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Liste de tous les co-auteurs des articles

1) Chantal Baron
2) Micheline Beauchamp
3) Janos G Filep
4) Alain Fournier
5) Sophie Ouellet
6) Mohamed Hachicha
7) Charles N Serhan
8) Nicos A Petasis

To whom it may concern

We, the undersigned, agree and consent that Madame Christine Zouki can use the following articles in her M.Sc. thesis entitled: "Régulation des interactions entre les leukocytes humains et les cellules endothéliales: mécanismes pro- et anti-adhésifs", to be submitted to the faculté des études supérieures, Université de Montréal.

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4. **Zouki C**, Ouellet S, Filep JG. The anti-inflammatory peptides, antiflammins, regulate the expression of adhesion molecules on human leukocytes and prevent neutrophil adhesion to endothelial cells. *FASEB J.* (accepted for publication, 1999)

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I. INTRODUCTION

1. L'inflammation

1.1 L'inflammation et les maladies coronariennes aigues

Depuis maintenant 60 ans, on associe l'inflammation à l'infarctus du myocarde (1). Des études récentes suggèrent que l'inflammation locale puisse être impliquée dans la pathogénèse des maladies cardiovasculaires ischémiques (2,3). L'événement déclencheur du développement de l'athérosclérose et des maladies cardiovasculaires semble être un dommage causé à l'endothélium (4-8). Le dommage peut être induit par une variété de facteurs tels les lipoprotéines de faible densité oxydées (LDL) (9,10), les lipoprotéines glycosylées (11,12), les produits finaux de glycosylation (13), l'hyperhomocystéinémie (14), les forces mécaniques (15) ou une infection virale (16). Bien que des élévations en LDL suffisent pour induire les composantes de la réaction athérosclérotique (17,18), une étude démontre que seulement 30% des patients ayant une athérosclérose ont un niveau élevé de lipides. Ce qui est intéressant c'est qu'une étude récente a rapporté la présence de *Chlamydia pneumoniae* dans des spécimens de plaque athérosclérotiques provenant de patients ayant subit une athérectomie coronarienne (19). Bien que d'autres études soient nécessaires pour confirmer un lien entre *Chlamydia* et athérosclérose, ces observations suggèrent que des infections pourraient contribuer au développement des maladies cardiovasculaires. Les neutrophiles sont les cellules les plus nombreuses au site inflammatoire lors des premières étapes de l'inflammation, et on pense qu'ils contribueraient au dommage causé à l'endothélium. La figure 1 représente un schéma des mécanismes qui peuvent promouvoir le dommage médié par les neutrophiles. Certains marqueurs inflammatoires ou hémostatiques constituent des facteurs de risques cardiovasculaires (20). En effet, les niveaux d'inflammation, tel que mesuré par la concentration plasmatique de protéine C-réactive par exemple, permettent de prédire le risque d'un premier infarctus chez des hommes apparemment en santé (21,22). Alors l'inhibition de l'inflammation dans la paroi artérielle pourrait diminuer les risques d'un second événement.

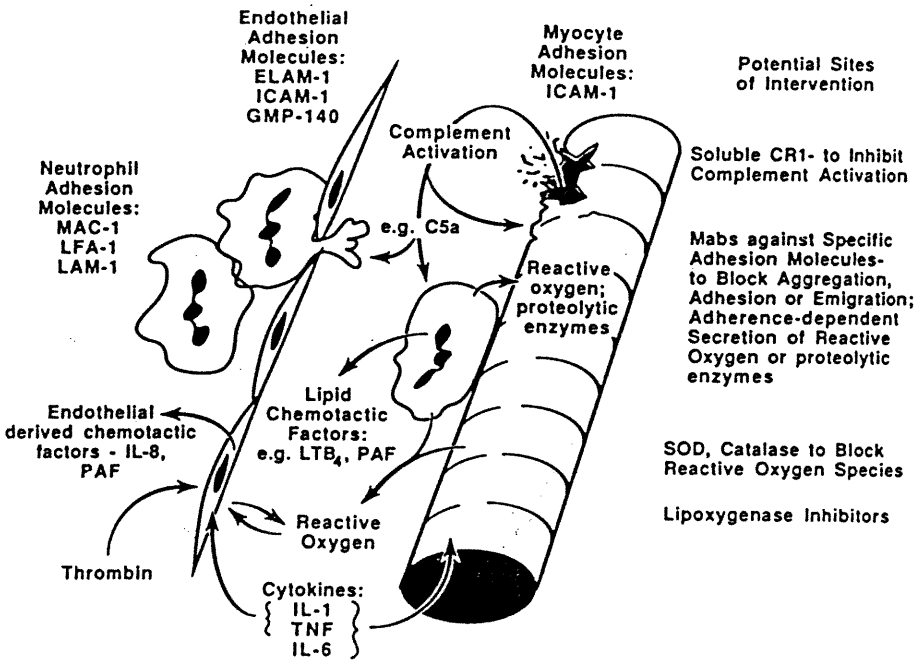

Figure 1 : Mécanismes qui peuvent mener à un dommage vasculaire médié par les neutrophiles. Pour une description détaillée des molécules d'adhésion, des médiateurs et des événements, voir les sections appropriées de cette thèse. **Entman et al. 1991 (23)**

1.2 La réponse inflammatoire

La défense de l'hôte est souvent caractérisée soit par une réponse inflammatoire aiguë ou chronique suite à un stimulus causé par une infection ou un dommage tissulaire. Les signes classiques de l'inflammation sont la douleur, la rougeur, la chaleur, l'oedème et l'altération de la fonction. Les études récentes portent sur les processus qui initient et amplifient l'inflammation, peut-être à cause des effets néfastes qu'elle peut apporter et son association avec plusieurs maladies (24). En effet si le dommage persiste, il y aura destruction du tissu et une inflammation chronique, ce qui mènera à un remodelage tissulaire et à une fibrose. Bien que les mécanismes moléculaires spécifiques qui mènent à une inflammation aiguë ou chronique ne soient pas complètement élucidés, il est maintenant clair que des médiateurs autocrines : paracrines produit par des cellules inflammatoires et non immunes, et l'interaction entre ces cellules, soient important pour bien comprendre le

dommage inflammatoire. Un événement clé de la réponse inflammatoire est l'accumulation locale de leucocytes. Sous des conditions normales, les leucocytes dans le sang périphérique sont distribués selon un pool circulatoire et un pool marginatif, puis ce dernier compte pour 60% du nombre total de cellules (25,26). Lors d'une inflammation le nombre de leucocytes qui roulent dans les veinules postcapillaires augmente considérablement (27). Après une adhésion sélective, ces leucocytes traversent la paroi vasculaire entre les cellules endothéliales et s'accumulent au site de lésion. Il existe une corrélation positive entre le degré d'infiltration des leucocytes et la sévérité de la maladie. (28)

2. La dysfonction endothéliale

2.1 Fonction endothéliale normale

Les cellules endothéliales accomplissent de multiples fonctions physiologiques. L'endothélium synthétise ou transforme et sécrète de nombreuses molécules, et fabrique plusieurs récepteurs à sa surface endoluminale dont il régit l'expression. Par toutes ces substances, l'endothélium module le tonus vasculaire, la coagulation, la fibrinolyse, la croissance et la différentiation cellulaire, puis les réactions immunitaires et inflammatoires (29,30). Les cellules endothéliales agissent en concert avec les tissus environnant pour intégrer les différentes réponses. La table 1 résume certaines des propriétés constitutives de l'endothélium.

Table 1 : Résumé de quelques propriétés constitutives de l'endothélium.

FONCTIONS	SUBSTANCES
Modulation du tonus vasculaire	
Vasodilatation	• Prostacycline (PGI_2) • Oxyde nitrique (NO) • Facteur hyperpolarisant
Vasoconstriction	• Endothéline-1 • Angiotensine-II
Modulation des réactions hémostatiques et thrombogènes	
Activation de l'hémostase	• Facteur Willebrand • Collagène • Facteur tissulaire • Récepteurs pour facteurs de la coagulation
Protection contre la thrombose	• Glycosamoniglycans sulfatés, thrombo-moduline • Prostacycline, NO, ADP diphospho-hydrolase (CD39), t-PA
Rôle dans l'inflammation	
Adhésion des leucocytes	• Molécules d'adhésion vasculaires • Cytokines
Croissance cellulaire	
Régulateurs de croissance et mitogènes	• NO

2.2 Réponse au dommage

2.2.1 Immédiate

À la suite d'un stimulus mécanique ou biochimique, les cellules endothéliales produisent une variété de réponses. Au site local d'inflammation, les cellules sont activées par une variété de cytokines et de facteurs de croissance. Les réponses endothéliales aiguës se produisent dans les premières minutes suivant la stimulation, car ils ne requièrent pas de synthèse de protéines. Des médiateurs dérivant de l'endothélium, tel la prostacycline, la PGE_2, les facteurs relaxants dérivés de l'endothélium (incluant le NO), le facteur hyperpolarisant dérivé de l'endothélium, l'endothéline-1 et les facteurs contractants dérivés de l'endothélium agissent pour moduler le tonus et la perméabilité vasculaire, la fonction plaquettaire et la relâche de neurotransmetteurs (29,30). En effet une activation ou un dommage endothélial résulte en un débalancement dans la production de substances dérivées de l'endothélium. Par exemple on aura une augmentation de la production d'ET-1 (31) endothéliale et de superoxide (32). La production de superoxide servirait de "scavenger" de NO produit par la NO synthase constitutive (33,34) et donc diminuerait la biodisponibilité du NO. On aurait alors une augmentation de la perméabilité vasculaire puisque ET-1 est un vasoconstricteur puissant (35). L'expression de la P-sélectine sur la surface endothéliale joue un rôle important dans l'activation et l'adhésion des leucocytes circulants (36).

2.2.2 Expression des gènes

Les cellules endothéliales répondent aussi au dommage en altérant l'expression de différents gènes dont les produits sont important dans l'inflammation. Les proto-oncogènes nucléaires, *c-fos* et *c-jun*, sont induit rapidement et de façon transitoire en réponse à plusieurs stimuli (37,38). Par contre l'expression des protéines de la famille de *c-fos* et *c-jun* dans les cellules endothéliales n'est pas encore bien comprise. Des études récentes ont identifié plusieurs gènes de réponse spécifiques aux cellules endothéliales, incluant *edg-1* qui semble-t-il coderait pour un récepteur membranaire couplé à une protéine G (39), le

facteur de transcription putatif *edg-2* (40) et *B12*, qui pourrait jouer un rôle dans l'activation des gènes (41).

Il y a aussi le gène qui code pour la cyclooxygénase II (COX II) (42), une enzyme clé dans la synthèse de prostacycline et de PGE_2. La COX II est induite par une variété de cytokines et de facteurs de croissance dans différents types cellulaires, incluant l'endothélium. De plus les cellules endothéliales produisent de la collagénase (participant à la dégradation de la matrice et le remodelage), différents facteurs de croissance (ex.: monocyte colony-stimulating factor (M-CSF) et granulocyte : macrophage colony-stimulating facror (GM-CSF)), des mitogènes (ex.: platelet-derived growth factor (PDGF), le transforming growth factor β (TGFβ) et le basic fibroblast growth factor (bFGF).

Le facteur de transcription nucléaire NF-kB régularise l'expression de plusieurs gènes impliqués dans la réponse inflammatoire. Plusieurs gènes cibles dans les cellules endothéliales contiennent des sites de liaison NF-kB dans leur région 5', incluant MHC I, MHC II, ICAM-1, VCAM-1, ELAM-1, GM-CFS et l'urokinase (30). Des sites de liaison pour le facteur de transcription AP-1 sont aussi retrouvé dans les promoteurs d'ICAM-1, de VCAM-1, d'ELAM-1 et de l'urokinase. Par contre, le facteur de transcription AP-1 ne semble pas suffire pour l'activation transcriptionnelle de ces gènes (30). À la table 2, on retrouve les gènes régulés dans les cellules endothéliales en réponse à un dommage ou des stimuli inflammatoires.

Table 2 : Propriétés inductibles de l'endothélium.

Produits de gène	Fonctions
Remodelage/thrombose/coagulation	
PAI-1	•Inhibiteur de uPA et tPA
uPA, tPA	•Transforme plasminogène en plasmine •Dégradation de la matrice, fibrinolyse •Activation de TGFβ latente
Collagénase	•Dégradation de la matrice, remodelage
Facteur tissulaire	•Cofacteur pour activer coagulation
Vitronectine	•Intégrine lie récepteur vitronectine
Interactions leucocytes/endothélium	
E-sélectine	•Se lie à ligand sur PMNs, monocytes, lymphocytes
ICAM-1	•Lie ligands sur PMNs, mono et lympho
VCAM-1	•Lie ligands sur mono et lympho
P-sélectine	•Lie ligand sur PMNs
MHC-I	•Présente antigènes solubles aux cellules T cytotoxiques
MHC-II	•Présente antigènes solubles aux cellules T cytotoxiques
IL-6	•Facteur de croissance pour cellules B
IL-8	•Facteur chimiotactique pour les PMNs
PAF	•facteur chimiotactique pour les PMNs
MCP	•facteur chimiotactique pour monocytes
IL-1β	•Cytokine inflammatoire
TNF-α	•Cytokine inflammatoire
Tonus vasculaire et interaction plaquettes	
COX	•synthèse de PGI_2 et PGE_2
NO-synthase	•synthèse de NO
Endothéline	•vasoconstricteur, mitogène
Facteurs de croissance	
M-CSF, GM-CSF	•induit formation de colonies de macrophages et de monocytes
PDGF	•mitogène pour cellules muscle lisse
TGFβ	•inhibiteur et activateur de croissance
bFGF	•mitogène pour plusieurs cellules

Modifiée de Gerritsen et Bloor, 1993 (30)

3. L'adhésion leucocytes-cellules endothéliales

L'adhésion cellulaire joue un rôle primordial dans les mécanismes de défense de l'hôte. Les neutrophiles sont la première ligne de défense, et ils sont rapidement mobilisés et recrutés aux sites d'infection ou de dommage tissulaire. Les molécules présentes à la surface des leucocytes et des cellules endothéliales participent au ralentissement des leucocytes lorsqu'ils sortent des capillaires et entrent dans les veinules postcapillaires, qui elles sont le site majeur de l'adhésion des leucocytes aux cellules endothéliales. Chacune des étapes du recrutement des leucocytes, i.e. le roulement, l'adhésion ferme et la migration transendothéliale, implique la participation de différentes familles de molécules d'adhésion.

3.1 Les molécules d'adhésion

Trois familles majeures de molécules d'adhésion participent à la régulation de la migration des leucocytes; soit les Sélectines, les Intégrines et les Immunoglobulines. Chacune de ces familles participent à des étapes différentes du processus. Mes études portent plus spécifiquement sur les molécules d'adhésion (et leurs ligands) à la surface des leucocytes et des cellules endothéliales. Les membres de ces familles sont distribués d'une part à la surface des leucocytes, et d'autre part à la surface des cellules endothéliales (voir figure 2).

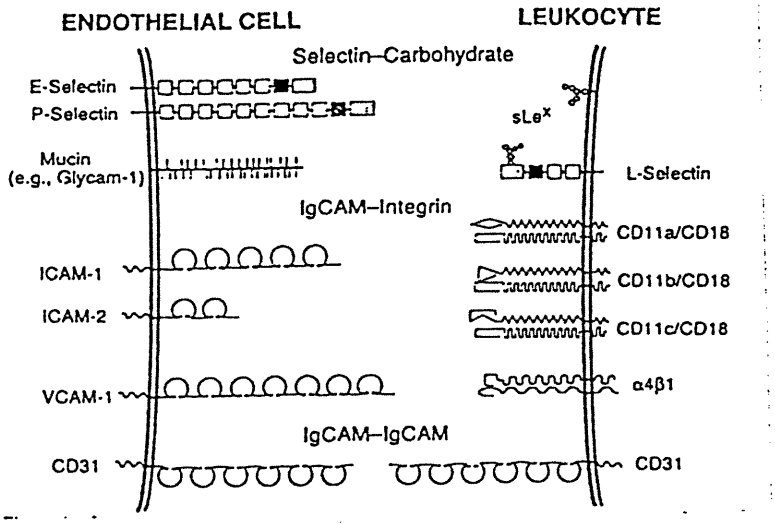

Figure 2 : Distribution des différentes molécules d'adhésion.
Oppeinheimer-Marks, N et Lipsky, PE, 1996 (43).

3.1.1 Les sélectines

Les sélectines ont un rôle très important dans l'attachement initial des leucocytes à l'endothélium. Cette étape se traduit par le roulement des leucocytes sur la paroi endothéliale grâce au flux sanguin, et elle est essentielle à l'adhésion ferme des leucocytes à la surface endothéliale. La famille des sélectines comprend trois membres, dont la structure est très similaire : la L-sélectine (CD62L), la E-sélectine (CD62E) et la P-sélectine (CD62P) (voir table 3).

Table 3 : Les membres de la famille des Sélectines qui médient l'interaction des leucocytes aux cellules endothéliales.

Récepteur	*Ligand*	*Distribution*	*Régulation*
L-sélectine	P-sélectine, E-sélectine, GlyCAM, CD14, MadCAM	Leucocytes au repos	Clivée suite à l'activation
E-sélectine	L-sélectine, sialyl Lewis X, CLA sialyl Lewis A, LFA-1	Cellules endothéliales activées	Induite par IL-1, TNF-α, LPS
P-sélectine	L-sélectine, PSGL-1, PSL, sialyl Lewis X	Cellules endothéliales activées, plaquettes activées	Induite par thrombine, histamine

Modifiée de Oppeinheimer-Marks, N et Lipsky, PE, 1996 (43)

3.1.1.1 Caractéristiques structurales des sélectines

Les sélectines présentent des caractéristiques structurales et fonctionnelles uniques. D'une part, leur fonction est restreinte au système vasculaire (exprimées par les leucocytes et les cellules endothéliales). D'autres part, toutes les sélectines sont des glycoprotéines transmembranaires de type 1, i.e. l'extrémité N-terminale est extracellulaire, représentant un seul passage transmembranaire et une courte extrémité C-terminale cytoplasmique. La partie extracellulaire comprend trois domaines : le domaine lectine, le domaine EGF et le domaine SRC. Dans ce dernier, le nombre de motifs varie d'un membre à l'autre; 2(L-sélectine); 6(E-sélectine); 9(P-sélectine). Les structures sont représentées à la figure 3.

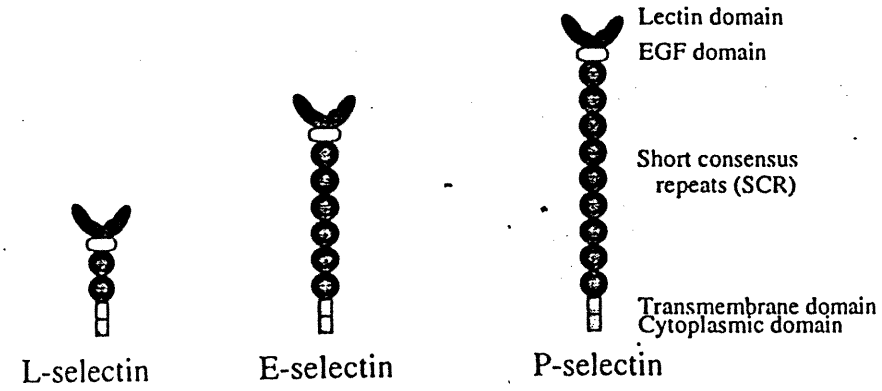

Figure 3 : Les membres de la famille des sélectines.
Kishimoto TK, Rothlein R, 1994 (44)

3.1.1.2 Caractéristiques particulières des différents membres

La production de souris transgéniques déficientes pour les sélectines a permis de bien comprendre la fonction de chaque sélectine et les interactions entre les sélectines lors de l'inflammation (45-47). Ces études suggèrent que les sélectines agissent de façon coordonnée pour médier le roulement et l'adhésion lâche.

L-sélectine

Son expression est constitutive à la surface des leucocytes, tandis que son ligand est seulement présent à la surface des cellules endothéliales activées. Quelques minutes suivant l'activation des leucocytes par une variété d'agents tels le FMLP, le PAF ou le LPS par exemple, la L-sélectine est clivée de la surface des leucocytes par un clivage protéolytique juste à l'extérieur du domaine transmembranaire entre la Lys^{321} et la Ser^{322} (48-50). Le clivage ne se produit pas seulement en réponse à une activation cellulaire, mais aussi suite à un traitement avec un cross-linker chimique (51) ou à certains anti-inflammatoires non-stéroidiens (52).

Même si la nature biochimique et moléculaire de l'enzyme protéolytique n'a pas encore été identifiée, elle semble être constitutivement active (53,54). De plus le clivage nécessiterait la formation d'une structure tridimensionnelle de la L-sélectine (53,54). Conséquemment, le clivage de la L-sélectine de la surface limite l'habileté des leucocytes à rouler sur les cellules endothéliales (50). Une étude récente a rapporté que la queue intracytoplasmique de la L-sélectine est phosphorylée sur une sérine suite à l'activation par des chémoattractants (55). De plus des inhibiteurs de calmoduline induisent directement le clivage de la L-sélectine (56). Ces observations suggèrent que l'expression constitutive de la L-sélectine est contrôlée par la calmuduline dans les cellules non stimulées.

La E-sélectine

La E-sélectine est exprimée à la surface des cellules endothéliales activées seulement (57). Plusieurs cytokines pro-inflammatoires dont l'IL-1β, le TNF-α ainsi que le LPS augmentent son expression (58). Celle-ci requiert la synthèse *de novo* de protéines (sous contrôle transcriptionnel) résultant en un pic d'expression à 4-6 heures après la stimulation, et diminue progressivement jusqu'au niveau basal en 24 heures (58).

La P-sélectine

La P-sélectine est exprimée à la surface des cellules endothéliales activées (59,60) et des plaquettes (61,62). Elle est emmagasinée dans les corps de Weibel-Palade des cellules endothéliales non-stimulées (59,60) et dans les granules α des plaquettes (63). Lorsque les cellules endothéliales sont activées (ex. avec de l'histamine ou de la thrombine), la P-sélectine est mobilisée à la surface des cellules activées en quelques minutes (63,64), puis elle est ensuite endocytosée ou elle sera dégradée ou recyclée dans les corps de Weibel-Palade (61). Lors d'une inflammation, la P-sélectine endothéliale agit pour recruter les leucocytes dans les veinules postcapillaires, tandis que la P-sélectine associée aux plaquettes promouvoit

l'aggrégation des leucocytes aux plaquettes pour former des thrombi (65). Les cellules endothéliales peuvent aussi exprimer la P-sélectine en réponse au LPS et aux cytokines. Par contre, il a été observé que les réponses sont différentes selon les espèces. Suite à une stimulation avec du TNF-α ou du LPS l'expression de la P-sélectine est augmentée à la surface des cellules endothéliales murines (66,67), mais à la surface des cellules endothéliales humaines (68). Ces différences de réponse seraient reliées à des différences dans le promoteur pour la P-sélectine (67).

3.1.1.3 Les ligands (contre-récepteurs) des sélectines

Les sélectines fonctionnent en se liant via leur domaine lectine à des glycoprotéines (O-glycosylées) appelées sialomucines. Les sialomucines présentent dans leur structure des oligosaccharides complexes (composés d'acide sialique, de fucose, de galactose) soit des polylactosamines [Galβ(1,4)GlcNacβ(1,4)Gal], qui sont fucosylées et/ou sulfatées (69). Entre autres, les ligands des sélectines présentent l'épitope du tétrasaccharide sialyl Lewis X (Sle^x) (CD45) ou de son isomère SLe^e (70). Trois sialomucines liant la L-sélectine ont été identifiée sur les cellules endothéliales (GlyCAM-1, CD34, MadCAM-1). Le PSGL-1 (P-selectin glycoprotein ligand-1), largement exprimé par les leucocytes, lie la P- et la E-sélectine (71).

3.1.1.4 Les sélectines et les mécanismes de transduction du signal

Des études suggèrent que la L-sélectine est impliquée dans la signalisation intracellulaire. En effet la liaison de la L-sélectine à son ligand, exprimé par les celulles endothéliales, active certaines fonctions biologiques des neutrophiles dont une augmentation de la production d'O^-_2 et une augmentation de l'adhésion médiée par les β_2-intégrines (augmente leur avidité) (71). Les voies effectrices activées ne sont pas encore bien définies. La réponse induite par les sélectines est inhibée par le genistein, un inhibiteur de tyrosine kinase, et par la staurosporin, un inhibiteur de PKC, suggérant l'activation de protéines tyrosine kinases et PKC (71). D'autres études suggèrent que la E-sélectine pourrait aussi être impliquée dans la signalisation intracellulaire des cellules endothéliales. Il semble y avoir un regroupement des

molécules de E-sélectines à la surface endothéliale et leur association avec certaines protéines du cytosquelette et protéines régulatrices dont la FAK, une tyrosine kinase cytoplasmique (72). Les voies effectrices restent à définir.

3.1.2 Les intégrines

Les étapes subséquentes de l'accumulation extravasculaire des leucocytes au foyer inflammatoire impliquent l'adhésion ferme des leucocytes à la surface endothéliale, leur diapédèse entre les jonctions interendothélilales et leur migration dans les tissus. Les intégrines jouent un rôle important dans ces étapes.

3.1.2.1 Caractéristiques structurales des intégrines

Les intégrines forment une superfamille de glycoprotéines transmembranaires. Ce sont des hétérodimères formés par l'association non covalente d'une chaîne α et d'une chaîne β. Chaque sous-unité α et β sont des protéines à un passage transmembranaire, présentant un large domaine extracellulaire intracytoplasmique. La figure 4 représente un schéma de la structure d'une intégrine. Chaque sous-unité α varie de 120 à 180 kDa et l'analyse de leur séquence d'ADNc révèle plusieurs caractéristiques communes, dont une séquence répétée en tandem qui constitue le site de liaison aux cations divalents (73). Ces sites sont essentiels à la liaison du ligand et à l'association des sous-unités. Chaque sous-unité β varie de 90 à 210 kDa. Les sous-unités β présentent 40 à 60% d'homologie, avec des caractéristiques structurales très conservées dans une grande variété d'espèces. L'extrémité N-terminale est riche en résidus Cys. Ce domaine est suivi du domaine de liaison du ligand et d'un second noyau riche en Cys impliqué dans la formation de ponts disulfures internes résistant aux protéinases et constitué de 4 domaines répétitifs de 40 acides aminés.

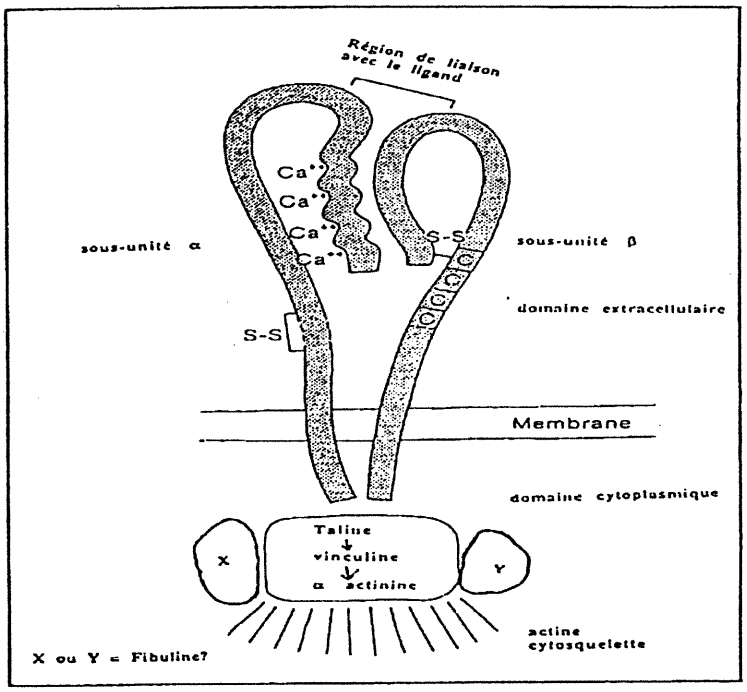

Figure 4: Représentation schématique de la structure d'une intégrine.
Labat-Robert J, 1992 (73)

3.1.2.2 Classification des intégrines

Les intégrines sont classifiées selon la natures de la chaîne β (β_1-β_7). Les sous-classes impliquées dans les interactions entre les leucocytes et les cellules endothéliales sont les β_2-intégrines, la β_1-intégrine et la β_7-intégrine. La table 3 présente les différents membres des intégrines. Mes études portent plus particulièrement sur les β_2-intégrines, donc je m'atarderait plutôt sur ces dernières.

Les β_2-intégrines

Les membres de cette famille contiennent une des quatre chaînes α désignées par CD11a, CD11b, CD11c et CD11d qui sont couplées à une chaîne β commune, la CD18 . L'hétérodimère CD11a/CD18 (LFA-1) est exprimé à la surface de la plupart des leucocytes et interagit avec ICAM-1 et ICAM-2 sur les cellules endothéliales pour causer l'adhésion ferme (74).

Table 3 : Classification des intégrines.

Sous-classe	Récepteur	Ligands	Distribution	Régulation
β_1 (CD29)	VLA-1	Laminine , collagène	Cellules T activées Fibroblastes	Induit par mitogène
	VLA-2	Collagène, laminine	Cellules T activées Plaquettes Cellules endothéliales	Augmenté par mitogène
	VLA-4	VCAM-1, fibronectine	Lymphocytes Monocytes Eosinophiles	Activation fonctionnelle par antigènes
	VLA-5	Fibronectine	Lymphocytes Monocytes Cellules endot. Fibroblastes	Activation fonctionnelle par antigènes
	VLA-6	Laminine	Lymphocytes Plaquettes Cellules endot. Fibroblastes	Activation fonctionnelle par antigènes
β_2 (CD18)	LFA-1 (CD11a)	ICAM-1, ICAM-2, ICAM-3, E-sélectine	Toutes les cellules hématopoiétiques sauf globule rouge	Activation fonctionnelle par différents stimuli
	Mac-1 (CD11b)	ICAM-1, fibrinogène, facteur X, iC3b	Monocytes Granulocytes Lymphocytes	Expression augmentée par facteurs chémotactiques
	p150/95 (CD11c)	Fibrinogène, iC3b	Monocytes Granulocytes Lymphocytes	Augmenté par TNF-α
β_7	$\alpha 4\beta 7$	MAdCAM-1,VCAM-1, fibronectine	Cellules B et T Macrophages	?
	$\alpha^E/\beta 7$	E-cadhérine	Lymphocytes dans l'intestin	induit par TGF-β1

Modifiée de Oppenheimer-Marks Lipsky, 1996 (43)

Cette intégrine ne semblerait pas être emmagasinée dans les leucocytes donc son augmentation résulterait plutôt d'un changement de conformation qui permettrait aux leucocytes d'interagir avec ICAM-1 (75). Les hétérodimères CD11b/CD18 (Mac-1) et CD11c/CD18, exprimés par les neutrophiles et les monocytes, sont emmagasinés

dans des granules et lorsque les leucocytes sont activés, ils sont rapidement mobilisés à la surface en se fusionnant avec la membrane cellulaire (75). L'activation par des médiateurs (ex. : PAF et IL-8) ou des cytokines (ex. : TNFα) augmente l'expression de ces intégrines à la surface des cellules. Le CD11b/CD18 interagit avec ICAM-1 sur l'endothélium tandis que le ligand pour le CD11c/CD18 est encore incertain. Le rôle pour le CD11d/CD18 dans le recrutement des leucocytes n'est pas vraiment établit (76). L'immunoneutralisation de ces intégrines par des anticorps spécifiques empêcherait l'adhésion des leucocytes activés à l'endothélium activé. L'adhésion des leucocytes non stimulés est médiée par l'interaction de CD11a/CD18 avec ICAM-1 tandis que l'adhésion des leucocytes activés se fait par les interactions de CD11a/CD18 et CD11c/CD18 à ICAM-1. L'activation régule l'adhésion en altérant soit l'affinité de chaque molécule par un changement de conformation ou en induisant l'aggrégation (clustering) des β_2-intégrines ce qui augmente alors l'avidité c'est-à-dire la force de liaison (77-79).

β_1- et β_7-intégrine

Ces hétérodimères contribuent aussi au recrutement de différentes populations de leucocytes. La β_1-intégrine $\alpha4\beta1$ (VLA-4) médie l'adhésion des lymphocytes, des monocytes, des éosinophiles et des cellules NK aux cellules endothéliales activées exprimant le contre-récepteur VCAM-1 (80). La β_7-intégrine $\alpha4\beta7$ est exprimée à la surface de certains lymphocytes B et T et fonctionne comme un "homing receptor" pour le tissu lymphoïde intestinal. Elle se lie à MadCAM-1 puis à VCAM-1 (81).

3.1.2.3 Les intégrines et les voies de signalisation

En plus de médier l'attachement des leucocytes aux cellules endothéliales, les intégrines modifient aussi des voies de signalisation intracellulaires associées à l'activation cellulaire. Donc les intégrines agissent comme récepteurs de régulation et fonctionnent en transmettant des signaux biochimiques vers l'intérieur de la cellule, ce qu'on appelle le "outside-in signaling"(82). Cette signalisation "outside-

in" régule la chimiotaxie, la sécrétion de cytokines, la phagocytose, la transcription de nombreux gènes via l'activation de NF-kB incluant les gènes des cytokines (IL-8, TNF-α, IL-1β) et autres (*c-fos, c-jun*...) (83,84). La FAK aurait un rôle important à jouer dans la signalisation intracellulaire induite par les intégrines (85). La kinase FAK est une tyrosine kinase cytoplasmique, rapidement phosphorylée sur des résidus tyrosine en réponse à l'adhésion cellulaire. Certaines études suggèrent que l'interaction FAK-β_1-intégrine ne serait pas directe mais impliquerait une interaction via la taline (86). D'autres part, la signalisation par les intégrines conduit à l'activation de la voie des MAPK (87), bien que le mécanisme exact ne soit pas encore bien compris.

3.1.3 Les molécules d'adhésion apparentées aux Ig

3.1.3.1 Caractéristiques structurales

Les membres de cette famille sont des glycoprotéines présentant une seule chaîne transmembranaire et qui présentent dans la partie extracellulaire de leur structure des domaines analogues à ceux constituant les chaînes lourdes et légères des immunoglobulines, ainsi qu'une courte séquence dans la partie cytoplasmique. Plus d'une trentaine de molécules sont regroupées dans cette superfamille. Les membres impliqués dans l'adhésion des leucocytes aux cellules endothéliales incluent ICAM-1, ICAM-2, VCAM-1, MadCAM-1 et PECAM, la molécule d'adhésion des plaquettes aux cellules endothéliales.(voir figure 5).

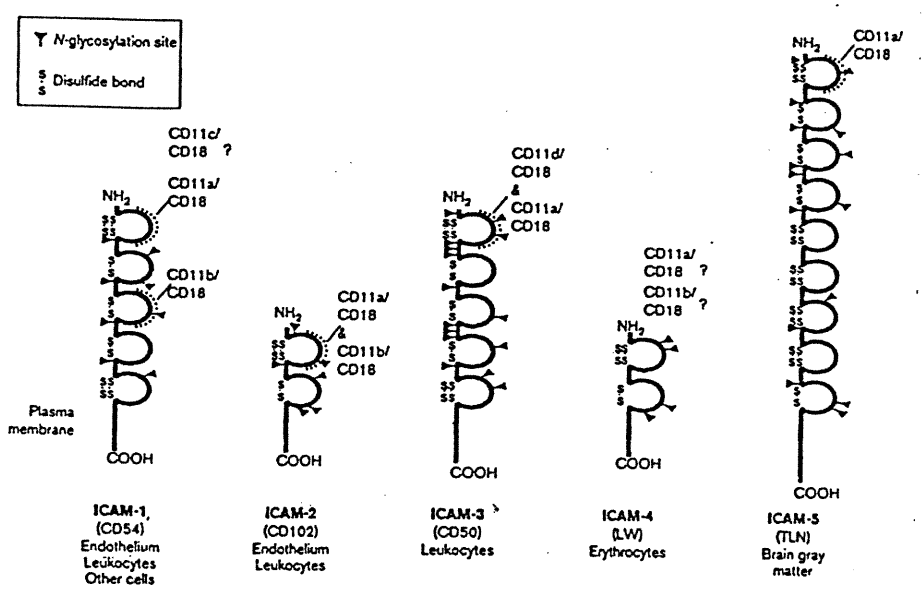

Figure 5 : Structure des molécules d'adhésion apparentées aux immunoglobulines. **Gahmberg, 1997 (88)**

3.1.3.2 Caractéristiques particulières

ICAM-1 est exprimé à la surface des leucocytes, des fibroblastes, des cellules épithéliales ainsi que des cellules endothéliales. Son expression constitutive est très faible, mais elle peut augmenter suite à une stimulation avec des cytokines (IL-1β, TNF-α), du LPS, de la thrombine et des esters de phorbol. La stimulation des cellules endothéliales induit la transcription et la translation *de novo*, résultant en un pic d'expression à environ 5h, et cette expression est maintenue élevée pendant 24h (89). Comme je l'ai mentionné plus tôt, ICAM-1 lie CD11a/CD18 et CD11b/CD18 sur les leucocytes, et médie l'adhésion ferme des leucocytes aux cellules endothéliales (90). Une forme soluble d'ICAM-1, qui représente un fragment clivé d'ICAM-1 endothéliale, peut être détectée dans le plasma lors d'une inflammation (91). Certaines évidences suggèrent que l'ICAM-1 soluble pourrait lier CD11/CD18 sur les leucocytes, mais les conséquences fonctionnelles de cette liaison ne sont pas encore claires.

ICAM-2 est exprimée constitutivement sur les cellules endothéliales et son expression n'est pas affectée par l'activation des cellules endothéliales. ICAM-2 se lie à CD11a/CD18, mais avec une affinité plus faible qu'ICAM-1 (75).

VCAM-1 peut lier $\alpha4\beta1$ et $\alpha4\beta7$ sur les leucocytes, et elle médie le trafic des monocytes et des lymphocytes. Son expression est augmentée suite à une stimulation par une variété d'agents. De façon similaire à ICAM-1, son augmentation d'expression requiert la synthèse *de novo*, résultant en un pic d'expression à environ 6h qui diminue en 48-72h (92).

PECAM-1 est exprimée constitutivement à la surface des plaquettes, des leucocytes et des cellules endothéliales. Elle médie l'adhésion des leucocytes et des plaquettes aux cellules endothéliales, la migration des leucocytes à travers les cellules endothéliales (93) ainsi que leur migration à travers la membrane basale périvasculaire (94). Son expression n'est pas affectée suite à une stimulation.

MadCAM-1 est exprimée sur les veinules endothéliales élevées. Elle sert de ligand pour la L-sélectine et l'intégrine $\alpha4\beta7$, puis est impliquée dans le "homing" des lymphocytes vers les plaques de Peyer (95).

3.2 Les mécanismes d'adhésion leucocytes-cellules endothéliales

Les neutrophiles interagissent avec les cellules endothéliales selon un processus à plusieurs étapes régulé par les différentes molécules d'adhésion. Les observations faites par plusieurs chercheurs, souvent en utilisant la microscopie intravitale, ont permis de prévoir que des mécanismes très précis gouvernent ces interactions cellule-cellule.

3.2.1 L'hypothèse du processus à plusieurs étapes

Il y a en effet un contrôle complexe des interactions entre les leucocytes et les cellules endothéliales *in vivo* (96-98), et on peut décrire ce processus actif par un modèle général en quatre étapes d'événements séquentiels, représentés à la figure 6. Ceci comprend trois étapes ou plus, incluant la capture et le roulement initial médié

par les sélectines, l'activation cellulaire et l'adhésion ferme, et conséquemment la transmigration.

Suite à une activation, l'endothélium exprime ses propriétés pro-inflammatoires. Ces propriétés incluent la production de cytokines inflammatoires, de chémokines, de facteurs de coagulation et d'agents vasoactifs, ainsi que l'expression des molécules d'adhésion spécifiques qui lierons les neutrophiles, ce qui les ralenti et cause alors leur roulement dans le sens du flot sanguin. Le contact initial et le roulement sont médiés par la L-sélectine exprimée à la surface des neutrophiles puis par la P- et la E-sélectine exprimées par les cellules endothéliales activées. En roulant le long de l'endothélium, les neutrophiles sont exposés à des signaux d'activation incluant des substances solubles dérivées de l'endothélium tel l'IL-8, et des agents présents à la surface des cellules endothéliales tel le PAF ou à des signaux de transduction provenant des molécules d'adhésion-mêmes. L'activation des neutrophiles mène au clivage de la L-sélectine de la surface avec une augmentation concommitante de l'expression ou de l'activation des β_2-intégrines (99). Les β_2-intégrines interagissent avec ICAM-1 sur les cellules endothéliales pour promouvoir l'adhésion ferme, ce qui permet l'arrêt du roulement des neutrophiles. Une fois que les neutrophiles sont adhérés fermement, leur forme change (ils s'applatissent), ils projettent des pseudopodes entre les cellules endothéliales, et émigrent dans les tissus.

Bien qu'en général il y ai un accord concernant les mécanismes impliqués dans les interactions entre les neutrophiles et les cellules endothéliales, les mécanismes responsables de la transmigration ne sont pas encore clairs. Les études *in vivo* d'inflammation aiguë ont démontré que les inhibiteurs de l'élastase préviennent l'accumulation des neutrophiles dans les tissus infectés (100-102). Ces observations suggèrent que les neutrophiles mobilisent leur pool endogène de protéases pour traverser l'endothélium protéolytiquement. Par contre les inhibiteurs d'élastase utilisés peuvent aussi interférer avec l'adhésion des leucocytes (101,102).

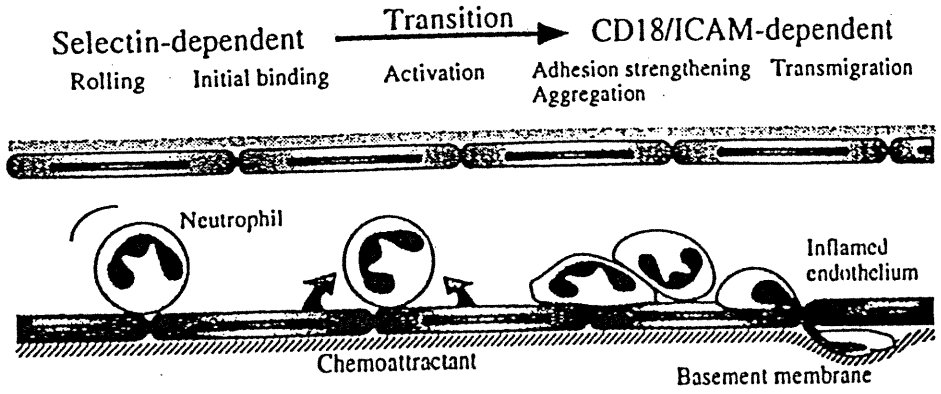

Figure 6 : Un modèle des interactions entre les neutrophiles et les cellules endothéliales. Dans ce modèle simplifié, les sélectines médient l'attachement initial et le roulement des neutrophiles, tandis que les molécules CD18/ICAM-1 sont impliquées dans l'adhésion ferme et la migration transendothéliales. **Kishimoto TK, Rothlein R, 1994 (103)**

Les maladies génétiques humaines nous ont fourni des informations importantes concernant la physiologie et la pathologie vasculaire. Un bon exemple est celui des patients LAD-1 (104) qui sont déficients en β_2-intégrines. Les neutrophiles de ces patients ne peuvent traverser l'endothélium et accumuler aux sites inflammatoires (105), et *in vitro* ils ne peuvent pas lier, ni migrer à travers des monocouches de cellules endothéliales activées ou non (106). De plus, la technologie des animaux transgéniques et en particulier le ciblage de mutations génétiques d'intérêt (knockout) a été très utile pour déterminer les fonctions des différentes molécules d'adhésion (107-109), et d'avoir plus de détail concernant les séquences d'événements. Des knockout pour la L-sélectine (46), la P-sélectine (45) et la E-sélectine (47), nous ont démontré les défauts inflammatoires associés à l'absence des sélectines. Par exemple, des souris knockout pour la L-sélectine ($L^{-/-}$) démontrent une baisse du recrutement des neutrophiles dans différents modèles d'inflammation (46,110). Récemment, des études avec des doubles et même des triples knockout de sélectines ont démontré que les sélectines possèdent des fonctions distinctes tout en

étant simultanées (111). Par contre, d'après certaines études, les molécules d'adhésion requises pour le recrutement des neutrophiles pourraient démontrer des différences selon l'organe affecté. Par exemple des souris knock-out pour ICAM-1, P-sélectine ou les deux ont un phénotype normal, mais l'accumulation des neutrophiles dans la cavité péritonéale induite par le LPS est grandement réduite (112). Par contre, au niveau des poumons, dans les mêmes circonstances, le recrutement des neutropiles n'est pas affecté. Non seulement les réponses pourraient être organes spécifiques, mais elles pourraient aussi dépendre du stimulus utilisé (113). Donc en appliquant le modèle proposé il faut tenir compte de ces différences.

4. Les mécanismes endogènes pro- et anti-adhésifs

4.1 L'endothéline

4.1.1 La structure et la synthèse des endothélines

L'endothéline (ET) a été isolée et séquencée pour la première fois à partir de cellules endothéliales en culture provenant de cochon (114). La famille des ETs comprend trois membres (isoformes), l'ET-1, l'ET-2 et l'ET-3, qui sont codés par trois gènes distincts (115). Chaque isoforme contient 21 acides aminés, 2 ponts disulfures intra-chaînes et une séquence C-terminale conservée nécessaire à l'activité biologique (115). Cette structure est unique parmi les mammifères, mais elle partage une similarité avec celle des sarafotoxines, des peptides provenant de venin d'un serpent d'Israël, *Atractaspis engaddensis* (116). Les structures sont représentées à la figure 7. Les ETs sont produites par une variété de tissus. L'ET-1 a été isolée pour la première fois de l'endothélium (114), mais elle est aussi exprimée dans d'autres organes non vasculaires tel le cerveau, les reins, les poumons et autres (117). L'ET-2 et l'ET-3 peuvent être détectées dans d'autres organes tels les intestins, la glande surrénales et le cerveau. L'ET-3 est relativement abondante dans les tissus neuronaux et on la considère comme la forme neuronale des endothélines (118-120). Bien que retrouvées dans une variété de tissus, seulement l'ET-1 se retrouve dans les cellules endothéliales vasculaires. L'ET-1 est un peptide vasoconstricteur puissant des artères coronariennes tant *in vitro* que *in vivo* (121) et a récemment été reconnue comme un médiateur clé de l'ischémie (122).

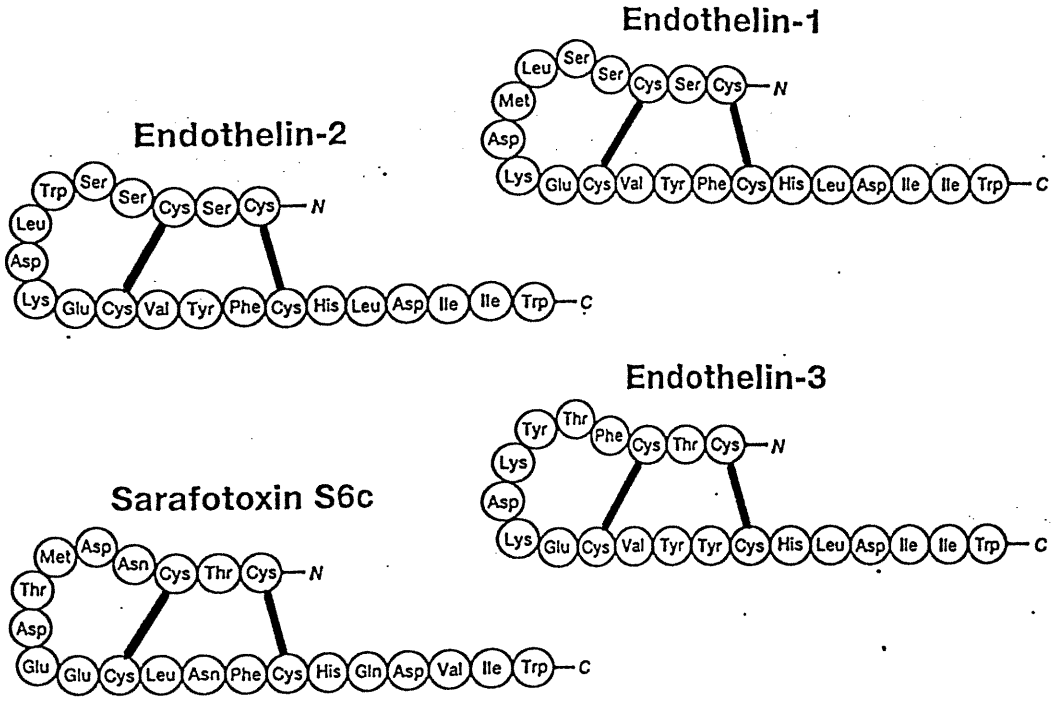

Figure 7 : Stuctures des peptides de la famille des endothélines et de la sarafotoxine S6c. **Webb DJ, 1997 (123).**

L'ET-1 est synthétisée à partir d'un précurseur initial, la preproendothéline, comprenant 212 acides aminés.. Ce précurseur est clivé par une endopeptidase pour former un autre précurseur biologiquement inactif de 38 acides aminés, la Big ET-1. Puis finalement la Big ET-1 est clivée par l'enzyme de conversion de l'endothéline (ECE) pour donner le peptide actif de 21 acides aminés. (voir figure 8). Deux gènes ont été clonés pour l'ECE, ECE-1 et ECE-2 (124). Les deux protéines ont une similarité de séquence avec l'endopeptidase neutre 24.11 (125), et elles peuvent être inhibées par le phosphoramidon (126).

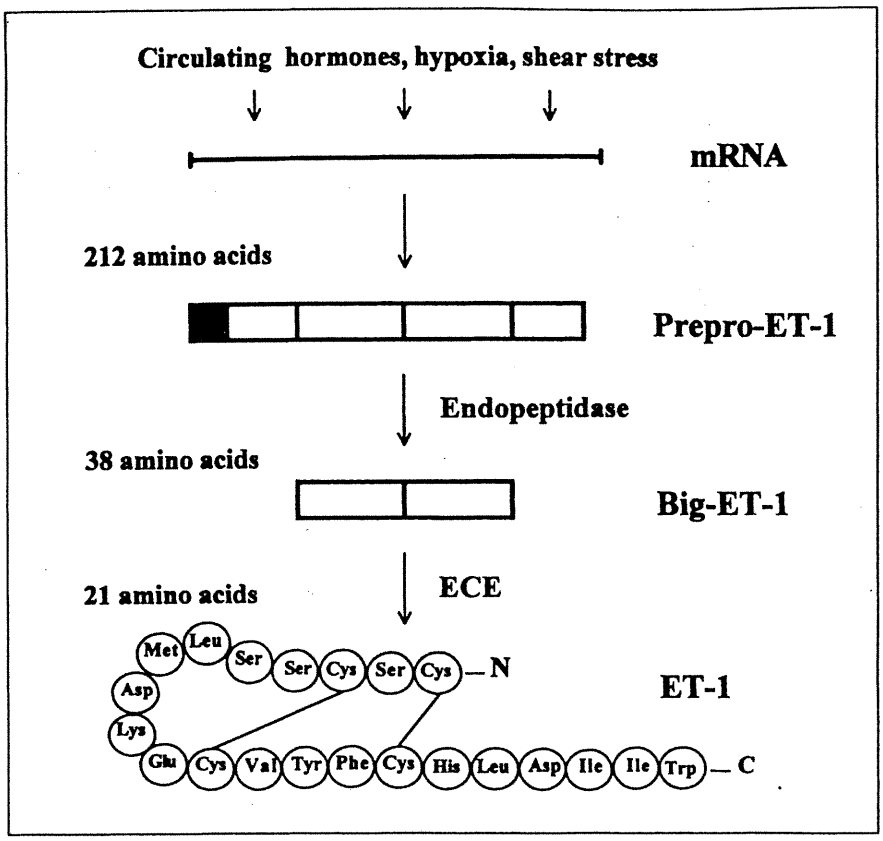

Figure 8 : Biosynthèse de l'endothéline-1 (ET-1). **Mateo et al (128)**

4.1.2 Les récepteurs à l'endothéline

Il existe au moins deux types de récepteurs à l'ET. Ces récepteurs appartiennent à la superfamille des récepteurs couplés aux protéines G (127), et ils sont classifiés selon leur affinité différente pour les trois isopeptides de l'endothéline. L'ordre d'affinité pour le récepteur ET_A est ET-1 > ET-2 > ET-3 (128). Tandis que pour le récepteur ET_B elles ont toutes la même affinité (non sélectif) (129). Le gène humain pour ET_A, présent sur le chromosome 4, contient 8 exons et 7 introns, et le gène pour ET_B, présent sur le chromosome 13, comprend 7 exons et 6 introns (130-132). La principale différence structurale entre ces deux gènes est la présence d'un codon de plus dans la région 5'- non codante de ET_A. D'autres part, les sites d'excision exon-intron sont conservés entre ces deux

récepteurs suggérant leur évolution d'un gène ancestral commun (130,132). Certaines évidences pharmacologiques suggèrent l'existence d'un troisième sous-type de récepteur, non-ET_A/non-ET_B, qui est nommé ET_C (133). Il a été cloné dans les mélanophores des nématodes, *Xenopus laevis* (133), mais l'existence d'un tel sous-type de récepteur chez les mammifères est encore inconnue.

4.1.3 Les antagonistes aux récepteurs à l'endothéline

Plusieurs techniques ont été utilisées pour la découverte d'antagonistes efficaces contre les récepteurs de l'endothéline (134). Lors du développement de ces composés, il a été découvert que les récepteurs ET_A reconnaissent la structure tertiaire des portions N-terminale et C-terminale de la molécule d'endothéline. Par contre les récepteurs ET_B ne reconnaissent que la structure de la portion C-terminale. Particulièrement la séquence Glu^{10}-Trp^{21} de l'ET. Il existe maintenant plusieurs types d'antagonistes sélectifs pour les récepteurs ET_A, dont les plus connus sont BQ-123 (135) et FR 139317 (136,137), des antagonistes sélectifs pour le récepteur ET_B tels BQ-788 (138) et IRL 1038 (139), ainsi que des antagonistes non sélectifs pour les récepteurs ET_A/ET_B dont le Bosentan (140) par exemple. La table 4 montre les différents antagonistes que l'on peut retrouver et leur affinité pour les types de récepteurs.

Table 5 : Les affinités des antagonistes de l'endothéline pour les récepteurs ET_A et ET_B.

	Structure	ET_A		ET_B	
		IC_{50}	K_i	IC_{50}	K_i
ET_A receptor-selective antagonists					
BQ-123	cyclo (-D-Trp-D-Asp-Pro-D-Val-Leu)	13-63 nM	17-25 nM	> 18 µM	11-31 µM
BQ-153	cyclo-D-sulphalanine-Pro-D-Val-Leu-D-Trp	9 nM	-	54 µM	-
BQ-485	perhydroazepin-1-yl-Leu-D-Trp-D-Trp	3.4 nM	-	26 µM	-
FR 139317	((*R*)2-[(*R*)-2-[(*S*)-2-[[1-(hexahydro-1*H*-azepinyl)]carbonyl]amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1*H*-indoyl)]-propionyl]amino-3-(2-pyridyl)propionic acid	6-13 nM	1 nM	>20 µM	7.3 µM
WS-7338B	cyclo (D-allo-Ile-Leu-D-Trp-D-Glu-Ala)	270 nM	500 nM	>130 µM	-
50-235	27-*O*-caffeoyl myricerone	1-3 nM	-	>1 µM	-
BMS 182874	(5-(dimethylamino)-N-(3,4-dimethyl-5-isoxazolyl)-1-napththalenesulphonamide	200 nM	61 nM	>10 µM	>200 µM
WS009 A, B	anthraquinone chromophores	5 µM	-	>800 µM	-
ET_B receptor-selective antagonists					
IRL-1038	[Cys^{11}-Cys^{15}]endothelin-1 (11-21)	-	500 nM	-	6-11 nM
BQ-788	*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylelucyl-D-1-methoxy-carbonyl-tryptophanyl-D-norleucin	1.3 µM	-	1.2 nM	-
RES-701-1	cyclic (Gly^1-Asp^9)(Gly-Asn,-Trp-His-Gly-Thr-Ala-Pro-Asp-Trp-Phe-Phe-Asn-Tyr-Tyr-Trp))	>5 µM	-	10 nM	-
Nonselective ET_A/ET_B antagonists					
	Thr^{18}γ-methyl[Leu^{19}]endothelin-1	0.7 nM	-	0.25 nM	-
PD 142893	acetyl-(3,3-D-diphenylalanine-Leu-Asp-Ile-Ile-Trp	15 nM	-	150 nM	-
PD 145065	Ac-D-5*H*-(dibenzyl[*a,d*]cycloheptane-10,11-dihydroglycine)Leu-Asp-Ile-Ile-Trp	4 nM	-	15 nM	-
TAK-044	cyclo[D-α-aspartyl-3-[(4-phenyl-piperazin-1-yl)carbonyl]Ala-Asp-D-2-(2-thienyl)-Gly-Leu-D-Trp	10 nM	-	32 nM	-
Ro 46-2005	4-*tert*-butyl-*N*-[6-(2-hydroxy-ethoxy)-5-(3-methoxy-phenoxy)-4-pyrimidinyl]-benz-ene-sulfonamide	220-430 nM	-	0.1-11 µM	-
Bosentan	4-*tert*-butyl-*N*-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl]-benzenesulfonamide	-	4.7 nM	-	5-95 nM
Pheophorbide a		80 nM	-	210 nM	-
CGS 27830	1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-4-(3-nitrophenyl)-3-pyridine-carboxylic acid	16 nM	-	295 nM	-
SB 209670	indane carboxylic acid	2 nM	0.43 nM	32 nM	15 nM

Filep JG, 1995 (141)

4.1.4 Action de l'ET-1 sur les leucocytes

Étant donnée que l'ET-1 est un vasoconstricteur puissant, ce n'est pas étonnant de voir que la plupart des recherches concernant l'ET-1 portent sur son effet au niveau du tonus vasculaire (142,143). Par contre, plusieurs études indiquent que l'ET-1 démontre des actions pro-inflammatoires (141). Dans certaines conditions pathologiques tel l'ischémie (144) et l'athérosclérose (145) qui sont caractérisée par une augmentation de l'adhésion des leucocytes aux cellules endothéliales (146), on retrouve une augmentation de l'expression tissulaire et des niveaux plasmatiques d'ET-1. Plusieurs études suggèrent que l'ET-1 puisse être un modulateur autocrine/paracrine important des fonctions des neutrophiles. L'ET-1 cause une leucocytopénie sélective des neutrophiles chez le cochon d'Inde (147) et induit l'accumulation des neutrophiles dans le coeur (148), le poumon (149) et le rein (150) isolés perfusés de rat. Des études ont rapporté que le blocage du récepteur ET_A empêche la leucocytopénie des neutrophiles induite par l'ET-1 (151), ainsi que l'accumulation des neutrophiles dans les poumons de rats perfusés (152). Les neutrophiles stimulés par l'ET-1 migrent de la lumière des veines à la matrice extracellulaire de corde ombilicale humaine, et induit une destruction massive du tissu (153). L'ET-1 promouvoit l'aggrégation des neutrophiles (154), stimule l'expression de CD11b/CD18 à la surface de neutrophiles humains et augmente leur adhésion à des cellules endothéliales bovines en culture (148). Par contre l'ET inhibe le roulement et l'adhésion des neutrophiles dans la circulation mésentérique chez le chat via la modulation de la synthèse de NO suivit de i'inhibition de l'expression de la P-sélectine sur les cellules endothéliales (155). Mais dans aucune étude on a questionné l'effet de l'ET-1 sur l'expression de la L-sélectine, d'ICAM-1 et de la E-sélectine, ou les sous-types de récepteurs impliqués dans les interactions entre les neutrophiles et les cellules endothéliales, ainsi que le ou les mécanismes impliqués.

4.2 La protéine C-réactive

Plusieurs changements, loin du site d'inflammation et impliquant différents organes, pourraient accompagner l'inflammation. Ces changements systémiques sont

communément appelés les réponses de phase aiguë, même s'ils peuvent accompagner aussi bien les désordres inflammatoires aigus que chroniques. Les cytokines produites lors du processus inflammatoire sont les stimulateurs de la production des protéines de phase aiguë.

4.2.1 Structure et synthèse de la protéine C-réactive

La protéine C-réactive est une de ces protéines de phase aiguë. Des études récentes de crystallographie et de mutagenèse (156) ont permis de comprendre la biologie structurale de la protéine, tandis que les expériences utilisant des souris transgéniques (156) ont confirmé sa fonction dans la défense de l'hôte.

La protéine C-réactive est une protéine d'environ 105 kDa qui appartient à la famille des pentraxines (157). Elle se caractérise par la présence de cinq sous-unités (158) identiques liées de façon non covalente formant une protéine cyclique symétrique. Elle est synthétisée par le foie (159), et sa concentration plasmatique chez des sujets sains est moins de 1 μg/ml. Son taux de synthèse (induit par l'IL-8 ou l'IL-6) et de sécrétion augmente dans les heures suivant un dommage ou une inflammation, et ses niveaux peuvent augmenter plus de milles fois dans les 24h suivantes (160). En général, le niveau de réponse de la protéine C-réactive est relié à la sévérité de l'inflammation ou l'état du dommage tissulaire (161).

4.2.2 Les actions biologiques de la protéine C-réactive

Plusieurs fonctions ont été attribuées à cette protéine, et on peut les classer en trois catégories principales (162) :

1) Elle possède des fonctions de reconnaissance et elle cible les mécanismes de défense.
2) Elle modifie le comportement des cellules effectrices dont les PMNL, les lymphocytes, les monocytes et les plaquettes.
3) Elle est clivée par des enzymes protéolytiques ou modifiées, et les fragments générés sont biologiquement actifs.

En effet la protéine C-réactive se lie avec une grande affinité à la phosphocholine (162). Elle active le système du complément (163). Elle se lie avec spécificité aux lymphocytes et modifie quelques-unes de leurs fonctions (164). Il a été rapporté que la protéine C-réactive bloque la mobilisation de Ca^{2+} induite par le PAF (165), la production la relâche de β-glucuronidase et de superoxide par les macrophages et les neutrophiles (166). Puis dans les plaquettes, elle inhibe la relâche d'acide arachidonique et l'agrégation (167,168).

4.2.3 La protéine C-réactive et les neutrophiles

Le recrutement et l'activation des neutrophiles étant essentiels lors de la réponse inflammatoire, l'effet de la protéine C-réactive sur ces cellules pourrait avoir une importance particulière. La protéine C-réactive se lie avec une grande affinité aux neutrophiles humains (169,170). Plusieurs chercheurs ont rapporté la présence d'un seul récepteur pour la protéine C-réactive sur les cellules mononucléaires et les neutrophiles, mais Tebo et al. (171) puis Zahedi et al. (172) ont conclue que deux récepteurs sont présents. Une étude récente a démontré que le récepteur principal sur les leucocytes est un récepteur Fcγ (FcγRII) (173). La protéine C-réactive inhibe l'activation des neutrophiles incluant leur chimiotaxie (174), leur influx dans les espaces alvéolaires (175,176), la production de superoxide et leur dégranulation en réponse au FMLP et au PAF (166). Les mécanismes par lesquels la protéine C-réactive régule la fonction des leucocytes sont inconnus. La protéine C-réactive n'affecte pas la liaison de ligands (ex. IL-8) à leur récepteur, mais elle augmente l'activité de Erk2 (177) et de PI-3 kinase (177). Par contre, la protéine C-réactive peut inhiber l'activité de p38 MAPK (178). De plus, elle peut augmenter les niveaux d'AMPc cytosoliques (179) et inhiber la mobilisation de Ca^{2+} intracellulaire (165).

4.2.4 Les peptides dérivés de la protéine C-réactive

La stimulation des neutrophiles active une protéase à sérine associée à la membrane qui mène au clivage de peptides biologiquement actifs de la protéine C-réactive (180). Il a été suggéré que la fonction biologique spécifique de la protéine C-réactive puisse résulter de cette dégradation (181). En effet, on retrouve trois régions "tuftsin-like" dans la molécule de la protéine C-réactive, et il a été démontré que les trois peptides synthétiques correspondants peuvent stimuler la chimiotaxie des neutrophiles (181). D'autres peptides correspondant à la séquence d'acides aminés 77-82 et 201-206 inhibent la production de superoxide et la chimiotaxie des neutrophiles humains en réponse à différents stimuli (182,183), ainsi que la chimiotaxie des éosinophiles (184). Ces peptides inhibent aussi l'influx des neutrophiles et l'infiltration de protéines dans les alvéoles suite à une inflammation induite par le fMLP chez des souris (185). Tandis que le peptide 77-82 peut augmenter la production de cytokines par les monocytes/macrophages alvéolaires et peut amplifier leur activité tumorale (186,187).

4.3 Les lipoxines

Les médiateurs lipidiques jouent un rôle critique dans l'inflammation et dans d'autres événements reliés au système vasculaire incluant l'athérosclérose et la thrombose (188). L'acide arachidonique est un substrat pour les cyclooxygénases et les lipoxygénases, menant à la formation des prostaglandines, de thromboxane, de prostacycline et des leukotriènes, respectivement (189). Contrairement à d'autres médiateurs lipidiques, qui sont surtout pro-inflammatoires, les lipoxines représentent une classe unique de médiateurs lipidiques avec des actions anti-inflammatoires puissantes (189-191).

4.3.1 Les voies de synthèse

Les lipoxines (LXs) sont des éicosanoïdes contenant un trihydroxytétraène (189,190). La lipoxine est générée lors d'interactions cellule-cellule, surtout celles impliquant les leucocytes. Chez l'humain la biosynthèse de la LX est un bon exemple d'interaction entre la lipoxygénase (LO) et les voies transcellulaires (190-192). Les LXs peuvent être générées par l'une des trois voies représentées aux figures 9a,b et c. Les voies de synthèse initiées par la 5-LO (figure 9a) et par la 15-LO (figure 9b) toutes deux mènent à la formation de LXA_4 et de LXB_4 (191). La troisième voie (figure 9c) est initiée par l'aspirine (ATL) et mène à la formation de 15-épi-LXA_4 et de 15-épi-LXB_4 (193), qui pourraient être responsables pour certains des effets thérapeutiques de cet agent anti-inflammatoire (191,194).

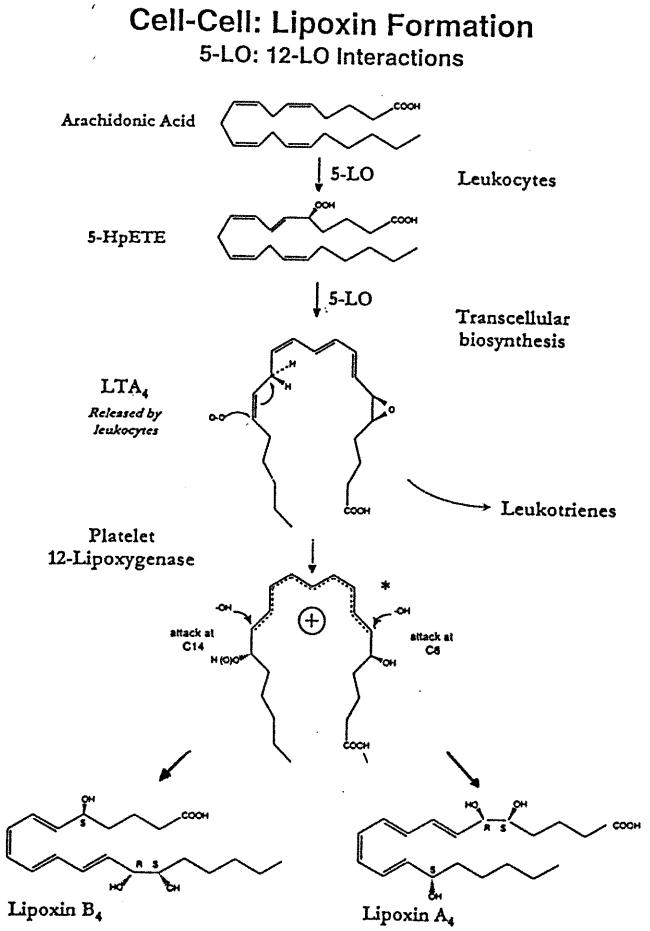

Figure 9a : Voie de synthèse initiée par la 5-LO, et menant à la formation de LXA_4 et de LXB_4. **Serhan, 1997 (191)**

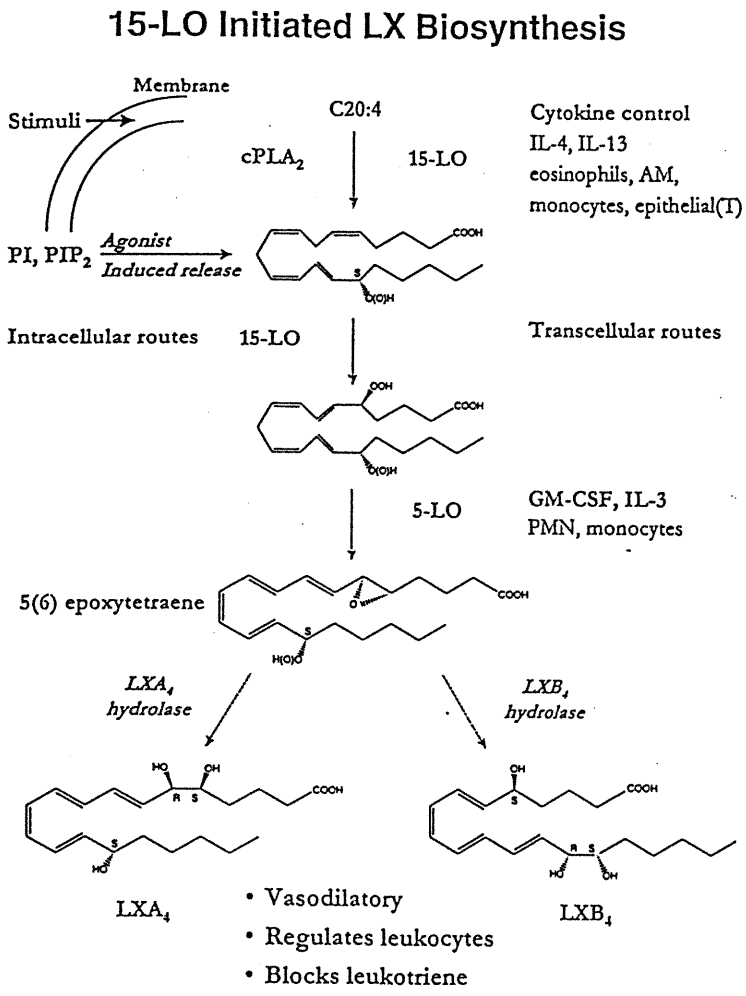

Figure 9b : Voie de synthèse des LXA_4 et LXB_4 initié par la 15-LO.
Serhan, 1997 (191)

Ces trois voies peuvent fonctionner indépendamment ou en concert (191). Par exemple, suite à un stimulus quelconque, les neutrophiles sont recrutés à un site d'inflammation ou ils pourront interagir avec les plaquettes. Une fois que les plaquettes adhèrent à la surface des neutrophiles, les neutrophiles activés généreront le leukotriène A_4 (figure 9b) qui sera relâché et il sera transformé par la LO-12 des plaquettes pour produire la lipoxine. En même temps dans le vaisseau la voie de ATL (figure 9c) pourrait fonctionner. Lorsque les cellules endothéliales sont activées, elles activent la COX-2 (PGHS-II), qui est acétylée par l'aspirine. Quand la cellule endothéliale est activée, il y a aussi relâche d'acide arachidonique qui est

ensuite transformé en 15*R*-HETE par la COX-2 acétylée. Puis le 15*R*-HETE est converti par les neutrophiles adhérents à l'endothélium en 15-epi-LXs (191).

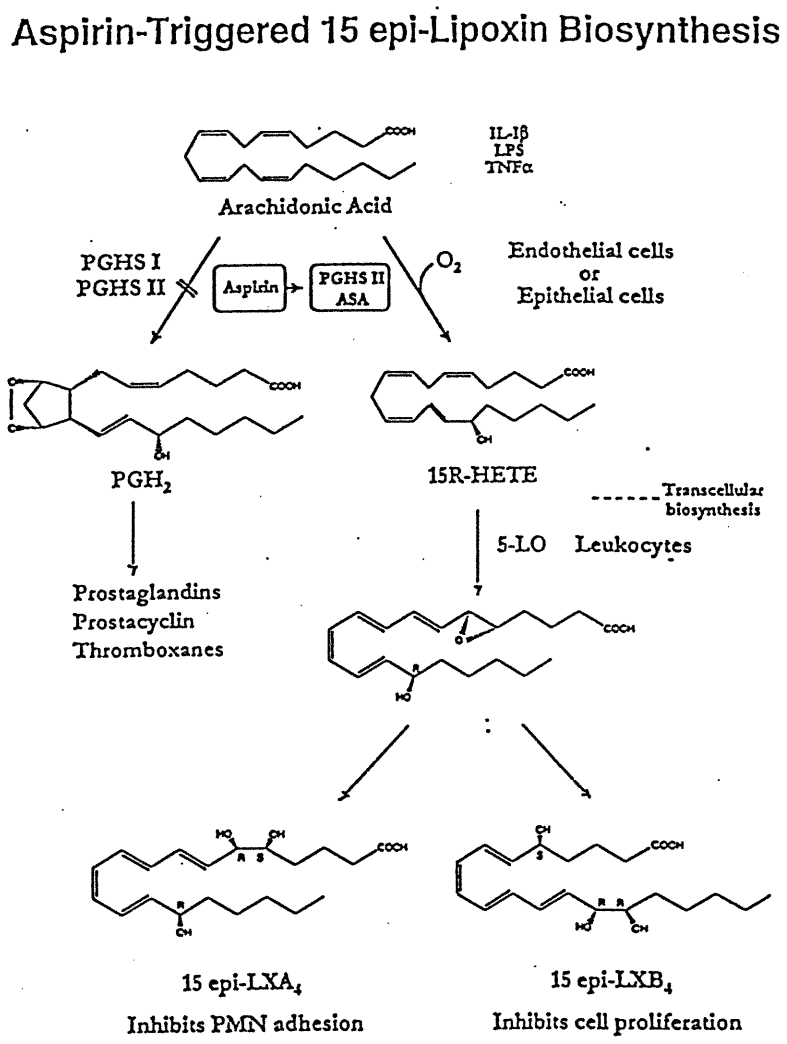

Figure 9c : Voie des ATL (aspirin-triggered lipoxin) induite par l'aspirine et menant à la formation de 15-epi-LXA_4 et de 15-epi-LXB_4. **Serhan, 1997 (191)**

4.3.2 Les analogues stables

Les lipoxines sont rapidement générées, produisent des réponses et sont régulés et métabolisés. Comme la plupart des autacoides et des médiateurs lipidiques, les lipoxines sont enzymatiquement inactivées en quelques secondes ou en quelques minutes. Ceci implique la déshydrogénation au carbone en position C-15 (191). Pour étudier et comprendre le rôle des lipoxines et des ATL, il a fallu fabriquer des analogues stables de la lipoxine. Ils résistent mieux à l'inactivation métabolique et retiennent l'activité biologique de la LX et des ATL (195) (figure 10). Par exemple, le 15-R/S-methyl-LXA_4 n'est métabolisé qu'à 10% après 3 heures dans du sang de souris, contrairement à la LXA_4 qui est métabolisée à 40% (196). La structure de quelques analogues stables de la LXA_4 est représentée à la figure 10.

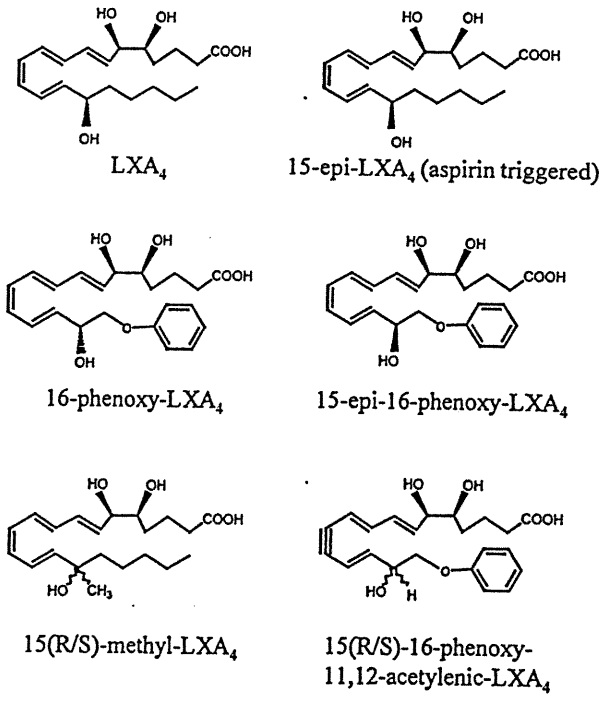

Figure 10 : Structure des analogues stables des lipoxines.
Serhan, 1997 (194)

4.3.3 Effet des lipoxines et leur action sur les leucocytes

Chez les mammifères, la production de LX a été détectée dans les poumons (197), les artères coronariennes (198), dans du sang total activé *ex vivo* (199) et dans les reins (200). Les LXs causent une vasodilatation (201) par la relâche de prostacycline (202,203) et probablement de NO par l'endothélium (204). LXA_4 diminue la constriction des voies respiratoires chez les asthmatiques (205), puis inhibe la chimiotaxie des neutrophiles et des éosinophiles (206,207). LXA_4 et ses analogues stables inhibent l'adhésion des PMNs (208) et leur migration à travers les cellules endothéliales et épithéliales (209,210), bloquent la diapédèse des PMNs des veinules postcapillaires (211) et inhibent l'accumulation de PMN dans les tissus inflammés dans des modèles animaux tels la glomérulonéphrite expérimentale chez le rat (200) et le modèle "air pouch" chez la souris (196). LXA_4 et LXB_4 diminuent l'augmentation de l'expression de CD11/CD18 à la surface des neutrophiles humains activés avec le FMLP (212) et inhibent le roulement des neutrophiles médié par la P-sélectine dans des microvaisseaux mésentériques de rats (213). Contrairement aux PMNs, les LXs stimulent la chimiotaxie et l'adhésion des monocytes (212). Des analogues de ATL inhibent l'accumulation de PMNs dans le modèle de "mouse air pouch" (196).

Dans les PMNs humains, LXA_4 interagit avec des récepteurs spécifiques couplés aux protéines G associés à la membrane (212,215) et encodés par le ADNc pINF114 (212). LXA_4 bloque aussi l'hydrolyse de la phosphoinositide (216). ATL induit l'accumulation de diphosphate presqualène, un composé de la voie de signalisation du phosphate polyisoprényle (PIPP) (217), ce qui mène à l'inhibition de la phospholipase D et de la production de superoxide (218). De plus les récepteurs de la LXA_4 sont aussi exprimés sur les entérocytes humains (219) et médient l'inhibition de la synthèse d'IL-8 (219). Bien que les cellules endothéliales ne possèdent pas de récepteurs pour les LXs, LXA_4 pourrait affecter la formation des cellules endothéliales via l'activation des récepteurs LTD_4 (220).

4.4 Les antiflammines

4.4.1 Les protéines anti-inflammatoires inductibles par les glucocorticoïdes : la lipocortine et l'utéroglobine

Il est maintenant bien connu que les glucocorticoïdes inhibent l'accumulation des leucocytes dans les tissus inflammés (221). Bien que communément utilisés pour traiter différentes maladies inflammatoires humaines, les mécanismes par lesquels ils réduisent l'influx des leucocytes et l'inflammation ne sont pas bien compris. Entre autres, ils peuvent inhiber les phospholipases (222,223), inhiber la transcription de diverses cytokines (224-226), et stabiliser les membranes lysosomales et autres (227). Des données récentes suggèrent qu'ils puissent modifier l'expression des molécules d'adhésion sur les cellules endothéliales (228) et aussi sur les leucocytes (221). Certains des effets des glucocorticoïdes sont médiés par des protéines inductibles (229,230). Une protéine, la lipocortine-1, a été découverte et caractérisée par son habileté à supprimer la production d'éicosanoïdes en agissant sur phospholipase A_2 dans la membrane. Elle est membre d'une grande famille de protéines, les annexines, qui lient le Ca^{2+} et les phospholipides. On retrouve présentement 13 membres de cette famille, mais seulement la lipocortine-1 et la lipocortine-5 semblent jouer un rôle dans l'inflammation (231). La protéine purifiée pouvait mimer l'action des glucocorticoïdes (223). La lipocortine-1 démontre plusieurs effets anti-inflammatoires dans différents modèles *in vivo* comme on peut le voir à la table 6. Une autre protéine induite par les glucocorticoïdes et ayant un effet inhibiteur sur la phospholipase A_2 est l'utéroglobine, une protéine sécrétée chez le lapin (230).

Table 6 : Résumé des effets anti-inflammatoires de la lipocortine 1 dans différents modèles *in vivo*.

Marqueur	Stimulus	Inhibition par la lipocortine1	Inversé par Ac anti-lipocortine1	Références
Migration PMN	IL-1β	oui	oui	(232)
	IL-8	-	oui	(233)
	Zymozan	oui	oui	(234)
	Carrageenine	-	oui	(235)
	Ischémie/Rep.	-	oui	(236)
Migration monocytes	Zymozan	-	oui	(237)
Migration eosinophiles.	Ovalbumine	-	non	(238)
	MIP-1α	-	non	(239)
Oedème patte	Carrageenine	oui	oui	(240)
Oedème peau	Stimulation électrique	-	oui	(241)
Douleur inflammatoire.	Bradykinine,	-	oui	(242)
	IL-1β	-	oui	(242)

Modifiée de Perretti, 1998 (231)

4.4.2 Découverte et séquences des antiflammines

Des études récentes ont démontré que des fragments de lipocortine 1 peuvent mimer les actions de la protéine native (243), dont un long fragment N-terminal correspondant aux acides aminés 1-188, le peptide Ac2-26, ainsi qu'un nonapeptide correspondant aux acides aminés 246-254, appelé antiflammine-2 (AF-2). La figure 11 montre les fragments biologiquement actifs provenant de la lipocortine-1. Si on regarde le modèle de l'interaction de la lipocortine-1 à son récepteur putatif (figure.12), on voit que deux portions de la molécule de lipocortine-1, le long fragment N-terminal et la région correspondant à l'AF-2, sont requises pour la liaison (243). Par conséquent, la région N-terminale et celle correspondant à l'AF-2 pourrait possiblement moduler la fonction des leucocytes séparément. La séquence de l'AF-2 (HDMNKVLDL) présente une grande homologie avec celle de l'AF-1

(MQMKKVLDS) qui est le fragment C-terminal de l'hélice 3 de l'utéroglobine. Ces nonapeptides ont plusieurs effets anti-inflammatoires.

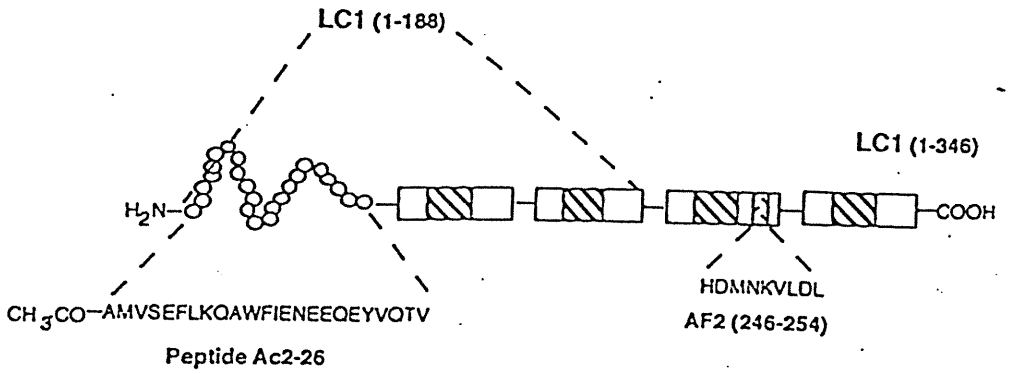

Figure 11 : Fragments biologiquement actifs de la lipocortine-1.

4.4.3 Action des antiflammines sur les leucocytes

Les antiflammines inhibent la synthèse de PAF par les neutrophiles humains (245), la chimiotaxie et l'agrégation des neutrophiles et des monocytes (245,246) et l'aggrégation des plaquettes induit par l'ADP ou le collagène (247). Elles atténuent aussi la formation d'oedème dans la patte de rat (248) ou l'oreille murine (249), et l'infiltration des leucocytes dans l'inflammation oculaire chez le rat (250).

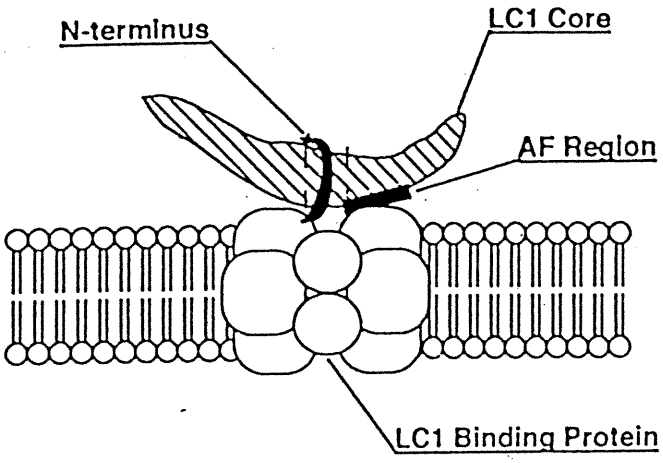

Figure 12 : Modèle de la liaison de la lipocortine-1 à son récepteur putatif.
Perretti, 1997 (251)

II. HYPOTHÈSES ET OBJECTIFS SPÉCIFIQUES

II. HYPOTHÈSES ET OBJECTIFS SPÉCIFIQUES

Principalement nous voulons étudier les mécanismes qui régulent les interactions entre les leucocytes et les cellules endothéliales, une étape clé de l'accumulation des neutrophiles aux sites d'inflammation et de dommage. Nous avons alors exploré les mécanismes d'action de certains médiateurs endogènes pro-inflammatoires (ET-1), ou anti-inflammatoires (protéine C-réactive, LX, AF).

1) Nous avons entrepris de caractériser le sous-type de récepteur à l'ET qui médie l'action de l'ET-1 sur l'expression des molécules d'adhésion à la surface de neutrophiles humains et des cellules endothéliales, ainsi que sur l'adhésion des neutrophiles aux cellules endothéliales d'artères coronariennes. D'autres part, nous voulions vérifier si le PAF pourrait médier ces actions de l'ET-1.

2) Nous voulions déterminer si la protéine C-réactive et les peptides dérivés de la protéine C-réactive pouvait affecter la première étape du recrutement des neutrophiles, c'est-à-dire l'interaction entre les neutrophiles et les cellules endothéliales médiée par la L-sélectine.

3) Nous avons vérifié l'impact d'analogues stables de la LXA_4 sur le sang humain et explorer leur mécanisme d'action cellulaire sur des cellules isolées. Nous avons étudié l'expression des molécules d'adhésion sur les leucocytes humains et sur les cellules endothéliales d'artères coronariennes en culture.

4) Puis nous avons étudié l'impact et le mécanisme cellulaire de l'action des antiflammines sur l'expression des molécules d'adhésion à la surface des leucocytes et des cellules endothéliales, puis sur l'adhésion des neutrophiles aux cellules endothéliales d'artères coronariennes.

III. RÉSULTATS

CHAPITRE I (Article I)

Zouki C, Baron C, Fournier A, Filep JG. Endothelin-1 enhances neutrophil adhesion to human coronary artery endothelial cells : role of ET_A receptors and platelet-activating factor. *British J Pharmacol.* 1999, 127, 969-979.

N.B. Dans mes publications, pour des raisons personnelles mon nom a été utilisé dans une forme simplifiée. Il a été remplacé par Zouki au lieu de Al-Zouki.

Introduction au chapitre I

L'endothéline-1, un constricteur puissant des artères coronariennes, tant *in vitro* que *in vivo* (121), a récemment été reconnu comme l'un des médiateurs clé de l'ischémie cardio-vasculaire (122). Des études récentes, incluant celles provenant de notre laboratoire, ont suggéré que l'ET-1 puisse être un modulateur autocrine/paracrine important dans la fonction des neutrophiles (141).

Nous avons entrepris de caractériser le sous-type de récepteur à l'ET qui médie l'action de l'ET-1 sur l'expression de la L-sélectine et de CD18 à la surface de neutrophiles humains, sur l'expression de la E-sélectine et d'ICAM-1 sur les cellules endothéliales, et sur l'adhésion des neutrophiles aux cellules endothéliales provenant d'artères coronariennes. Pour ceci nous avons fait des études de liaison au récepteur et en utilisant des agonistes et des antagonistes sélectifs aux récepteurs ET_A et ET_B. De plus nous voulions vérifier si le PAF médie ces actions de l'ET-1 parce que L'ET-1 stimule la synthèse de PAF par les neutrophiles et aussi parce que le PAF agit en tant que signal pour la liaison des neutrophiles aux cellules endothéliales.

Nos résultats ont démontré que l'augmentation de l'adhésion des neutrophiles aux cellules endothéliales d'artères coronariennes en culture est tout d'abord médié par l'activation du récepteur ET_A sur les neutrophiles et subséquemment par la relâche de PAF.

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Endothelin-1 enhances neutrophil adhesion to human coronary artery endothelial cells: role of ET_A receptors and platelet-activating factor

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1 The potent coronary vasoconstrictor, endothelin-1 (ET-1) may also regulate neutrophil traffic into tissues. The aim of the present study was to characterize the endothelin receptors responsible and to investigate the underlying mechanisms.

2 ET-1 (1 nM – 1 μM) markedly enhanced attachment of human neutrophils to lipopolysaccharide-, and to a lesser extent, to ET-1-activated human coronary artery endothelial cells (HCAEC). This can partially be blocked by monoclonal antibodies against E-selectin, L-selectin or CD18, whereas combination of the three antibodies inhibited adhesion by ~83%. Increases in neutrophil adhesion evoked by ET-1 were also blocked by the platelet-activating factor (PAF) antagonists, BN 52021 (50 μM) and WEB 2086 (10 μM).

3 ET-1 downregulated the expression of L-selectin and upregulated expression of CD11b/CD18 and CD45 on the neutrophil surface and induced gelatinase release with EC_{50} values of ~2 nM. These actions of ET-1 were almost completely prevented by the ET_A receptor antagonist FR 139317 (1 μM) and the ET_A/ET_B receptor antagonist bosentan (10 μM), whereas the ET_B receptor antagonist BQ 788 (1 μM) had no effect. ET-1 slightly increased the expression of E-selectin and ICAM-1 on HCAEC, that was prevented by BQ 788, but not by FR 139317.

4 Receptor binding studies indicated the presence of ET_B receptors (K_D: 40 pM) on phosphoramidon-treated HCAEC and the predominant expression of ET_A receptors (K_D: 38 pM) on neutrophils.

5 These results indicate that promotion by ET-1 of neutrophil adhesion to HCAEC is predominantly mediated through activation of ET_A receptors on neutrophils and subsequent generation of PAF.

Keywords: Neutrophil granulocytes; endothelin-1; ET_A and ET_B receptors; adhesion molecules; selectins; integrins; ICAM-1; neutrophil-endothelial adhesion; platelet-activating factor; human coronary artery endothelial cells

Abbreviations: ET-1, endothelin-1; HBSS, Hanks' balanced salt solution; HCAEC, human coronary artery endothelial cells; LPS, lipopolysaccharide; mAb, monoclonal antibody; PAF, platelet-activating factor; PBS, phosphate buffered saline

Introduction

Endothelin-1 (ET-1), a potent constrictor of coronary arteries both *in vitro* and *in vivo* (reviewed by Rubanyi & Polokoff, 1994) has recently been recognized as one of the key mediators of myocardial ischaemia (Lüscher, 1991). Elevated tissue expression and plasma levels of ET-1 were found to occur in pathological conditions such as myocardial ischaemia (Miyauchi *et al.*, 1989; Watanabe *et al.*, 1991) and atherosclerosis (Lerman *et al.*, 1991), which are also characterized by increased adhesiveness of leukocytes to endothelial cells (Lefer & Lefer, 1993).

Recent studies from several laboratories, including ours have suggested that ET-1 may be an important autocrine/paracrine modulator of neutrophil functions. ET-1 causes a selective neutrophil leukocytopenia in guinea-pigs (Filep *et al.*, 1995b) and induces neutrophil accumulation in the isolated perfused heart (López-Farré *et al.*, 1993), lung (Helset *et al.*, 1996; Khimenko *et al.*, 1996) and kidney (Espinosa *et al.*, 1996). Neutrophils challenged with ET-1 migrate from the venous lumen into the tissue matrix of the human umbilical cord, and induce a massive tissue destruction (Halim *et al.*, 1995). ET-1 promotes neutrophil aggregation (Gómez-Garré *et al.*, 1992; López-Farré *et al.*, 1995), stimulates surface expression of CD11b/CD18 on human neutrophils and augments their adhesion to cultured bovine endothelial cells (López-Farré *et al.*, 1993). No data, however, are available on the effects of ET-1 on the expression of L-selectin (CD62L), or the ET receptor subtype(s) and the underlying mechanism(s) that mediate neutrophil-endothelial adhesion. To date two distinct mammalian ET receptor subtypes have been cloned, ET_A (which is highly selective for ET-1) and ET_B (non-isopeptide selective) (Arai *et al.*, 1990; Sakurai *et al.*, 1990) and pharmacological evidence suggest the existence of a third receptor subtype (termed non-ET_A/ET_B) on mammalian cells (Harrison *et al.*, 1992). Previous studies reported that ET_A receptor blockade prevents ET-1-induced neutrophil leukocytopenia (Filep *et al.*, 1995b) and neutrophil accumulation in rat perfused lung (Khimenko *et al.*, 1996).

Accordingly, this study was undertaken to characterize the endothelin receptor subtypes which mediate the action of ET-1 on the surface expression of L-selectin and CD11b/CD18 on human neutrophils, expression of E-selectin and

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ICAM-1 on human coronary artery endothelial cells (HCAEC) and on adhesion of neutrophils to HCAEC by receptor binding assays and by using selective ET_A and ET_B receptor antagonists and agonists. Since multiple adhesion receptors are involved in neutrophil adhesion to activated endothelial cells (Spertini *et al.*, 1991; Zouki *et al.*, 1997), we assayed the contribution of L-selectin, E-selectin and CD18 to the attachment elicited by ET-1. In addition, we also investigated whether platelet-activating factor (PAF) mediates these actions of ET-1, because ET-1 stimulates PAF synthesis in neutrophils (Gómez-Garré *et al.*, 1992) and PAF may function as a signal for neutrophils to bind to the endothelium (Zimmerman *et al.*, 1990; Kuijpers *et al.*, 1991).

Methods

Materials

ET-1 and IRL-1620 (Suc-[Glu^9,Ala^{11,15}]endothelin-1(18-21)) were synthesized in our laboratory by solid-phase method. The purity of peptides were greater than 98%, as analysed by high performance liquid chromatography. Bosentan (4-*tert*-butyl-N-[6-(2 hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bi-pyrimidin-4-yl]-benzene-sulphonamide) was a gift from Dr M. Clozel (Hoffmann LaRoche Ltd. Basel, Switzerland), FR 139317 ((R)2-[(R)- 2 -[[1-(hexahydro-1H-azepinyl)]-carbonyl]-amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1H-indoyl)]-propionyl]amino-3-(2-pyridyl) propionic acid) was a gift from Fujisawa Pharmaceutical Co. (Osaka, Japan). BQ 788 (N-*cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine) was obtained from Calbiochem-Novabiochem (San Diego, CA, U.S.A.). BN 52021 (ginkgolide B) was a gift from Dr P. Braquet (Institut Henri Beaufour, Le Plessis Robinson, France). WEB 2086 (3-[4-(2-chlorphenyl)-9-methyl-6H-thienol[3,2-f][1,2,4]triazolo-[4,3-a] [1,4]-diazepine-2-yl]-1-(morpholinyl)-1-propanon) was a gift from Dr H. Heuer (Boehringer-Ingelheim KG, Ingelheim, Germany). Drugs were dissolved in 0.9% NaCl with the exception of BN 52021 which was dissolved in dimethylsulphoxide and was diluted further in with 0.9% NaCl as appropriate. Lipopolysaccharide (LPS, *E. coli*, serotype O111:B4) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Phosphoramidon was purchased from Peptides International (Louisville, KY, U.S.A.).

Whole-blood incubation

Venous blood (anticoagulated with sodium heparin, 50 U ml^{-1}) was obtained from non-smoking healthy volunteers (male and female, 25–44 years old) who had not taken any drugs for at least 10 days before the experiments. Informed consent was obtained from each volunteer, and the protocol was approved by the Clinical Research Committee. White blood cells counts were between 4500 and 8500 cells per μl. Whole blood aliquots were transferred to polypropylene tubes, placed on a rotator, and preincubated with one of the following antagonists for 10 min at 37°C: the ET_A receptor selective antagonist FR 139317 (1 μM) (Aramori *et al.*, 1993), the ET_A/ET_B antagonist bosentan (10 μM) (Clozel *et al.*, 1994), the ET_B receptor selective antagonist BQ 788 (1 μM) (Ishikawa *et al.*, 1994), or the PAF receptor antagonist BN 52021 (50 μM) (Braquet *et al.*, 1985) or WEB 2081 (10 μM) (Casals-Stenzel *et al.*, 1987), and then challenged with either ET-1 or the selective ET_B receptor agonist IRL 1620 (Takai *et al.*, 1992) (both 1 pM–1 μM) for 30 min at 37°C in 5% CO_2 in air.

Flow cytometry analysis

Direct immunofluorescence labelling of control or treated neutrophil granulocytes in whole blood were performed as described previously (Filep *et al.*, 1997). Leukocytes were stained with saturating concentration of FITC-labelled anti-human L-selectin monoclonal antibody (mAb) DREG-56 (IgG_1, PharMingen, San Diego, CA, U.S.A.), R-phycoerythrin-conjugated mouse anti-human CD11b mAb Leu-185 (IgG_1, Becton Dickinson Immunocytometry Systems, Mountain View, CA, U.S.A.) or PerCP-labelled anti-human CD45 mAb 2D1 (IgG_1, Becton Dickinson). Appropriately labelled, class-matched irrelevant mouse IgG_1 was used as a negative control for each staining. Single-colour immunofluorescence staining was analysed by a FACScan Flow Cytometer (Becton Dickinson) with Lysis II software.

Isolation and treatment of neutrophil granulocytes

Neutrophil granulocytes were isolated from peripheral blood by centrifugation through Ficoll-Hypaque gradients (Pharmacia Diagnostics AB, Uppsala, Sweden), sedimentation through dextran (3%, w v^{-1}), and hypotonic lysis of erythrocytes. The resultant cell preparation contained >97% neutrophils. Neutrophils (5×10^6 cells ml^{-1}) were suspended in a modified Hanks' balanced salt solution (HBSS) consisting of (mM): NaCl 145, K_2PO_4 10, $CaCl_2$ 1.4, $MgCl_2$ 1.2, glucose 10, and 250 μg ml^{-1} human serum albumin, pH 7.4, preincubated with one of the antagonists for 10 min and then challenged with ET-1 or IRL 1620 for 30 min at 37°C. Then the cells were pelleted, and the supernatants were collected for further analysis.

Measurement of granule enzyme release

Lysozyme, β-glucuronidase and gelatinase were assayed as described previously (Filep *et al.*, 1997; Zouki *et al.*, 1997). Enzyme release was determined as the percentage of total enzyme units released from neutrophils treated with 0.1% Triton X-100.

Culture of endothelial cells

Normal human coronary artery endothelial cells obtained from Clonetics Corp. (San Diego, CA, U.S.A.) were cultured as described (Zouki *et al.*, 1997). HCAEC (passages 4–6) seeded into 24-well or 96-well microplates and grown to confluence were used in the experiments.

Neutrophil-endothelial cell adhesion assay

The adhesion assay was performed as described previously (Zouki *et al.*, 1997). In brief, monolayers of HCAEC in 96-well microplates were stimulated with LPS (1 μg ml^{-1}) or ET-1 (100 nM) for 6 h at 37°C in a 5% CO_2 atmosphere. The wells were then washed three times, and 2×10^5 ^{51}Cr-labelled neutrophils in 100 μl were added. Radiolabelled neutrophils were preincubated for 10 min with one of the antagonists at the concentration indicated. In another set of experiments, LPS or ET-1-activated HCAEC were incubated for 15 min with the function blocking anti-E-selectin mAb ENA-2 (IgG_1, purified $F(ab')_2$ fragments; Monosan, Uden, The Netherlands) at 10 μg ml^{-1} (Leeuwenberg *et al.*, 1990)

or the irrelevant mAb MOPC-21 (IgG_1, PharMingen) before addition of neutrophils. The mAb reactive with E-selectin was also added back to the neutrophil suspensions so that mAb ENA-2 was present throughout the assay. Radiolabelled neutrophils were incubated with the anti-L-selectin mAb DREG-56 (IgG_1, PharMingen) at 20 $\mu g\ ml^{-1}$ (Kishimoto *et al.*, 1991) or the anti-CD18 mAb L130 (IgG_1, Becton Dickinson) at 10 $\mu g\ ml^{-1}$ (Zouki *et al.*, 1997) for 15 min at 37°C before addition to HCAEC. After incubation of endothelial cells plus neutrophils in the absence or presence of ET-1 (100 nM) for 30 min at 37°C on an orbital shaker at 90 r.p.m., loosely adherent or unattached neutrophils were washed three times, and the endothelial monolayer plus the adherent neutrophils were lysed in 200 μl of 0.1 M NaOH. The number of adhered neutrophils in each experiment was estimated from the radioactivity of a control sample. Treatment of HCAEC with any of the antibody used in these studies did not affect the integrity of viable endothelial monolayers.

Expression of E-selectin and ICAM-1

Monolayers of HCAEC in 24-well microplates were incubated for 4 h at 37°C in a 5% CO_2 atmosphere with LPS (1 $\mu g\ ml^{-1}$) or ET-1 (100 nM) in the absence or presence of ET receptor antagonists. The cells were then detached by exposure to EDTA (0.01%) in PBS for 10 min at 37°C followed by gentle trituration. The cells were resuspended in ice-cold NaCl solution (150 mM) containing 0.02% sodium azide, incubated with saturating concentrations of FITC-labelled mouse anti-human E-selectin mAb 1.2B6 (Serotec, Kidlington, England) and R-phycoerythrin-conjugated mouse anti-human ICAM-1 mAb HA58 (PharMingen) for 30 min at 4°C, washed and fixed in formaldehyde (3.9% in PBS). Appropriately labelled, class-matched irrelevant mouse IgG_1 was used as a negative control

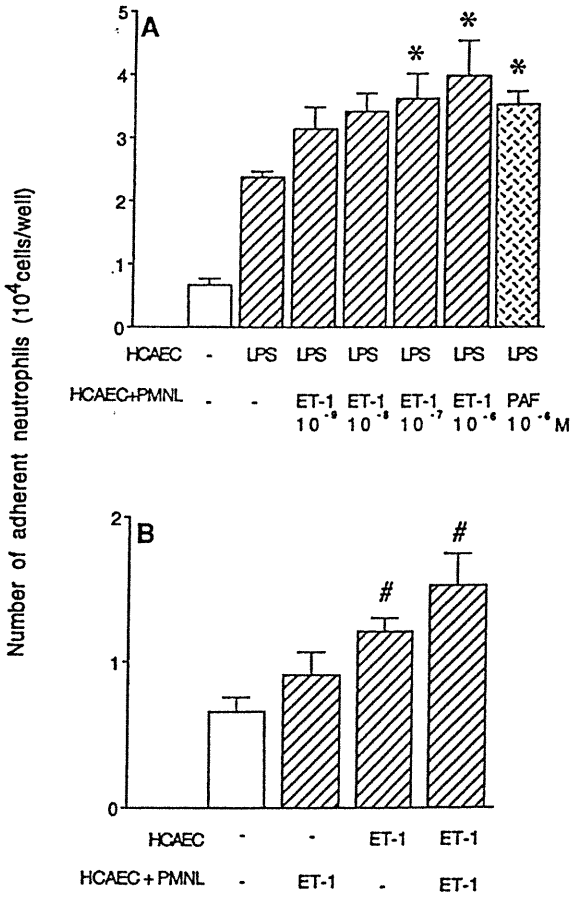

Figure 1 Endothelin-1 (ET-1) augments adhesion of neutrophils to coronary artery endothelial cells (HCAEC). Confluent HCAEC monolayers were cultured for 6 h with (A) 1 $\mu g\ ml^{-1}$ lipopolysaccharide (LPS) or (B) 100 nM ET-1. Neutrophils (PMNL) together with ET-1 or PAF (1 μM) were then added and incubated with HCAEC for 30 min at 37°C (PMNL+HCAEC). Values are expressed as mean±s.e.mean of three experiments in triplicate using neutrophils from different donors. *$P<0.05$ vs LPS; #$P<0.05$ vs unstimulated (open column).

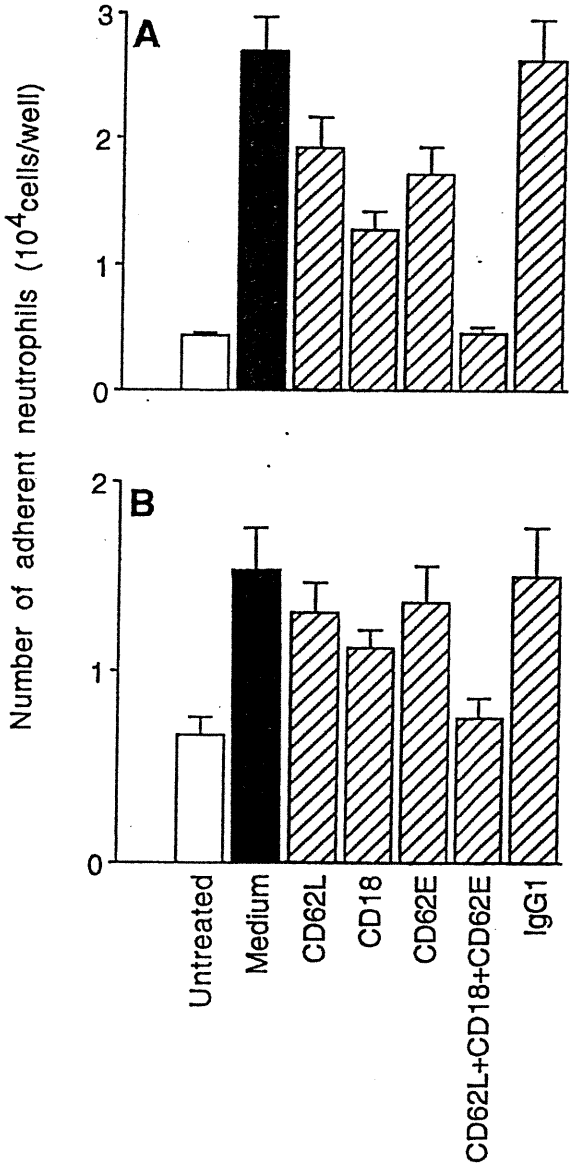

Figure 2 Inhibition of endothelin-1 (ET-1)-stimulated neutrophil attachment to coronary artery endothelial cells (HCAEC) by function-blocking monoclonal antibodies (mAbs) directed against E-selectin (CD62E) expressed on HCAEC, and against L-selectin (CD62L) and CD18 expressed by neutrophils. Radiolabelled neutrophils were added to (A) untreated or lipopolysaccharide (1 $\mu g\ ml^{-1}$)-activated HCAEC or (B) ET-1 (100 nM)-activated HCAEC and incubated for 30 min at 37°C in the absence (medium) or presence of mAbs. The specificity of mAbs is indicated on the X-axis. The irrelevant mAb MOPC-21 (IgG_1) was used as a negative control. Results are expressed as mean±s.e.mean of three experiments in triplicate using neutrophils from different donors.

for each staining. Immunofluorescence of HCAEC was then analysed with a FACScan flow cytometer.

Radioligand binding studies

Isolated neutrophil granulocytes (2×10^6 cells per ml) were resuspended in HBSS supplemented with 2 μM leupeptin, 1 μM pepstatin A and 0.1 mM phenylmethylsulphonyl fluoride. ET receptor antagonists BQ 788 and FR 139317 were added 10 min before addition of 92 pM ^{125}I-labelled ET-1 (specific activity: 2200 Ci mmol^{-1}, New England Nuclear, Cambridge, MA, U.S.A.). For competition studies, ET-1 was mixed with [^{125}I]-ET-1 before addition to cells. Binding assays were performed at 4°C for 60 min. Free and bound ligand were separated using a silicone oil centrifugation method (Filep & Földes-Filep, 1989). Pellets were solubilized with 0.5 ml 2% Triton X-100 and radioactivity was measured using a Wallac 1470 Wizard Automatic Gamma Counter (Turku, Finland). ET-1 binding to HCAEC was performed in accordance to the method of Fujitani *et al.* (1992). In brief, confluent monolayers of HCAEC in 24-well plates were cultured in the absence or presence of phosphoramidon (4 mM) for 2 days. Following extensive washing with HBSS, the cells were incubated in HBSS with [^{125}I]-ET-1 (50 pM) and the desired concentrations of unlabelled ET-1, BQ 788 and FR 139317. After incubation at 37°C for 60 min, which was sufficient to reach maximum binding, cells were washed with HBSS, harvested into 0.1 N NaOH and the cell associated radioactivity was counted. In all assays, non-specific binding was determined in the presence of 100 nM unlabelled ET-1. Specific binding was calculated as total binding minus nonspecific binding. Binding data were analysed with the EBDA-LIGAND software (Elsevier Biosoft Cambridge, U.K.) (Munson & Rodbard, 1980).

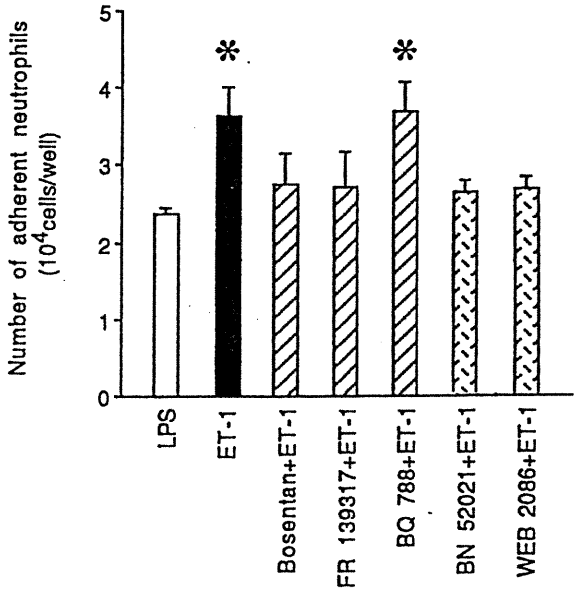

Figure 3 Inhibition of neutrophil adhesion to LPS-activated human coronary artery endothelial cells (HCAEC) by endothelin and platelet-activating factor receptor antagonists. Neutrophils preincubated with one of the antagonists for 10 min were cultured with lipopolysaccharide (LPS, 1 μg ml^{-1})-activated HCAEC in the presence of medium (LPS) or ET-1 (100 nM) for 30 min at 37°C. The following antagonists were used: bosentan (10 μM), FR 139317 (1 μM), BQ 788 (1 μM), BN 52021 (50 μM) and WEB 2086 (10 μM). Results are mean±s.e.mean for three experiments performed in triplicate using neutrophils from different donors. *$P<0.05$ vs LPS.

Statistical analysis

Results are expressed as means±s.e.mean. Statistical comparisons were made by ANOVA using ranks (Kruskal-Wallis test) followed by Dunn's multiple contrast hypothesis test to identify differences between various treatments or by the Mann-Whitney *U*-test. P values <0.05 were considered significant for all tests.

Results

Endothelin-1 enhances neutrophil adhesion to human coronary artery endothelial cells

Only a few neutrophils were able to bind to unstimulated HCAEC. Neutrophil adherence was enhanced 3.6 fold by activation of HCAEC with LPS (Figure 1A). ET-1 produced further, concentration-dependent increases in the number of adhering neutrophils onto LPS-activated HCAEC (Figure 1A). ET-1 did not enhance neutrophil adhesion to unstimulated HCAEC. However, when neutrophils were added to HCAEC cultured with ET-1 (100 nM) for 6 h, on average 1.8 fold more neutrophils adhered to stimulated than to unstimulated HCAEC (Figure 1B). The number of adhering neutrophils was further enhanced when the adhesion assay was performed in the presence of ET-1

Table 1 E-selectin and ICAM-1 expression on human coronary artery endothelial cells challenged with ET-1 and LPS

	E-selectin		*ICAM-1*	
	MFI	*Positive cells* (%)	*MFI*	*Positive cells* (%)
Control	1.7±0.2	4.1±0.2	28.5±6.1	57.9±5.3
LPS, 1 μg ml^{-1}	43.9±7.1**	81.0±2.1**	364.0±18.4**	96.6±0.7*
ET-1, 10^{-9} M	3.1±0.3	5.0±0.7	31.7±8.2	52.8±6.1
ET-1, 10^{-8} M	4.3±0.6*	7.2±0.5	44.5±7.5*	63.3±2.5
ET-1, 10^{-7} M	5.0±0.5*	8.0±0.6*	68.5±4.8*	70.0±2.7*
ET-1, 10^{-6} M	4.7±0.7*	7.3±0.5*	64.4±2.5*	70.7±2.8*
BQ 788+ET-1, 10^{-7} M	2.4±0.1#	7.2±0.4	37.0±4.3#	68.7±1.9
FR 139317+ET-1, 10^{-7} M	4.7±0.4	7.6±0.4	68.8±4.8	67.9±2.1

Monolayers of HCAEC were cultured with LPS or ET-1 for 4 h at 37°C in the absence or presence of BQ 788 (1 μM) or FR 139317 (1 μM). E-selectin and ICAM-1 expression was assessed by flow cytometry following detachment and staining of the cells with fluorescent dye-labelled monoclonal antibodies. Antibody staining is expressed as mean fluorescence intensity (MFI) after subtracting nonspecific immunostaining. Positive cells represent the percentage of the total cells which stained positive for the indicated adhesion molecule. Values are expressed as mean±s.e.mean of 3–4 independent measurements. *$P<0.05$; **$P<0.01$ vs control (i.e. cells cultured in medium only); #$P<0.05$ vs ET-1, 10^{-7} M.

(1.17 ± 0.05 vs $1.53 \pm 0.22 \times 10^4$ adherent neutrophils per well, $n=3$, $P<0.05$) (Figure 1B). These results indicate that ET-1 by itself is capable of enhancing neutrophil adhesion to HCAEC.

Since multiple receptors are involved in neutrophil adhesion to LPS-stimulated HCAEC even under nonstatic conditions (Spertini *et al.*, 1991; Zouki *et al.*, 1997), we assayed the contribution of L-selectin, CD18 integrins and E-selectin to the ET-1-stimulated binding interaction. A significant proportion of neutrophil-HCAEC attachment was blocked by mAb binding to E-selectin ($36 \pm 2\%$, $n=3$), L-selectin ($29 \pm 2\%$) and CD18 ($45 \pm 5\%$) (Figure 2A). The combination of these mAbs inhibited the neutrophil adhesion by ~83% (Figure 2A). ET-1-stimulated neutrophil adhesion to ET-1-activated HCAEC was blocked by mAb binding to E-selectin ($25 \pm 3\%$, $n=3$), L-selectin ($28 \pm 2\%$) and CD18 ($39 \pm 2\%$) (Figure 2B). The combination of these three mAbs inhibited the neutrophil adhesion by ~70% (Figure 2B).

Inhibition of neutrophil-endothelial attachment by ET_A receptor and platelet-activating factor receptor antagonists

Preincubation of neutrophils with the selective ET_A receptor antagonist, FR 139317 (1 μM) or with the ET_A/ET_B receptor antagonist, bosentan (10 μM) markedly attenuated the ET-1-stimulated neutrophil adherence to LPS-activated HCAEC (Figure 3). No significant difference could be detected between the degree of inhibition with FR 139317 and bosentan. Furthermore, the ET_B receptor selective antagonist BQ 788 (1 μM) had no detectable effect on the number of adhering neutrophils (Figure 3), further indicating ET_A receptors as the relevant ET receptor subtype responsible for the adhesion enhancing action of ET-1. Since ET-1 induces the synthesis and release of PAF from neutrophils (Gómez-Garré *et al.*, 1992), we next investigated the role of PAF in mediating ET-1-induced neutrophil adherence. The PAF receptor antagonists BN 52021 (50 μM) and WEB 2086 (10 μM) significantly attenuated ET-1-stimulated neutrophil adherence (Figure 3). The degree of inhibition observed with BN 52021 or WEB 2086 did not differ from those seen with FR 139317 or bosentan. The vehicle of these antagonists had no effect on the adhesion (data not shown).

Endothelin-1 induces E-selectin and ICAM-1 expression via ET_B receptors

The expression of adhesion molecules by treated and untreated HCAEC was quantitated by flow cytometry analysis. Under our experimental conditions, approximately 60 and 4% of untreated HCAEC expressed ICAM-1 and E-selectin, respectively (Table 1). Treatment of HCAEC for

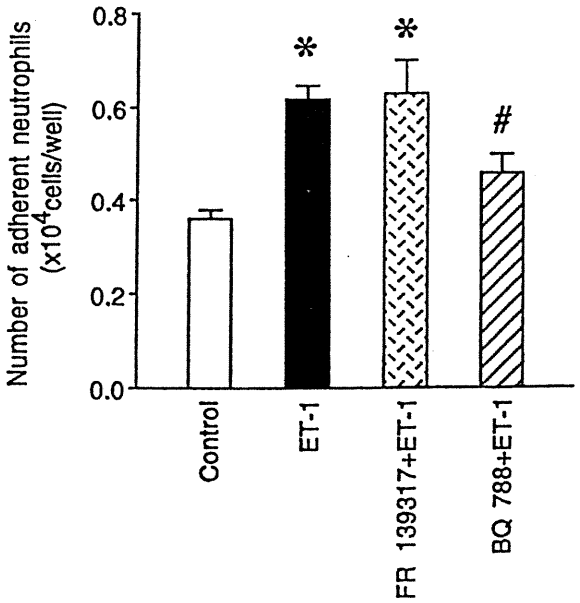

Figure 4 Effects of endothelin receptor antagonists on adhesion of neutrophils to human coronary artery endothelial cells (HCAEC) cultured with ET-1. HCAEC were left unstimulated (control) or activated with ET-1 (100 nM) for 6 h in the absence (ET-1) or presence of the ET_A-selective antagonist FR 139317 (1 μM) or the ET_B-selective antagonist BQ 788 (1 μM). Radiolabelled unstimulated neutrophils were then added and incubated with HCAEC for 30 min at 37°C. Values are expressed as mean±s.e.mean of three experiments in triplicate using neutrophils from different donors. *$P<0.05$ vs control: #$P<0.05$ vs ET-1.

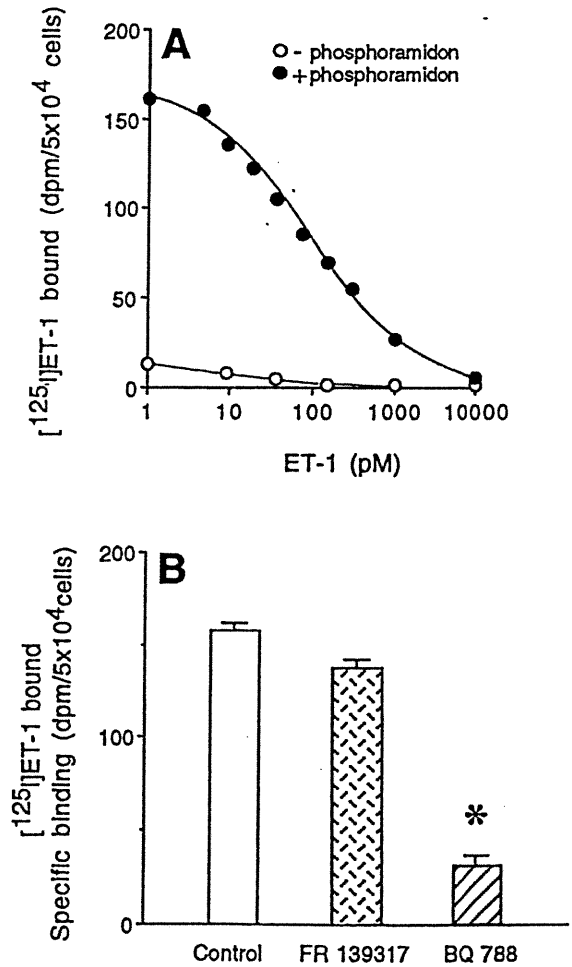

Figure 5 Endothelin receptor expression by human coronary artery endothelial cells (HCAEC). (A) Competitive binding of 50 pM [^{125}I]-ET-1 to HCAEC cultured in the absence (open circles) and presence of phosphoramidon (closed circles). Phosphoramidon was added at a concentration of 4 mM for 48 h. (B) Specific binding of 90 pM [^{125}I]-ET-1 to phosphoramidon (4 mM for 48 h)-treated HCAEC in the absence (control) and presence of FR 139317 (100 nM) or BQ 788 (100 nM). Nonspecific binding was determined in the presence of 100 nM ET-1. Results are expressed as mean±s.e.mean for three experiments performed in duplicate. *$P<0.05$ vs control.

4 h with ET-1 increased the overall expression of E-selectin and ICAM-1 in a concentration-dependent fashion, apparent maximum changes were observed at 10^{-7} M (Table 1). Increasing the concentration of ET-1 did not further enhance adhesion molecule expression. ET-1 treatment also increased the percentage of cells expressing these adhesion molecules (Table 1). As a positive control, LPS treatment evoked approximately 26 and 13 fold increases in E-selectin and ICAM-1 expression, respectively, and 81 and 97% of cells stained positive for these adhesion molecules, respectively (Table 1). The ET-1 (10^{-7} M)-induced increases in E-selectin and ICAM-1 expression were effectively inhibited by BQ 788 (1 μM), whereas FR 139317 (1 μM) had no detectable effects (Table 1). Consistently, the increases in the number of adherent neutrophils following activation of HCAEC with ET-1 were prevented by treatment with BQ 788, but not by FR 139317 (Figure 4).

Endothelin-1 binding to human coronary artery endothelial cells

Receptor binding studies were performed to characterize the ET receptors expressed on HCAEC. The specific binding of [^{125}I]-ET-1 to untreated HCAEC was too low to allow competition experiments (Figure 5). Treatment of the cells with phosphoramidon for 2 days increased the maximal specific binding by 12 fold (Figure 5A). Previous studies have shown that phosphoramidon does not effect the affinity of ET-1 (Fujitani *et al.*, 1992; Clozel *et al.*, 1993). Unlabelled ET-1 displaced specific [^{125}I]-ET-1 binding with an IC_{50} value of 90 pM (Figure 5A). The estimated dissociation constant (K_D) and maximum binding (B_{max}) were 40 pM and 0.06 fmol per 5×10^4 cells, respectively. Specific binding of [^{125}I]-ET-1 was markedly reduced in the presence of BQ 788, whereas ET_A receptor blockade with FR 139317 was without effect (Figure 5B), indicating that HCAEC predominantly express ET_B receptors.

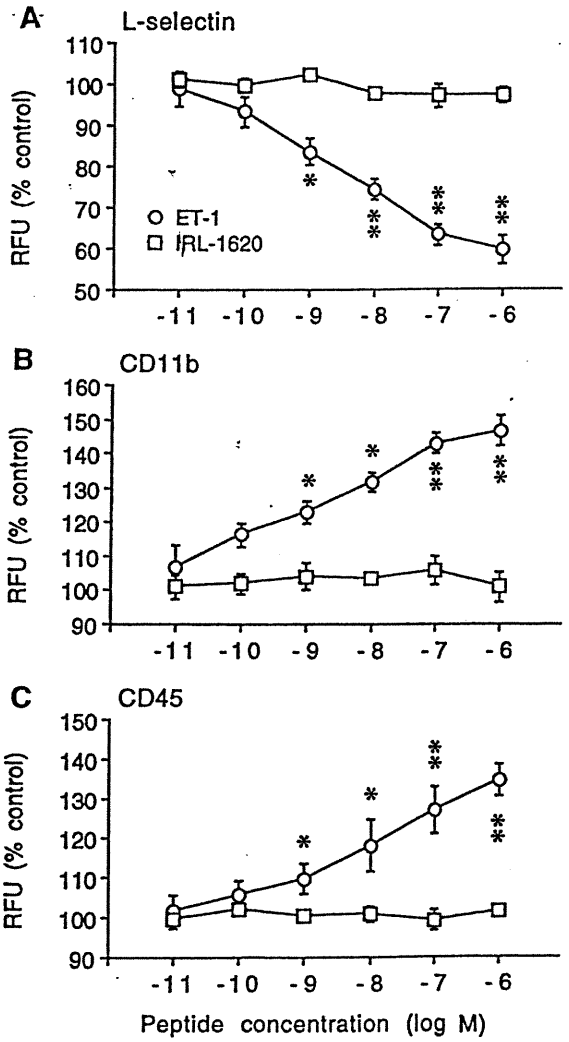

Figure 6 Concentration-dependent effect of endothelin-1 (ET-1) on surface expression of L-selectin (A), CD11b (B) and CD45 (C) on neutrophil granulocytes. Aliquots of whole blood were challenged with ET-1 or IRL-1620 for 30 min at 37°C. Fluorescence intensity (relative fluorescence unit, RFU) is presented as percentage of control, i.e. mean fluorescence intensity of neutrophils cultured in medium only. Results are expressed as mean±s.e.mean for 5–6 experiments using blood from different donors. *$P<0.05$; **$P<0.01$ vs control.

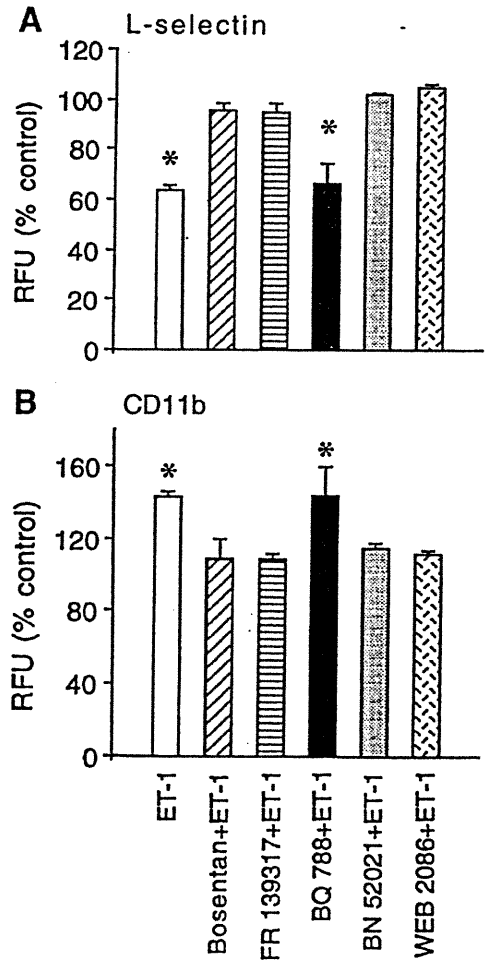

Figure 7 Inhibition of endothelin-1-induced downregulation of L-selectin (A) and upregulation of CD11b expression (B) by endothelin and platelet-activating factor receptor antagonists. Whole blood aliquots were preincubated with one of the antagonists for 10 min and then challenged with 100 nM ET-1 for 30 min at 37°C. Fluorescence intensity (relative fluorescence unit, RFU) is presented as percentage of control, i.e. mean fluorescence intensity of neutrophils cultured in medium only. Results are expressed as mean±s.e.mean for 5–6 experiments using blood from different donors. *$P<0.05$ vs control.

Table 2 Granule enzyme release from human neutrophils by endothelin-1 (ET-1)

	Enzyme release		
	Gelatinase	*β-Glucuronidase*	*Lysozyme*
Control	18.1±2.0	2.6±0.3	5.8±0.5
PAF, 1 μM	59.1±3.0*	2.7±0.5	6.4±0.7
ET-1, 1000 nM	37.0±1.7*	2.5±0.4	6.1±0.6
ET-1, 100 nM	31.6±1.9*	2.8±0.4	5.9±0.7
ET-1, 10 nM	27.7±2.4*	2.7±0.5	6.0±0.6
ET-1, 1 nM	19.8±1.8	2.5±0.4	5.9±0.4
Bosentan+ET-1, 100 nM	20.5±1.9	2.8±0.3	5.7±0.5
FR 139317+ET-1, 100 nM	21.4±2.3	2.9±0.3	5.9±0.7
BQ 788+ET-1, 100 nM	33.3±1.0*	3.0±0.4	6.3±0.8
BN 52021+ET-1, 100 nM	21.9±1.4	2.9±0.5	6.2±0.7
WEB 2086+ET-1, 100 nM	21.4±1.2	2.7±0.4	6.3±0.6

Values are expressed as percentage of total cellular enzyme activity released by neutrophils to the culture medium after incubation for 30 min at 37°C with ET-1 or platelet-activating factor (PAF) in the absence and presence of one of the following inhibitors: bosentan (10 μM), FR 139317 (1 μM), BQ 788 (1 μM), BN 52021 (50 μM) and WEB 2086 (10 μM). Values are means±s.e.mean of six independent experiments. *$P<0.05$ vs control (unstimulated neutrophils).

Endothelin-1 regulates expression of L-selectin and CD18 on neutrophils through activation of ET_A receptor and platelet-activating factor

Flow cytometry analysis of whole blood challenged with ET-1 showed that the neutrophil surface expression of L-selectin was downregulated (Figure 6A), whereas expression of CD11b was upregulated by ET-1 in a concentration-dependent fashion (Figure 6B) with an apparent EC_{50} concentration of ~2 nM. Similar increases were detected in CD18 expression (data not shown). No significant changes were detected with IRL-1620 over the concentration range studied (Figure 6A and B). The maximum changes that could be achieved with ET-1 were smaller than those evoked by 1 μM PAF (40±4 and 57±4% decreases in L-selectin expression by ET-1 and PAF, respectively; $n=6$, $P<0.05$; 47±4 vs 73±7% increase in CD11b expression by ET-1 and PAF, $P<0.05$). Furthermore, ET-1 also upregulated expression of CD45 (Figure 6C), indicating neutrophil activation.

None of the receptor antagonists used in this study on their own had an effect on expression of adhesion molecules on resting neutrophils. Both FR 139317 and bosentan markedly attenuated ET-1 (100 nM)-induced changes in L-selectin and CD11b expression, whereas BQ 788 had no detectable effects (Figure 7). The inhibitory actions of FR 139317 and bosentan did not differ significantly (Figure 7). Downregulation of L-selectin and upregulation of CD11b expression by ET-1 were also inhibited by the PAF receptor antagonists, BN 52021 and WEB 2086 (Figure 7).

Endothelin-1-induced granule enzyme release

To provide further evidence for neutrophil activation by ET-1, we studied degranulation of isolated neutrophils in response to ET-1. ET-1 did not induce release of β-glucuronidase (a marker for azurophil granules) or lysozyme (a marker for specific plus azurophil granules), whereas it evoked gelatinase release (a marker for tertiary granules) in a concentration-dependent fashion (Table 2). Similar changes were observed with PAF, albeit PAF released higher amounts of gelatinase than those observed with ET-1. The ET-1-induced gelatinase release was inhibited by FR 139317, bosentan, BN 52021 and WEB 2086, but not by BQ 788 (Table 2). IRL-1620 did not induce gelatinase release (data not shown). Lactate dehydrogenase release never exceeded 3% of the total cell content (data not shown), indicating that neutrophil integrity was not affected.

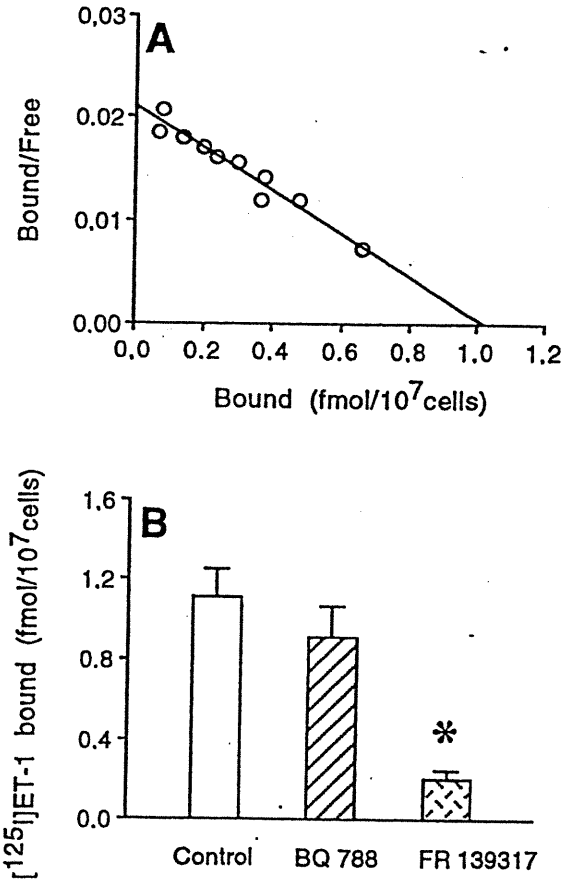

Figure 8 Endothelin receptor expression by human neutrophils. (A) Scatchard analysis of the specific binding of [^{125}I]-ET-1 to neutrophils. Specific binding was calculated as the difference between total binding and binding in the presence of 100 nM ET-1 (non-specific binding). This is a representative result for six experiments. (B) Specific binding of [^{125}I]-ET-1 to neutrophils was evaluated in the absence (control) and presence of the ET_A-selective antagonist FR 139317 (100 nM) or the ET_B-selective antagonist BQ 788 (100 nM). Nonspecific binding was determined in the presence of 100 nM ET-1 and represent approximately 10–15% of total binding. Values are mean±s.e.mean for seven experiments. *$P<0.05$ vs control.

Endothelin-1 binding to neutrophil granulocytes

Scatchard analysis of ET-1 displacement of [^{125}I]-ET-1 binding to neutrophils indicated a single class of high-affinity binding sites with a K_D value of 38±5 pM and a maximum binding of 1.03±0.08 fmol · per 10^7 cells ($n=6$, Figure 8A). Hill coefficients calculated with the LIGAND program were 0.97±0.03 ($n=6$). Specific binding of [^{125}I]-ET-1 was reduced markedly in the presence of the ET_A-selective antagonist FR 139317. In contrast, the ET_B-selective antagonist BQ 788 did not inhibit [^{125}I]-ET-1 binding (Figure 8B). These data

indicate predominant expression of ET_A receptors on human neutrophils.

Discussion

The novel finding of the present study relevant to the role of ET-1 in regulating leukocyte-endothelial interaction is that ET-1 promotes neutrophil adhesion to HCAEC predominantly through activation of ET_A receptors and subsequent release of PAF. This adhesion enhancing action can be attributed to the effects of ET-1 on neutrophils, and, to a lesser extent, to effects on the endothelium and involves multiple adhesion molecules, L-selectin, E-selectin and CD18 integrins.

Our study showed that ET-1 markedly enhances neutrophil adhesion to HCAEC stimulated with either LPS or ET-1, whereas it slightly increased neutrophil adherence to non-activated HCAEC and to feline coronary artery segments (Murohara & Lefer, 1996). López-Farré *et al.* (1993) reported that 30 min of incubation of bovine endothelial cells alone with ET-1 had no stimulatory effect on neutrophil adhesion. In our study, culture of HCAEC alone with ET-1 for 4–6 h resulted in increases in the expression of the endothelial adhesion molecules, E-selectin and ICAM-1, and significantly increased the number of adhering neutrophils. Therefore, it is conceivable that, like with other stimuli, such as LPS, induction by ET-1 of expression of ICAM-1 and E-selectin may require a long exposure time. However, ET-1 is a less potent activator of HCAEC than LPS, as evidenced by analysis of E-selectin and ICAM-1 expression and adhesion assays. Since in the adhesion assays, neutrophils were incubated with activated HCAEC in the presence of ET-1 for 30 min, stimulation of neutrophil adhesion by ET-1 can primarily be attributed to the effects of this peptide on neutrophils. No adhesion experiments were performed with neutrophils alone preincubated with ET-1 since ET-1 may induce neutrophil aggregation (Gómez-Garré *et al.*, 1992; López-Farré *et al.*, 1995), therefore making interpretation of the results difficult.

Leukocyte-endothelial cell interaction involves a complex interplay among adhesion molecules (Butcher, 1991; Springer, 1994). Indeed, the experiments with function-blocking mAbs revealed the involvement of CD18 integrins, E-selectin and L-selectin in mediating ET-1-induced adhesion of neutrophils to activated HCAEC. However, the relative contribution of these adhesion molecules varied when HCAEC was stimulated with LPS or ET-1. Thus, neutrophil adhesion to ET-1-stimulated HCAEC was only slightly inhibited by the anti-E-selectin mAb. This is consistent with the observation that 4 h incubation of HCAEC with ET-1 increased E-selectin expression only in 8% of cells compared to 71% cells positive for ICAM-1, the counterreceptor for CD18 integrins. Increases in both the intensity of staining for adhesion molecules and the percentage of HCAEC expressing E-selectin and ICAM-1 indicate that in addition to upregulating the expression on cells already expressing E-selectin and ICAM-1, ET-1 also induced expression of these adhesion molecules on previously negative cells. The findings that ET-1 increases E-selectin expression on about 20% of human brain microvascular endothelial cells (McCarron *et al.*, 1993) suggest the existence of regional differences in the ET-1 induction of adhesion molecule expression. The marked difference in the degree of inhibition of neutrophil adhesion with anti-CD18 mAb to HCAEC (40–50% inhibition) and to bovine endothelial cells (80–90% inhibition) (López-Farré *et al.*, 1993), most likely indicates species differences in the role of CD18 integrins in adhesion.

Consistent with previous results with human umbilical vein endothelial cells (Fujitani *et al.*, 1992; Clozel *et al.*, 1993), we also found that ET receptors on HCAEC became detectable by binding assay only after pretreatment of the cells with phosphoramidon, an inhibitor of ET-1 converting enzyme (Ikegawa *et al.*, 1991). Thus, it is plausible to assume that autocrine production of ET-1 by HCAEC decreases, either by binding or by downregulation, the number of ET-1 binding sites. Although the limited availability of HCAEC did not allow us to perform binding studies with the ET isopeptides, our results indicate the presence of a single class of high affinity ET receptor on HCAEC. The selective inhibition of [^{125}I]-ET-1 binding in the presence of BQ 788, but not FR 139317, is consistent with constitutive expression of ET_B receptors on HCAEC. Furthermore, the ET-1 induced expression of E-selectin and ICAM-1 was mediated through ET_B receptor activation, for this was also selectively blocked by BQ 788.

The present study showed that ET-1 at concentrations two order of magnitude lower than those used in the study of López-Farré *et al.* (1993) is capable of upregulating CD11b expression on neutrophils. Furthermore, at nanomolar concentrations, ET-1 downregulated L-selectin expression, upregulated CD45 expression on neutrophils and released gelatinase from tertiary granules, reflecting neutrophil activation. However, ET-1 up to a concentration of 1 μM did not induce β-glucuronidase and lysozyme release. Because the most readily mobilizable store of CD11b/CD18 is in a granule distinct from the classic azurophil and secondary granules Borregaard *et al.*, 1987), but may be associated with tertiary granules, upregulation of CD18 can occur without degranulation of azurophil and specific granules. Furthermore, ET-1 (over the range of 1 pM–1 μM) does not induce respiratory burst and superoxide production (Ishida *et al.*, 1990). A possible interpretation of these apparently discordant observations is that ET-1 may function as a quite selective neutrophil agonist, i.e. it is capable of enhancing adhesive properties of neutrophils without major increases in respiratory burst and superoxide production.

The actions of ET-1 on neutrophil-HCAEC adhesion and neutrophil expression of L-selectin and CD11b, and gelatinase release were significantly inhibited by the selective ET_A receptor antagonist FR 139317, but not by the ET_B receptor antagonist BQ 788, and were not mimicked by selective ET_B receptor stimulation with IRL-1620. These findings clearly point to the ET_A receptor as being the relevant receptor subtype responsible for these actions of ET-1. Indeed, receptor binding studies revealed the presence of a single class of high affinity ET-1 binding site on neutrophils. The observations that ET-1 binding was inhibited in the presence of FR 139317, whereas it was insensitive to BQ 788 are consistent with predominant expression of ET_A receptors by human neutrophils. Previous studies on experimental animals also showed that ET_A receptor activation augments leukocyte trafficking into tissues (Filep *et al.*, 1995b; Khimenko *et al.*, 1996). Further insight into the characteristics of the ET-1 effect was obtained by studying the involvement of PAF. Previous studies have suggested that the effects of ET-1 on microvascular permeability (Filep *et al.*, 1991), renal mesangial cell contractility (López-Farré *et al.*, 1991) and homotypic aggregation of human neutrophils (Gómez-Garré *et al.*, 1992; López-Farré *et al.*, 1995) are related to an increase in PAF synthesis by those cells. Indeed, ET-1 stimulates both the synthesis and release of PAF from human neutrophils (Gómez-Garré *et al.*, 1992) and rat brain microvessels (Catalán

et al., 1996). The ET-1-induced augmentation of neutrophil adhesion to HCAEC can be blocked by BN 52021 and WEB 2086, two potent and specific PAF receptor antagonists (Braquet *et al.*, 1985; Casals-Stenzel *et al.*, 1987) to a similar extent as observed with ET_A receptor blockade. Moreover, exogenous PAF produced similar changes as those observed with ET-1. Therefore, it appears that PAF plays a pivotal role in mediating the proadhesive action of ET-1. These findings provide further support to the notion that PAF, an autocrine/paracrine regulator of neutrophil activation, also functions as a signal for neutrophils to bind to the endothelium (Zimmerman *et al.*, 1990; Kuijpers *et al.*, 1991).

Recent studies showed that ET-1 may also exert an antiadhesive action. Thus, short-term (10 min) incubation of feline coronary artery segments with ET-1 was found to inhibit adhesion of neutrophils to thrombin-stimulated endothelium (Murohara & Lefer, 1996). This is a result of inhibition of P-selectin-mediated leukocyte-endothelial interaction by nitric oxide released upon activation of endothelial ET_B receptors (Murohara & Lefer, 1996). In our neutrophil-HCAEC binding assay, P-selectin-dependent adhesion was not studied, since P-selectin expression occurs within 10–20 min after application of inflammatory stimuli and is sustained for about 60 min (Weyrich *et al.*, 1995). Autocrine production of nitric oxide by human monocytes triggered by ET_B receptor activation has also been reported to reduce monocyte adhesion to the endothelium (King *et al.*, 1997). Based on these and the present results, we propose a model in which ET-1 exerts a dual action on neutrophil adhesion; a transient antiadhesive action, which is mediated through ET_B receptor-coupled NO production and a proadhesive action which is predominantly mediated through ET_A receptor activation-induced changes in surface expression of adhesion molecules on neutrophils. In addition, ET-1 could also upregulate expression of E-selectin and ICAM-1 by HCAEC through the activation of ET_B receptors, as discussed above. These may also lead to an increase in the number of adherent neutrophils. However, only a small portion of neutrophil attachment could be attributed to ET_B-receptor-mediated adhesion under our assay conditions. By contrast, activation of the ET_B receptor on rat aortic endothelial cells markedly enhanced neutrophil attachment (Hayasaki *et al.*, 1996). It is possible that short-term and long-term exposure of endothelial cells to ET-1 might effect endothelial adhesiveness differently, although the underlying mechanisms remain to be investigated.

The ET-1 concentrations required to evoke significant increases in neutrophil-endothelial adhesion were higher than those detected in the venous plasma or coronary sinus (1–23 fmol ml^{-1}) of patients with acute ischaemic coronary artery diseases (Yasuda *et al.*, 1990; Matsuyama *et al.*, 1991; Ray *et al.*, 1992). However, local ET-1 levels might be even higher than those detected in the plasma. Neutrophils themselves can also influence local levels of ET-1, either by increasing (i.e. enhanced conversion of big ET-1 to ET-1) or decreasing (increased degradation of ET-1) the amount of bioactive ET-1, depending on their state of activation (Caramelo *et al.*, 1997). Our observation that ET-1 at concentrations as low as 0.1–1 nM, is capable of inducing changes in expression of adhesion molecules on neutrophils would suggest that local ET-1 concentrations might be sufficiently high to promote neutrophil adhesion. The close association of elevated plasma ET-1 levels and increased neutrophil infiltration in vascular lesions in skin biopsies from patients with mixed connective tissue disease (Filep *et al.*, 1995a) would lend further support to this notion.

In conclusion, the present study documents the existence of ET_A receptors on neutrophils and the expression of ET_B receptors on HCAEC. Our results indicate that ET_A receptor-coupled PAF production and release by human neutrophils may provide a novel mechanism for autocrine or paracrine regulation of adhesion, resulting in augmented neutrophil adherence to HCAEC. These data taken together with rapid release of ET-1 from the sites of vascular injury (see Rubanyi & Polokoff, 1994), and the role of PAF in strengthening neutrophil adhesion to the endothelium (Zimmerman *et al.*, 1990; Kuijpers *et al.*, 1991) may provide important mechanisms for regulation of neutrophil-endothelial interactions at sites of vascular injury. Endothelin antagonists may therefore be of therapeutical value to reduce neutrophil accumulation and consequently tissue damage in myocardial diseases associated with elevated ET-1 production.

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CHAPITRE II (Article II)

ZOUKI C, Beauchamp M, Baron C, Filep JG. Prevention of in vitro neutrophil adhesion to endothelial cells through shedding of L-selectin by C-reactive protein and peptides derived from C-reactive protein. *J Clin Invest.* 1997, 100 : 522-529.

Introduction au chapitre II

La protéine C-réactive est une protéine de phase aiguë dont la concentration plasmatique augmente de 1000 fois lors d'une inflammation ou d'un dommage tissulaire (160), ce qui suggère un rôle probable de cette protéine dans la réponse inflammatoire. Le recrutement des neutrophiles étant essentiel dans la réponse inflammatoire, les effets de la protéine C-réactive sur ces cellules est un aspect important.

Cette étude nous a permis de suggérer un nouveau mécanisme d'action de la protéine C-réactive. Cette protéine pourrait empêcher la migration des neutrophiles aux sites inflammatoires en diminuant l'expression de la L-sélectine à la surface des neutrophiles. En effet, nous avons démontré d'une part qu'elle inhibe l'adhésion L-sélectine-dépendante des neutrophiles à des cellules endothéliales stimulées avec du LPS, et d'autre part qu'elle diminue l'expression de la L-sélectine à la surface des neutrophiles sans induire d'activation. Donc en diminuant le trafic des leucocytes vers les tissus infectés ou endommagés, la protéine C-réactive atténuerait ou limiterait la réponse inflammatoire.

Prevention of In Vitro Neutrophil Adhesion to Endothelial Cells through Shedding of L-Selectin by C-Reactive Protein and Peptides Derived from C-Reactive Protein

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Abstract

C-Reactive protein (CRP), the classic acute-phase reactant in humans, diminishes accumulation of neutrophils at inflammatory sites. To evaluate the underlying mechanisms, we have studied whether CRP and peptides derived from CRP could affect the first step of neutrophil extravasation, the L-selectin–mediated interaction of neutrophils with endothelial cells. CRP markedly attenuated attachment of human neutrophils to cultured LPS-activated human coronary artery and pulmonary microvascular endothelial cells with apparent IC_{50} values of 20 and 22 μg/ml, respectively. At similar concentrations, CRP rapidly downregulated the expression of L-selectin on the neutrophil surface, whereas it failed to affect expression of CD11b and CD45 or to induce granule enzyme release. The loss of L-selectin was due to cleavage and shedding of the molecule from the cell surface, as quantitated by the soluble form of L-selectin in cell-free supernatants. The effects of CRP could be prevented by an anti-CRP antiserum and by KD-IX-73-4, which inhibits shedding of L-selectin. Inhibition of adhesion with CRP was additive with function-blocking anti–E-selectin and anti-CD18 antibodies, but was not additive with anti–L-selectin antibody. Neutrophil attachment and L-selectin expression were also diminished by CRP peptides 174–185 and 201–206, but not peptide 77–82, albeit these peptides showed a weaker inhibitory effect than the parent protein. These studies indicate a specific activation-independent action of CRP and CRP peptides 174–185 and 201–206 on expression of L-selectin, and suggest that by attenuating neutrophil adhesion to the endothelium and consequently neutrophil traffic into tissues, native CRP and peptides 174–185 and 201–206 may be major mechanisms to attenuate or limit the inflammatory response. (*J. Clin. Invest.* 1997. 100:522–529.) Key words: C-reactive protein • adhesion molecules • L-selectin • neutrophils • inflammation

Introduction

C-Reactive protein (CRP)[1] is a prototypical acute–phase reactant. In healthy subjects, serum concentration of CRP is < 1 μg/ml. However, serum levels can increase as much as 1,000-fold within 24 h after the onset of inflammation or tissue damage (1), suggesting that this protein may initiate and/or modulate multiple responses of the host. In general, the magnitude of the CRP response is related to the severity of inflammation or the extent of tissue injury (2). Since extravasation and activation of neutrophil granulocytes are essential in the inflammatory response, the effects of CRP on these cells are of particular importance. CRP binds with high affinity to human neutrophils (3, 4) and inhibits neutrophil activation, including chemotaxis (5), influx into alveolar space (6, 7), and superoxide production and degranulation in response to FMLP and platelet-activating factor (8). Stimulation of neutrophils activates a membrane-associated serine protease which leads to cleavage of biologically active peptides from CRP (9). CRP peptides 77–82 and 201–206 have been found to inhibit neutrophil chemotaxis to FMLP in vitro (10) and to diminish neutrophil influx and protein leakage into alveoli after FMLP-induced inflammation in mice (11).

Neutrophil extravasation into inflamed or injured areas involves a complex interaction of leukocytes with endothelial cells via regulated expression of surface adhesion molecules (12, 13). The initial attachment of neutrophils to endothelium is mediated by L-selectin (CD62L) (12–15). L-Selectin is constitutively expressed by neutrophils and is released from neutrophils by a proteolytic cleavage within minutes after activation with a concomitant upregulation of Mac-1 (CD11b/CD18) (16, 17). The CD18 integrins, Mac-1 and LFA-1 (CD11a/CD18), are largely responsible for subsequent tightening of the adhesion and transendothelial migration of neutrophils via interactions with their endothelial counterreceptors, ICAM-1 and ICAM-2 (12, 13).

This study was undertaken to examine whether CRP and CRP peptides could affect the first step of neutrophil extravasation, the L-selectin–mediated interaction of neutrophils with endothelial cells. We found that CRP at clinically relevant concentrations and CRP peptides 174–185 and 201–206, but not peptide 77–82, strongly inhibited adhesion of neutrophils to cultured endothelial cells. This was due to the loss of L-selectin expression in neutrophils through a shedding mechanism.

Methods

Antibodies and reagents. In these studies, the monoclonal antibodies (mAbs) used included FITC-conjugated mouse anti–human L-selectin mAb DREG-56 (PharMingen, San Diego, CA), R-phycoerythrin–conjugated mouse anti–human CD11b mAb Leu-15 (Becton Dickinson Immunocytometry Systems, Mountain View, CA) and PerCP-

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1. *Abbreviations used in this paper:* CRP, C-reactive protein; FU, fluorescence units; HCAEC, human coronary artery endothelial cells HMVEC-L, human lung microvascular endothelial cells; sL-selectin soluble L-selectin.

conjugated mouse anti-human CD45 mAb 2D1 (Becton Dickinson). Appropriately labeled, class-matched irrelevant mouse IgG_1 was used as a negative control for each staining. The following murine mAbs were used in neutrophil-endothelial cell adhesion assays: anti-L-selectin mAb DREG-56 (IgG_1; PharMingen) at 20 μg/ml (18, 19); anti-E-selectin mAb ENA-2 (IgG_1, purified $F(ab')_2$ fragments, Monosan, Uden, The Netherlands) at 10 μg/ml (20), and anti-CD18 mAb L130 (IgG_1, Becton Dickinson) at 10 μg/ml. The irrelevant mAb MOPC-21 (IgG_1, PharMingen) at 20 μg/ml was used as a negative control.

Human CRP was obtained from Calbiochem-Novabiochem Corp. (La Jolla, CA). Purity of the protein was ascertained as a single silver-stained protein band of 22 kD. Peptides corresponding to amino acid sequences 77–82 (Val-Gly-Gly-Ser-Glu-Ile), 174–185 (Ile-Tyr-Leu-Gly-Gly-Pro-Phe-Ser-Pro-Asn-Val-Leu), and 201–206 (Lys-Pro-Gln-Leu-Trp-Pro) were purchased from Sigma Chemical Co. (St. Louis, MO). Purity of the peptides, as analyzed by the manufacturer, was 99%. The peptides were stored in distilled water at −20°C. Goat anti-human CRP antiserum and LPS (*Escherichia coli* O111:B4) were obtained from Sigma Chemical Co.

Compound KD-IX-73-4 (*N*-L-(2-hydroxyamino-carbonyl)methyl)-4-methylpentanoyl)-L-3-(2'-naphthyl)-alanyl-L-alanine amide), which inhibits shedding of L-selectin (21), was a gift from Dr. Takashi Kei Kishimoto (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT), and was prepared in DMSO (final concentration: 66 μmol/ml) and stored at −20°C.

Whole blood incubation. Venous blood (10 ml, anticoagulated with sodium heparin, 50 U/ml) was obtained by antecubital venipuncture from nonsmoking healthy volunteers (male and female, 32–54 yr) who had not taken any drugs for at least 10 d before the experiments. Informed consent was obtained from each volunteer, and the protocol was approved by the Clinical Research Committee. White blood cell counts were between 5,000 and 9,000 cells/μl. Whole blood aliquots were transferred to polypropylene centrifuge tubes, placed on a rotator, and incubated with CRP or one of the CRP peptides (3.1–200 μg/ml) for 30 min at 37°C, 95% air/5% CO_2. Preliminary experiments showed that the maximum effects that can be observed with CRP were achieved within 30 min of preincubation. In additional experiments, aliquots of blood were preincubated for 10 min with various concentrations of KD-IX-73-4 before addition of CRP or CRP peptides.

Flow cytometry analysis. Resting and treated neutrophils in whole blood were incubated with saturating concentration of fluorescent dye–conjugated anti-human L-selectin, anti-human CD11b, or anti-human CD45 mAb for 30 min at 22°C, erythrocytes were lysed, and leukocytes were fixed with 2 ml of a lysing medium (FACS® Lysing Solution; Becton Dickinson). The samples were then centrifuged, washed, and resuspended in PBS containing 0.1% sodium azide. Negative controls were obtained by omitting monoclonal antibodies. Nonspecific binding was evaluated by using appropriately labeled mouse IgG_1. Single-color immunofluorescence staining was analyzed by a cytofluorometer (FACScan®; Becton Dickinson) with Lysis II software. Data from 10,000 events per sample were acquired. Antibody binding was determined as mean fluorescence intensity after gating for neutrophils by their characteristic forward and side scatter properties. The results are presented as relative fluorescence units (RFU): $RFU = (FU_{experimental} - FU_{isotype}) \times 100/(FU_{control} - FU_{isotype})$, where $FU_{experimental}$ and $FU_{control}$ are the CD11b or L-selectin fluorescence intensity of treated cells and cells cultured in medium only, respectively, and $FU_{isotype}$ is the fluorescence intensity of class-matched irrelevant antibody. Likewise, in additional experiments, suspensions of isolated neutrophils were incubated under identical conditions with the conjugated antibodies, and fluorescence was assessed as described above.

Isolation and treatment of neutrophil granulocytes. Neutrophil granulocytes were isolated from peripheral blood by centrifugation through Ficoll-Hypaque gradients (Pharmacia Diagnostics AB, Uppsala, Sweden), sedimentation through dextran (3%, wt/vol), and hypotonic lysis of erythrocytes. The resultant cell preparation contained > 97% neutrophils. Neutrophils were suspended in a modified HBSS (in mmol/liter: NaCl, 145; K_2PO_4, 10; $CaCl_2$, 1.4; $MgCl_2$, 1.2; glucose, 10; and bovine serum albumin, 250 μg/ml, pH 7.4). The experiments were carried out in 1.5 ml polypropylene tubes. Neutrophils (5×10^6 cells/ml) were incubated in HBSS with CRP or CRP peptides (3.1–200 μg/ml) for 30 min at 37°C. Then the cells were pelleted, and the supernatants were collected for further analysis.

Enzymatic activity of supernatants from treated neutrophils. Lactate dehydrogenase (8), β-glucuronidase (8), lysozyme (8), and gelatinase (22) were assayed as described. Enzyme release was determined as the percentage of total enzyme units released from neutrophils treated with 0.1% Triton X-100.

Neutrophil-endothelial cell attachment assay. Normal human coronary artery endothelial cells (HCAEC) and lung microvascular endothelial cells (HMVEC-L) were obtained from Clonetics Corp. (San Diego, CA) and grown in endothelial cell growth medium containing 5% FBS and supplemented with human recombinant epidermal growth factor (10 ng/ml), hydrocortisone (1 mg/ml), gentamycin (50 mg/ml), amphotericin B (50 μg/ml), and bovine brain extract (3 mg/ml). HCAEC or HMVEC-L (passages 5 and 6) were seeded into 96-well microplates and grown to confluence. Cell monolayers were carefully washed, overlaid with 250 μl RPMI 1640 medium containing 10% FBS, and stimulated with LPS (1 μg/ml) for 6–8 h at 37°C in a 5% CO_2 atmosphere. The cells were then washed three times with 200 μl RPMI 1640 medium containing 10% FBS and 2×10^5 ^{51}Cr-labeled neutrophils in 100 μl were added. In another set of experiments, LPS-activated HCAEC were incubated for 15 min with ENA-2 or MOPC-21 mAbs before addition of neutrophils. The mAb reactive with E-selectin was also added back to the neutrophil suspensions so that mAb ENA-2 was present throughout the assay. Radiolabeled neutrophils were incubated with DREG-56, L130, or MOPC-21 mAb for 15 min at 37°C before addition to the endothelial cell monolayers. After incubation of endothelial cells with neutrophils for 30 min at 4 or 37°C on an orbital shaker at 90 rpm, loosely adherent or unattached neutrophils were washed three times with RPMI 1640 medium containing 10% FBS, and the endothelial monolayer plus the adherent neutrophils were lysed in 200 μl of 0.1 mol/liter NaOH. The number of neutrophils in each experiment was estimated from the radioactivity of a control sample. Inhibition of neutrophil attachment was calculated using the level of attachment to unstimulated endothelial cells as the baseline, and the level of attachment of LPS-stimulated endothelial cells as the maximal value. Neither treatment of HCAEC with any of the antibody used in these studies nor carrying out the assays at 4 or 37°C altered the integrity of viable endothelial monolayers.

sL-Selectin ELISA. Soluble L-selectin (sL-selectin) was quantitated in the supernatant of CRP-treated neutrophils by a specific ELISA (Bender MedSystems, Vienna, Austria). The detection limit of the assay was 0.4 ng/ml. Intraassay coefficient of variation was 4.6% at the midpoint (3.2 ng/ml) of the standard curve. There was no cross-reactivity with CRP or any of the CRP peptides studied.

Statistical analysis. Results are expressed as means±SEM. Statistical comparisons were made by ANOVA using ranks (Kruskal-Wallis test) followed by Dunn's multiple contrast hypothesis test to identify differences between various treatments, or by the Wilcoxon signed rank test and Mann-Whitney U test for paired and unpaired observations, respectively. P values < 0.05 were considered significant for all tests.

Results

CRP and peptides derived from CRP attenuate adhesion of neutrophils to LPS-stimulated endothelial cells. To study whether CRP or CRP peptides could affect neutrophil adhesion, we analyzed the attachment of neutrophils to HMVEC-L and HCAEC under nonstatic conditions. Only a few neutrophils were able to bind to unstimulated endothelial cells. However,

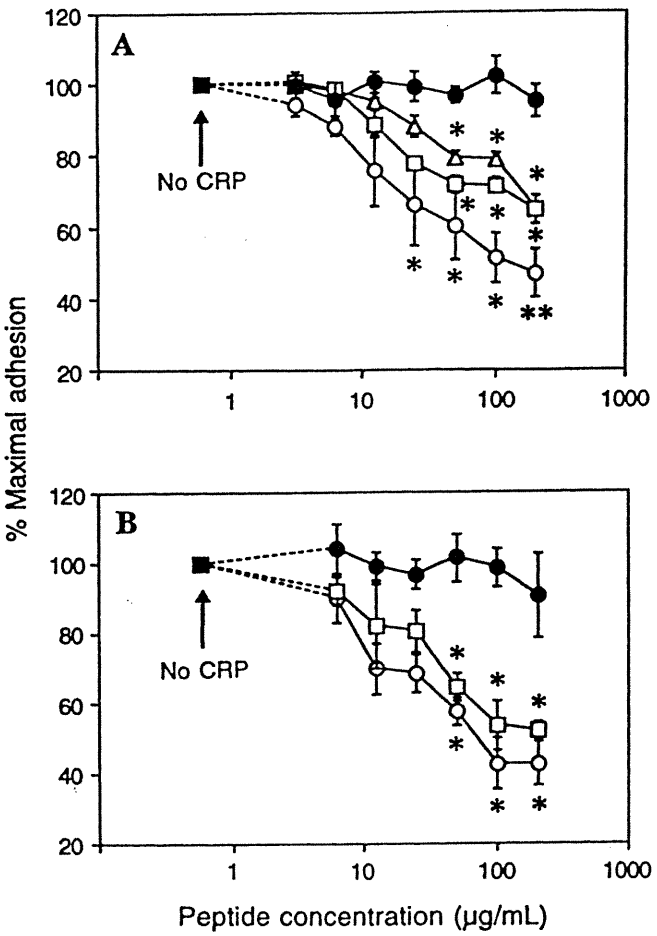

Figure 1. Effect of CRP and peptides derived from CRP on neutrophil attachment to human coronary artery (*A*) and pulmonary microvascular (*B*) endothelial cells. Confluent endothelial cell monolayers cultured for 6–8 h with 1 μg/ml LPS were used for neutrophil adhesion assays. Neutrophil granulocytes were incubated with CRP (*open circles*), peptide 77–82 (*closed circles*), peptide 174–185 (*open triangles*), or peptide 201–206 (*open squares*) for 30 min at 37°C before addition to endothelial cells. Adherence of neutrophils to unstimulated endothelium was determined, subtracted from the neutrophil adherence to stimulated endothelium, then expressed as a percentage of net adherence of neutrophils to LPS-stimulated endothelial monolayers. Neutrophil adhesion to unstimulated and LPS-stimulated HCAEC was $1.3\pm0.5 \times 10^4$ and $4.2\pm1.3 \times 10^4$ cells per well, respectively ($n = 3$). Neutrophil adhesion to unstimulated and LPS-stimulated HMVEC-L was $0.6\pm0.1 \times 10^4$ and $4.3\pm0.6 \times 10^4$ cells per well, respectively ($n = 3$). Values are expressed as mean±SEM of three experiments in duplicate using neutrophils from different donors. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. positive control (*No CRP*).

when endothelial cells were activated with LPS (1 μg/ml), on average 3.3- and 6.8-fold more neutrophils adhered to treated than to untreated HCAEC and HMVEC-L, respectively (Fig. 1). Preincubation of neutrophils with CRP inhibited neutrophil adhesion to LPS-activated HCAEC and HMVEC-L in a concentration-dependent manner with apparent IC_{50} values of 20 and 22 μg/ml, respectively (Fig. 1). No significant decreases in adhesion could be detected when untreated neutrophils were added to HCAEC cultured with LPS in the presence of 200 μg/ml CRP ($4.2\pm1.3 \times 10^4$ cells vs. $3.9\pm1.1 \times 10^4$ cells attached per well, $n = 3$). Preincubation of neutrophils with peptide 174–185 or 201–206 also attenuated neutrophil adherence to HCAEC, albeit these peptides showed a weaker inhibitory effect than CRP (Fig. 1 *A*). Similar results were obtained with peptide 201–206 using HMVEC-L (Fig. 1 *B*). In contrast, peptide 77–85 did not inhibit neutrophil adhesion (Fig. 1).

Preincubation of neutrophils with anti-CRP antiserum did not affect their adhesion to HCAEC. However, when CRP was preincubated with CRP antiserum for 20 min at 37°C before addition to neutrophils, neutrophil adhesion was nearly completely restored (number of neutrophils attached to LPS-activated HCAEC: medium only, $4.2\pm1.3 \times 10^4$ cells per well; CRP, $2.6\pm0.7 \times 10^4$ cells per well; anti-CRP antiserum plus CRP, $4.0\pm1.4 \times 10^4$ cells per well, $n = 6$).

Inhibition of L-selectin–mediated neutrophil adhesion to activated endothelial cells by CRP. Since multiple receptors are involved in neutrophil adhesion to LPS-stimulated endothelium even under nonstatic conditions (23), we assayed the contribution of L-selectin, E-selectin, and CD18 to the binding interaction and studied the possibility that CRP could affect E-selectin or CD18 function by using function-blocking mAbs. The binding assays were carried out at 37°C as well as at 4°C, where L-selectin shedding and CD18-dependent adhesion are minimal (23).

At 37°C, a significant proportion of neutrophil-HCAEC attachment was blocked by mAb binding to E-selectin (40±8%, $n = 3$), L-selectin (47±6%), or CD18 (22±2%) (Fig. 2 *A*). The combination of these mAbs inhibited the binding of neutrophils by 80–86% (Fig. 2 *A*). Adhesion of neutrophils treated with CRP (200 μg/ml) and anti–L-selectin mAb was similar to those observed with neutrophils treated with CRP or anti–L-selectin mAb alone (Fig. 2 *B*). Combination of CRP with either anti–E-selectin or anti-CD18 mAb resulted in inhibition of neutrophil binding similar to those observed with combinations of anti–L-selectin mAb and anti–E-selectin or anti-CD18 mAb, respectively (Fig. 2 *A*). Combining CRP, anti–E-selectin, and anti-CD18 mAbs blocked ~ 85% of adhesion. Thus, CRP acts in concert with anti–E-selectin and anti-CD18 mAb, but not with anti–L-selectin mAb, to inhibit neutrophil attachment to LPS-activated HCAEC, and in this assay system, L-selectin, E-selectin, and CD18 in combination can account for nearly all neutrophil attachment.

At 4°C, neutrophil adhesion was inhibited by anti–L-selectin mAb (54±3%, $n = 3$) and anti–E-selectin mAb (55±5%), whereas anti-CD18 mAb had little effect (9±2% inhibition) (Fig. 2 *B*). Again, combining these mAb blocked ~ 80% of adhesion. CRP in combination with anti–L-selectin mAb produced inhibition of neutrophil adhesion similar to that observed with CRP or anti–L-selectin mAb alone (Fig. 2 *B*). The inhibitory actions of CRP and anti–E-selectin or anti-CD18 mAb were additive, and did not differ from those observed with a combination of anti–L-selectin and anti–E-selectin or anti-CD18 mAb, respectively (Fig. 2 *B*). The combination of CRP with anti–E-selectin and anti-CD18 mAbs inhibited 78–85% of neutrophil adhesion. Assayed at either 4 or 37°C, qualitatively similar results were obtained when peptide 174–185 or 201–206 was combined with anti–E-selectin or anti-CD18 mAb (data not shown), and the control mAb (MOPC-21) gave results similar to medium alone (Fig. 2, *A* and *B*).

KD-IX-73-4 prevents the effects of CRP and peptides 174–185 and 201–206 on neutrophil-endothelial cell attachment. To

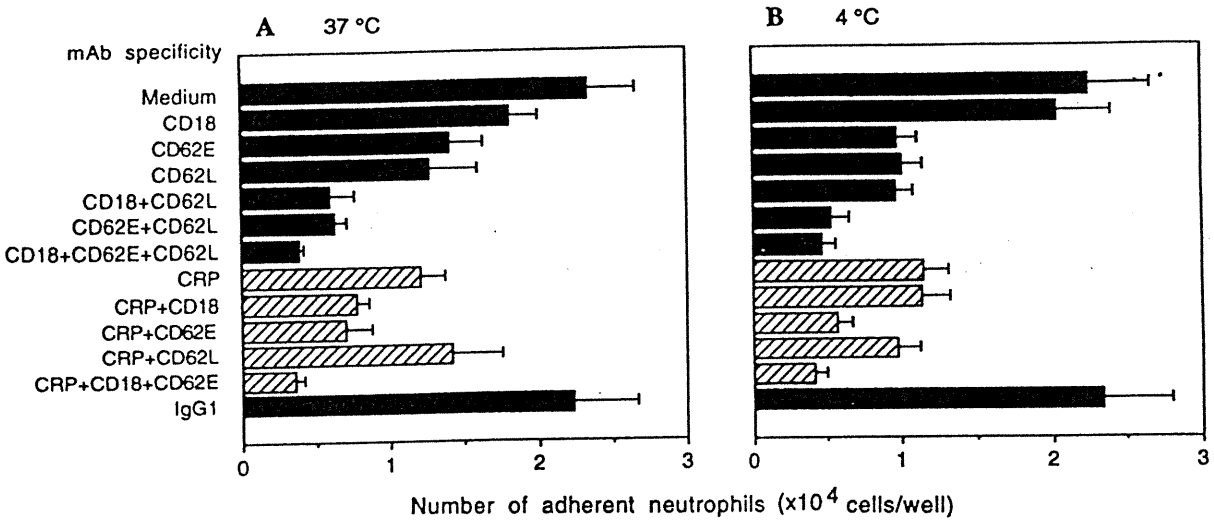

Figure 2. Inhibition of neutrophil binding to endothelial cells by anti-E-selectin, anti-L-selectin, and anti-CD18 mAbs, and CRP. Confluent HCAEC monolayers were activated for 6 h with 1 μg/ml LPS. Neutrophils and HCAEC were treated with CRP (200 μg/ml) or the indicated mAbs before and during the assay. Neutrophils were incubated with endothelial cells for 30 min at 37 (*A*) or 4°C (*B*). Neutrophil adhesion to unstimulated HCAEC was $0.57\pm0.08 \times 10^4$ and $0.34\pm0.05 \times 10^4$ cells per well at 37 and 4°C, respectively ($n = 3$). The irrelevant mAb MOPC-21 (IgG_1) was used as a negative control. Results are expressed as mean±SEM of three experiments using neutrophils from different donors.

study further the L-selectin–dependent component of neutrophil-endothelial attachment, we examined whether inhibition of L-selectin shedding would alter the actions of CRP or CRP-derived peptides on adhesion of neutrophils to HCAEC. Pretreatment of neutrophils with the metalloprotease inhibitor KD-IX-73-4 (44 nmol/ml) before challenging them with CRP or peptides 174–185 or 201–206 (each at 200 μg/ml) almost completely restored neutrophil binding to HCAEC (Fig. 3). KD-IX-73-4 alone did not affect neutrophil adhesion to activated endothelial cells (Fig. 3).

L-Selectin expression on neutrophils is downregulated by CRP and peptides derived from CRP. We next determined whether the effect of CRP and CRP peptides on neutrophil adhesiveness resulted from diminished expression of L-selectin. Flow cytometry analysis of whole blood treated with CRP showed that the neutrophil surface expression of L-selectin was downregulated by CRP in a concentration-dependent fashion with an apparent IC_{50} concentration of 24 μg/ml (Fig. 4). CRP showed a stronger effect than peptides 174–185 and 201–206, while peptide 77–82 had no effect on L-selectin expression

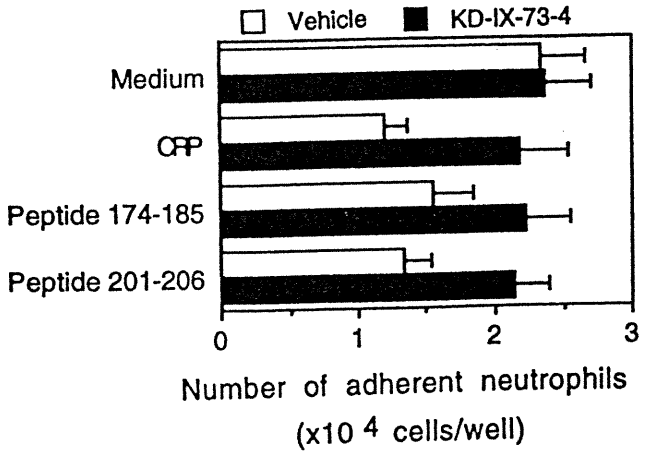

Figure 3. KD-IX-73-4 prevents the effects of CRP on neutrophil-endothelial cell attachment. Chromium-51–labeled neutrophils were incubated for 15 min with KD-IX-73-4 (44 nmol/ml, *solid columns*) or its vehicle (*open columns*), and challenged for 30 min with CRP, peptide 174–185, or 201–206 (all at 200 μg/ml) at 37°C before addition to LPS-activated HCAEC. The adhesion assay was performed at 37°C. Neutrophil binding to unstimulated HCAEC was $0.57\pm0.08 \times 10^4$ cells per well. Values are shown as mean±SEM of three experiments in duplicate using neutrophils from different donors.

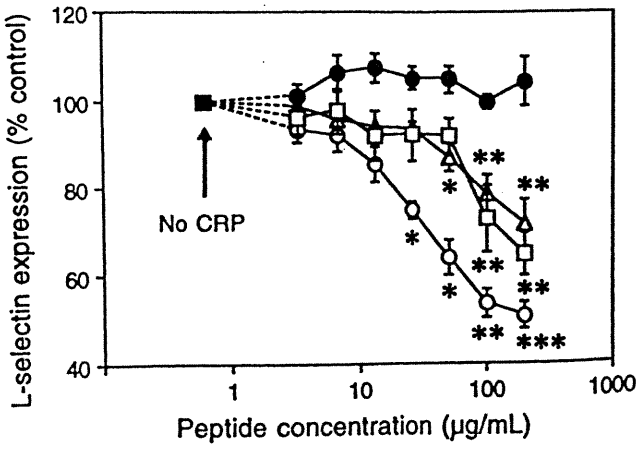

Figure 4. Concentration-dependent effect of CRP and CRP-derived peptides on surface expression of L-selectin by neutrophils. Aliquots of whole blood were incubated with CRP (*open circles*), peptide77–82 (*closed circles*), peptide 174–185 (*open triangles*), or peptide 201–206 (*open squares*) for 30 min at 37°C. L-Selectin expression is presented as percentage of control, i.e., mean fluorescence-intensity of neutrophils cultured in medium only for 30 min. Values are expressed as mean±SEM for five to six experiments using blood from different donors. $*P < 0.05$; $**P < 0.01$ vs. positive control (*No CRP*).

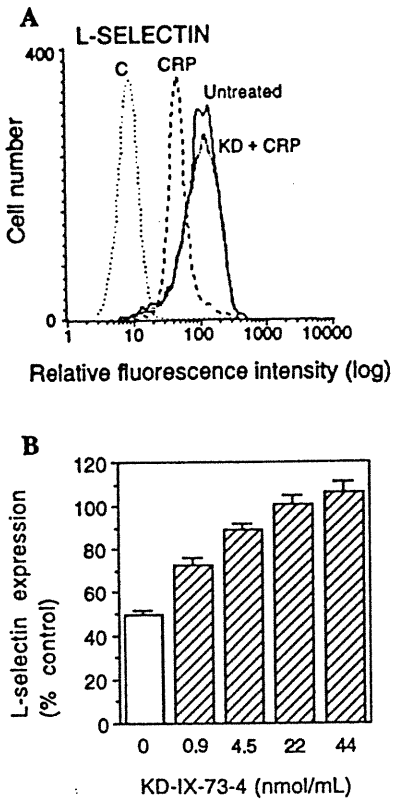

Figure 5. KD-IX-73-4 inhibits downregulation of neutrophil surface expression of L-selectin. (*A*) Aliquots of whole blood were preincubated for 15 min with KD-IX-73-4 (44 nmol/ml), and challenged for 30 min with CRP (200 μg/ml) at 37°C. In a representative histogram, the basal expression of the cell surface antigen (*Untreated*) and the negative control (*C*) immunostaining is also shown. (*B*) Concentration-dependent inhibition of CRP-induced downregulation of L-selectin expression. Aliquots of whole blood were preincubated for 15 min with various concentrations of KD-IX-73-4 (*hatched columns*) or its vehicle (*open column*), and challenged for 30 min with CRP (200 μg/ml) at 37°C. Values represent the mean±SEM obtained in duplicate determination for each sample. The results are representative of three independent experiments.

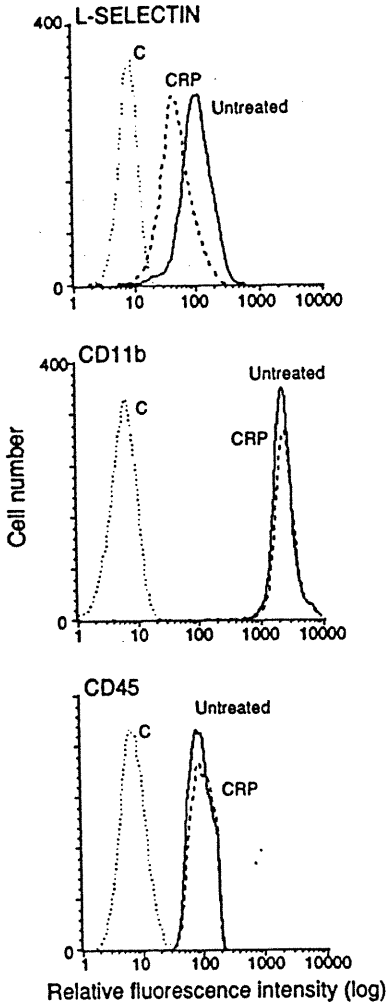

Figure 6. Effect of CRP on neutrophil surface expression of L-selectin, CD11b, and CD45. Aliquots of whole blood were incubated for 30 min in the presence of 200 μg/ml CRP at 37°C. In each histogram, the basal expression of the cell surface antigen (*Untreated*) and the negative control (*C*) of immunostaining is also shown. One representative experiment ($n = 6$) is shown.

(Fig. 4). Neither CRP nor any of the CRP peptides studied affected basal expression of L-selectin when the experiments were performed at 4°C (data not shown). Anti-CRP antiserum almost completely prevented the effect of CRP on L-selectin expression (mean fluorescence intensity for L-selectin: medium only, 84±7; CRP, 200 μg/ml, 47±4; anti-CRP antiserum plus CRP, 80±6, $n = 6$). Qualitatively and quantitatively similar effects on L-selectin expression were seen after incubation of isolated neutrophils with CRP or CRP peptides (data not shown).

KD-IX-73-4 prevents downregulation of neutrophil L-selectin expression elicited by CRP and peptides 174–185 and 201–206. Preincubation of whole blood aliquots with KD-IX-73-4 inhibited CRP-induced downregulation of L-selectin expression in a concentration-dependent fashion with an apparent IC_{50} concentration of ~ 1.6 nmol/ml (Fig. 5). Similarly, the effects of peptides 174–185 and 201–206 were also completely inhibited by 44 nmol/ml KD-IX-73-4 (data not shown). KD-IX-73-4 did not affect the characteristic forward- and side-light scatter profiles of neutrophils challenged with CRP or CRP peptides (data not shown).

Effect of CRP and peptides derived from CRP on neutrophil activation. Since L-selectin is rapidly downregulated from the surface of neutrophils upon cell activation, we investigated the possibility that CRP or CRP-derived peptides could activate neutrophils. We analyzed neutrophil surface expression of CD11b and CD45, which is upregulated upon activation and degranulation. CRP at a concentration of 200 μg/ml failed to modify baseline expression of CD11b or CD45 upon 30 min of whole blood treatment (Fig. 6). Similar results were obtained with CRP-derived peptides studied (data not shown).

Incubation of isolated neutrophils with CRP or any of the CRP peptides for 30 min did not induce a significant release of β-glucuronidase (a marker for azurophil granules), lysozyme (a marker for specific plus azurophil granules), or gelatinase (a marker for tertiary granules) (Table I). As a positive control, neutrophils challenged with platelet-activating factor secreted high amounts of gelatinase (Table I). Lactate dehydrogenase release never exceeded 2% of the total cell content, and was similar in the absence and presence of CRP or CRP peptides (data not shown), indicating that cellular integrity was not affected.

Shedding of L-selectin by CRP and peptides derived from CRP. To determine whether CRP and CRP peptides exert their inhibitory effect on neutrophil-endothelial cell adhesion by the cleavage and shedding of L-selectin, we measured sL-selectin in the cell-free supernatants of neutrophils incubated with CRP or CRP peptides for 30 min. CRP at 200 μg/ml produced more than a threefold increase in sL-selectin concentrations, which can be prevented by anti-CRP antiserum (Fig. 7). The maximum amount of sL-selectin detected after CRP was ~ 76% of the amount measured in the supernatant from neutrophils challenged with platelet-activating factor (Fig. 7).

Table I. Granule Enzyme Release from Human Neutrophils by CRP and Peptides Derived from CRPs

	Enzyme release		
	Gelatinase	β-Glucuronidase	Lysozyme
Control	18.7±1.6	2.3±0.4	6.0±0.8
PAF	57.0±5.6*	2.5±0.5	6.2±0.9
CRP	20.6±2.0	2.7±0.4	5.5±0.7
Peptide 77–82	21.4±1.4	2.3±0.4	6.2±0.8
Peptide 174–185	24.9±0.9	2.2±0.3	5.8±1.5
Peptide 201–206	22.7±2.6	2.3±0.5	5.7±1.1

Values are expressed as percentage of total cellular enzyme activity released by neutrophils to the culture medium after incubation with CRP (200 μg/ml), CRP-derived peptides (200 μg/ml), or platelet-activating factor (*PAF*, 1 μmol/l) for 30 min at 37°C. Total cell enzyme activity was measured in unstimulated neutrophils disrupted by treatment with 0.1% Triton X-100. Values are means±SEM of four independent experiments. $*P < 0.05$ compared to control.

Lower amounts of sL-selectin were detected in the supernatant medium of neutrophils incubated with peptides 174–185 and 201–206 at 200 μg/ml concentration (Fig. 7), corresponding to their potency to downregulate surface expression of L-selectin. In contrast, peptide 77–82 did not increase sL-selectin concentration (Fig. 7).

Discussion

We describe in this report that a novel mechanism by which CRP and CRP-derived peptides 174–185 and 201–206 may affect the inflammatory response is the modulation of adhesion of neutrophils to the endothelium. CRP or CRP-derived peptides 174–185 and 201–206 acting on neutrophils diminish attachment of resting neutrophils to LPS-stimulated endothelial cells via downregulation and shedding of L-selectin from the surface of neutrophils.

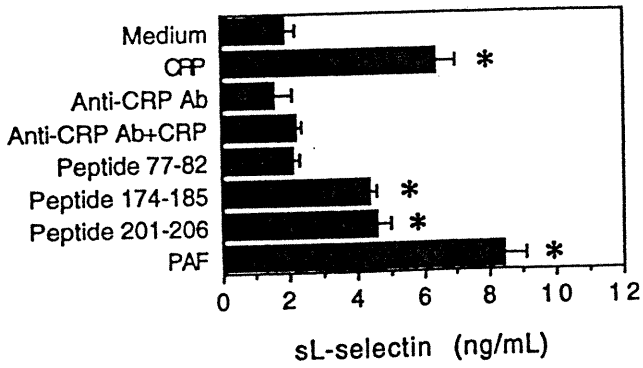

Figure 7. CRP and CRP-derived peptides induce shedding of L-selectin from neutrophils. Isolated neutrophils were incubated for 30 min in the presence of CRP (200 μg/ml), CRP plus anti-human CRP antiserum (*Anti-CRP Ab*), peptide 77–82, 174–185, or 201–206 (all at 200 μg/ml), or platelet-activating factor (*PAF*, 1 μmol/l) at 37°C. Supernatant fluids were collected and tested for sL-selectin by a specific ELISA. Values are means±SEM for three donor cell preparations. $*P < 0.05$ compared to control.

The neutrophil-endothelial cell attachment assay was used to study the first step of neutrophil migration. To simulate blood flow, the experiments were performed under nonstatic conditions (24). In agreement with results previously reported by other authors (23–25), mAbs against L-selectin, E-selectin, and CD18 each partially inhibited the adhesion of neutrophils to HCAEC at 37°C, whereas together they inhibited 80–86% of neutrophil adhesion. These results suggest that each of these adhesion molecules function independently and interact in a cooperative fashion to promote optimal neutrophil adhesion to HCAEC. To our knowledge, this study demonstrates for the first time that CRP can effectively attenuate the neutrophil attachment to LPS-activated human endothelial cells. This inhibition can be attributed to an effect of CRP on neutrophils rather than endothelial cells, as no significant decreases in neutrophil attachment were observed after culture of endothelial cells with LPS in the presence of CRP. Indeed, this study provides three lines of evidence indicating that CRP-induced inhibition of neutrophil adhesion to endothelial cells is predominantly attributable to the shedding of L-selectin. First, the shedding of L-selectin correlated with reduced neutrophil adhesion. Second, CRP or a function-blocking anti–L-selectin mAb resulted in similar reductions in neutrophil adhesion to HCAEC. Furthermore, the inhibitory actions of CRP and anti–L-selectin mAb were not additive, as assayed at both 37 and 4°C, where the L-selectin–dependent component of adhesion is larger (23, and this study). On the other hand, the inhibition with CRP was additive with anti–E-selectin and anti-CD18 mAbs, and did not differ from the inhibition observed when anti–L-selectin mAb was combined with anti–E-selectin or anti-CD18 mAb. These findings would indicate that CRP neither affected E-selectin or CD18 function nor interfered with the E-selectin ligand or ICAM-1/ICAM-2 under our experimental conditions. Third, the L-selectin shedding inhibitor KD-IX-73-4 (21) at a concentration which completely inhibited CRP-induced downregulation of L-selectin expression also prevented the effects of CRP on neutrophil adhesion. The finding that CRP inhibits L-selectin–dependent adhesion may be of particular importance, because L-selectin may function as the initial attachment receptor to activated endothelium, as it occurs during both in vitro and in vivo rolling at physiologic flow rates (14, 15). CRP inhibited neutrophil adhesion to both HCAEC and HMVEC-L with similar potency, indicating its ability to attenuate neutrophil adhesion in different parts of the vasculature. Our observations would explain, at least in part, the mechanism by which CRP prevented leukocyte accumulation in experimental alveolitis (6, 7).

It has been suggested that the specific biologic function of CRP may reside in its subsequences, with the function expressed upon degradation of the parent protein (26). Indeed, three tuftsin-like (Thr-Lys-Pro-Arg) regions are present in the CRP molecule, and the corresponding three synthetic peptides have been found to stimulate neutrophil chemotaxis (26). Other peptides homologous to amino acid sequences within regions 77–82 and 201–206 have been reported to inhibit human neutrophil superoxide production and chemotaxis to several stimuli (10, 27) as well as eosinophil chemotaxis (28), whereas peptide 177–182 has been shown to augment cytokine production by alveolar monocytes/macrophages and to enhance their tumoricidal activity (29, 30). Extending previous findings with peptide 201–206 (10, 11, 27), this study shows that this peptide can also effectively attenuate neutrophil at-

tachment to both HCAEC and HMVEC-L. We were surprised that peptide 77–82, which was shown to inhibit neutrophil influx into alveoli after FMLP-induced inflammation in mice (11), even at a concentration as high as 200 μg/ml, failed to affect neutrophil adhesion to endothelial cells. However, it should be noted that significant inhibition of neutrophil chemotaxis was only observed with peptide 77–82 concentrations higher than 200 μg/ml (11). Apart from differences in the assays used, the reason for this discrepancy is not readily apparent. Interestingly, peptide 177–182, which activates monocytes/macrophages (29, 30), showed an inhibitory potency similar to that of peptide 201–206 in attenuating adhesion of neutrophils to HCAEC. These observations suggest cell-specific effects of CRP peptides on various inflammatory cells.

Our results indicated that larger concentrations of peptides 174–185 and 201–206 than native CRP were required to obtain a similar degree of inhibition. Even accounting for the fivefold molar difference between monomeric synthetic peptides and the pentameric native CRP, on a molar concentration basis, 100–150-fold more peptide than native CRP was needed to achieve a similar degree of inhibition of neutrophil attachment. It is likely that higher concentrations of CRP peptides may be needed to occupy the CRP receptor. Although we cannot rule out that CRP degradation occurred in these experimental conditions, the differences in the concentrations of native CRP and CRP peptides required to inhibit neutrophil attachment would not lend support to the hypothesis that the biologic functions of CRP may entirely reside in its degradation products (26).

Activation of neutrophils uniformly results in downregulation of L-selectin expression as well as in upregulation of both CD11b/CD18 and CD45 expression (15, 31, 32). Within minutes of activation, leukocytes release L-selectin from their surface by a proteolytic cleavage just outside of the transmembrane domain (15, 33, 34). However, neither native CRP nor CRP peptides at the concentrations studied affected basal expression of CD11b/CD18 and CD45, indicating that neutrophils were not activated. This notion is further supported by the failure of CRP and CRP peptides to induce release of granule enzymes. L-Selectin shedding not only occurs in response to cell activation, but also after treatment with a chemical cross-linker (35) or certain nonsteroidal antiinflammatory drugs (25). Although the biochemical and molecular nature of the proteolytic enzyme remains to be identified, it appears to be a constitutively active enzyme (36, 37). Formation of an appropriate three-dimensional structure of L-selectin near the membrane has been implicated in the regulation of this proteolytic process (36, 37). It is tempting to speculate that CRP and peptides 174–185 and 201–206 could activate this process. However, the enzyme(s) and signaling mechanisms that regulate the conformation of L-selectin in response to CRP or CRP peptides remain key areas of inquiry. Inactivation of the proteolytic enzyme by KD-IX-73-4 would block both activation-dependent (21) and independent L-selectin shedding (this study). Our results indicate that downregulation of L-selectin expression is also due to the shedding of the molecule from the neutrophil surface, as quantitated by the soluble form of L-selectin in the cell-free supernatants of neutrophils cultured with CRP or CRP peptides. Although an inherent proteolytic activity of CRP itself on L-selectin cannot be completely ruled out, the observations that this phenomenon does not occur at 4°C coupled with the demonstration of L-selectin shedding by CRP peptides 174–185 and 201–206 would argue against this possibility.

The concentrations of CRP required in our in vitro experiments for the L-selectin shedding are below the peak plasma levels detected during the acute-phase response, suggesting that this event may also occur in vivo. However, it is difficult to establish a strict correlation between our in vitro observations and the in vivo behavior of CRP, which also depends on factors other than plasma concentrations, such as deposition at sites of tissue injury (38) and plasma metabolism (39). To our knowledge, no information is available on the local tissue or plasma levels of CRP peptides.

These experiments permit us to suggest a novel mechanism of action for CRP. This acute-phase protein may prevent migration of neutrophils into inflammatory sites by downregulating the neutrophil surface expression of L-selectin. This hypothesis rests on two separate lines of evidence: functional, at clinically relevant plasma concentrations native CRP inhibits L-selectin–dependent adhesion of neutrophils to LPS-activated endothelial cells; and phenotypic, at similar concentrations it selectively downregulates neutrophil expression of L-selectin expression without inducing cell activation. At the site of tissue injury or inflammation, CRP may undergo conformational changes, resulting in altered forms of CRP which may promote inflammation (40). On the other hand, considerable neutrophil-mediated degradation of CRP occurs at the same sites, resulting in peptides which either up- or downregulate leukocyte activity. Among these products, peptides 174–185 and 201–206 might also inhibit neutrophil migration, thereby contributing to demarcation of the inflammatory locus. These findings suggest that by diminishing leukocyte traffic into infected or injured tissues, native CRP and CRP peptides 174–185 and 201–206 may be a major mechanism to attenuate or limit the inflammatory response.

Acknowledgments

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In experiments which are unpublished, we studied further the effect of CRP derived peptides on neutrophil adhesion. In these studies we used two hydroxamate-based metalloproteases inhibitors, KD-IX-73-4, and compound 3 from Immunex corporation.

First we compared the inhibitory potentials of the two inhibitors on the L-selectin shedding induced by a variety of agonists (figure 8). Both KD-IX-73-4 and compound 3 reversed both cell activation-dependent and –independent shedding of L-selectin from human neutrophils and monocytes. The calculated IC_{50} values for KD-IX-73-4 and for compound 3 were in the range of 0.4 to 6.9 μM and did not differ significantly. By contrast, upregulation of CD18 expression by human neutrophils challenged with PAF, LPS, fMLP or PMA was not affected by the metalloprotease inhibitors (figure 9).

That inhibition of L-selectin shedding responsable for the actions of KD-IX-73-4 and compound 3 was assessed by measuring soluble L-selectin in the culture medium. As expected, both metalloprotease inhibitors prevented increases in soluble L-selectin concentrations. (figure 10).

Next we studied whether KD-IX-73-4 and compound 3 could also reverse the effects of CRP peptides, 174-185 and 201-206, on neutrophil adhesion to HCAEC. While none of the metalloprotease inhibitors alone affected neutrophil adhesion to LPS-activated HCAEC, they reversed the decreases in the number of adherent neutrophils observed with the CRP derived peptides (figure 11). The inhibitory action of the CRP derived peptides on neutrophil adhesion was due to downregulation of L-selectin, because they have an additive action with antibodies directed against CD18 and E-selectin, but not with an L-selectin antibody (figure 12).

This confirms that CRP peptides 174-185 and 201-206 can entirely mimic the actions of the parent compound CRP on neutrophil-endothelial interactions.

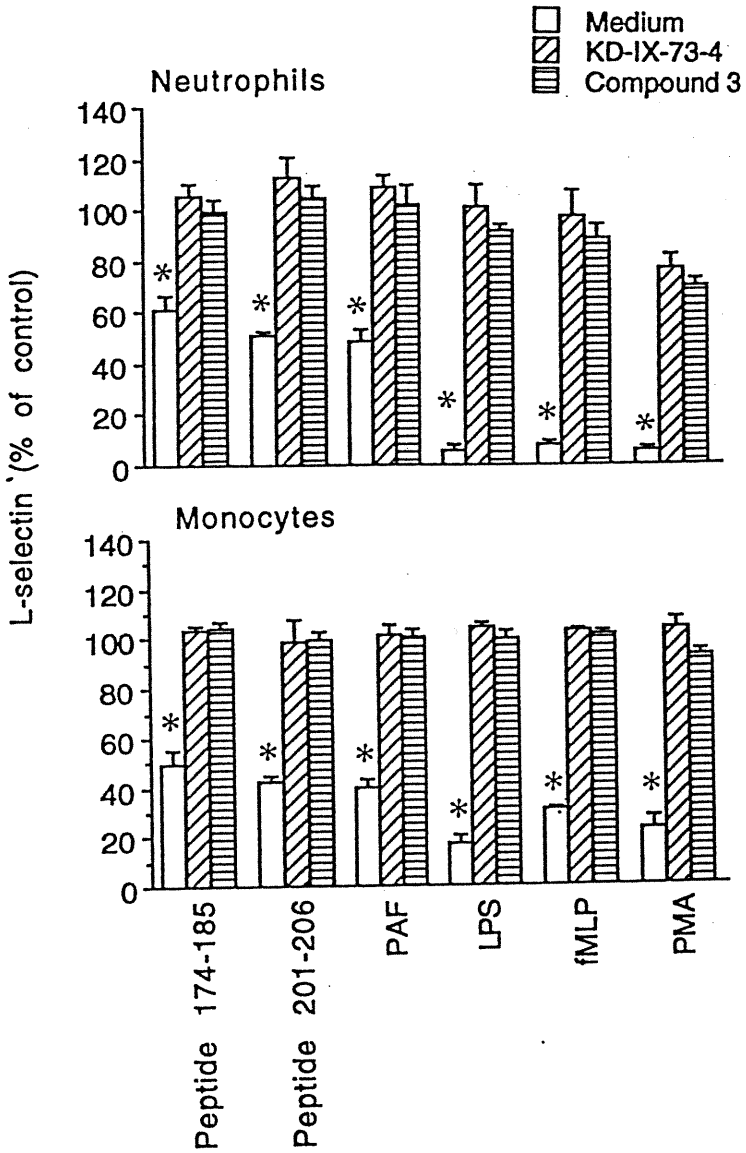

Figure 8. Inhibition of downregulation of L-selectin expression on human neutrophils and monocytes by metalloprotease inhibitors. Aliquots of blood were preincubated with KD-IX-73-4 (50 μM), compound 3 (50 μM) or their vehicle (medium) for 10 min at 37°C, and then challenged with CRP peptide 174-185 (200 μg/ml), peptide 201-206 (200 μg/ml), PAF (1 μM), LPS (1 μg/ml), fMLP (1 μM) or PMA (0.16 μM) for 30 min at 37°C. L-selectin expression is presented as percentage of control, i.e. mean fluorescence intensity of neutrophils and monocytes, respectively, in whole blood incubated in medium for 30 min. Values are expressed as mean$\pm$SEM of 3-5 experiments in duplicate using blood from different donors. * $P<0.05$ compared with control (control= 100%).

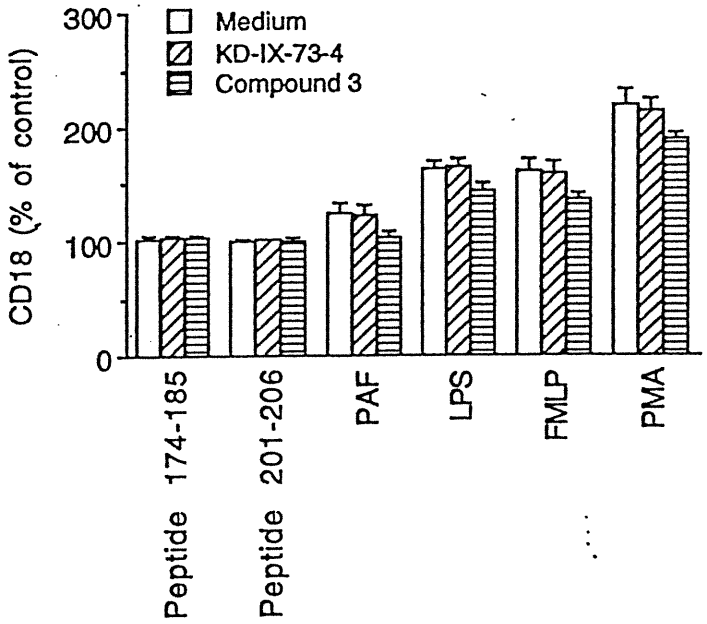

Figure 9. Effect of KD-IX-73-4 and compound 3 on CD18 expression on human neutrophils. Aliquots of human blood were preincubated with KD-IX-73-4 (50 μM), compound 3 (50 μM) or their vehicle (medium) for 10 min at 37°C, and then challenged with CRP peptide 174-185 (200 μg/ml), peptide 201-206 (200 μg/ml), PAF (1 μM), LPS (1 μg/ml), fMLP (1 μM) or PMA (0.16 μM) for 30 min at 37°C. CD18 expression is presented as percentage of control, i.e. mean fluorescence intensity of neutrophils in whole blood incubated in medium only for 30 min. Values are expressed as mean$\pm$SEM of 3 experiments in duplicate using blood from different donors.

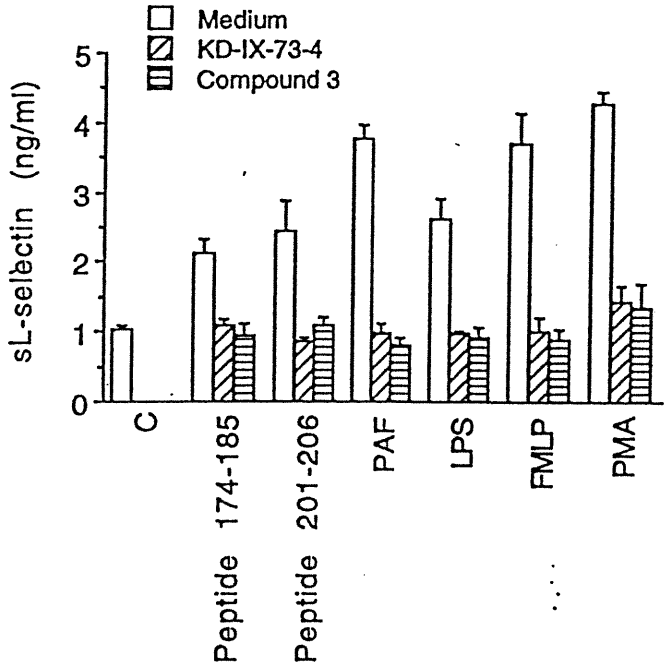

Figure 10. Inhibition of L-selectin shedding from neutrophils by metalloprotease inhibitors. Isolated neutrophils were incubated for 10 min in the presence of KD-IX-73-4 (50 μM) or compound 3 (50 μM), and then challenged with CRP peptide 174-185 (200 μg/ml), peptide 201-206 (200 μg/ml), PAF (1 μM), LPS (1 μg/ml), fMLP (1 μM) or PMA (0.16 μM) for 30 min at 37°C. Supernatant fluids were collected and tested for L-selectin by a specific ELISA. Values are expressed as mean$\pm$SEM for three different donor cell preparations.

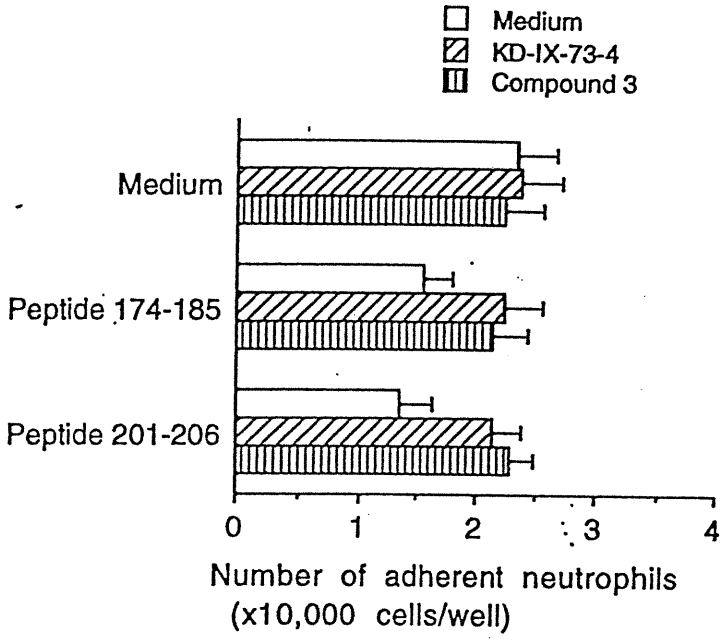

Figure 11. Prevention of the inhibitory effects of CRP peptide 174-185 and peptide 201-206 on adhesion of human neutrophils to coronary artery endothelial cells by the metalloprotease inhibitors KD-IX-73-4 and compound 3. Chromium-51-labeled neutrophils were incubated for 10 min with KD-IX-73-4 (50 μM), compound 3 (50 μM) or their vehicle (open columns), and challenged for 30 min with CRP peptide 174-185 or peptide 201-206 (both at 200 μg/ml) before addition to LPS-activated HCAEC. Neutrophil binding to unstimulated HCAEC was $0.57 \pm 0.08 \times 10^4$ cells/well (n=3). Values are shown as mean$\pm$SEM of three experiments in triplicate using neutrophils from different donors.

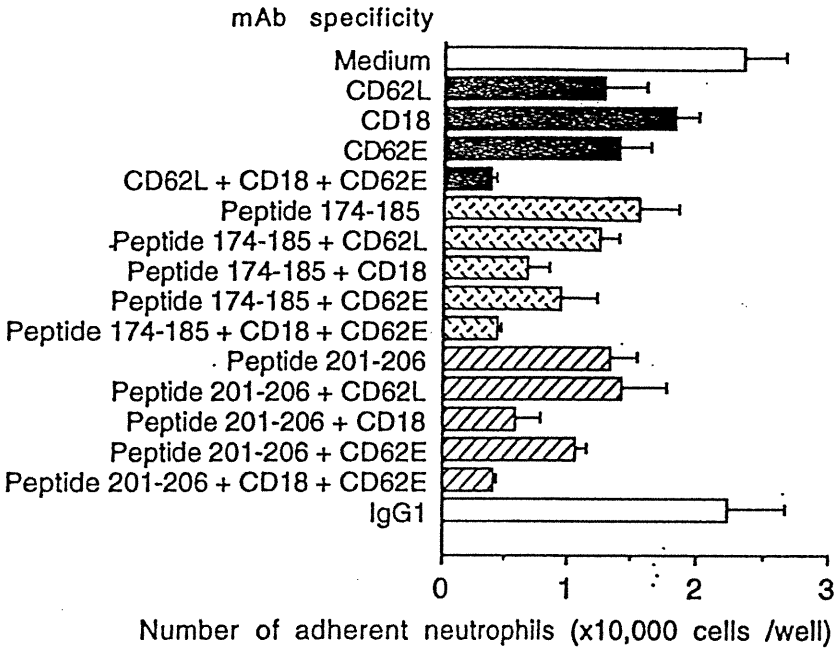

Figure12. Effects of mAb and CRP peptide 174-185 and peptide 201-206 on neutrophil adhesion to coronary artery endothelial cells. Confluent HCAEC monolayers were activated for 6 h with 1 μg/ml LPS. Neutrophils and HCAEC were treated with one of the CRP peptides (each at 200 μg/ml) or the indicated mAbs before and during the assay. Neutrophil adhesion to unstimulated HCAEC was $0.57\pm0.08\times10^4$ cells/well (n=3). The irrelevant mAb MOPC-21 (IgG1) was used as a negative control. Results are expressed as mean$\pm$SEM of three experiments using neutrophils from different donors.

Table 2. Inhibition by the metalloprotease inhibitors, KD-IX-73-4 and compound 3 of agonists induced changes in L-selectin expression on human neutrophils and monocytes in whole blood.

	Agonist	Agonist+ KD-IX-73-4 (50 μM)	IC_{50} (μM)	Agonist+ compound 3 (50 μM)	IC_{50} (μM)
Neutrophils					
Peptide 174-185	-34.2±4.2	5.5±4.5	1.49	-0.7±4.7	0.80
Peptide 201-206	-49.1±4.4	12.7±8.0	1.58	4.7±4.7	0.78
PAF	-48.1±3.0	8.4±5.1	0.79	-1.7±8.1	0.40
LPS	-95.9±1.1	0.9±8.9	1.18	-8.2±1.9	2.90
fMLP	-95.1±1.1	-3.4±10.4	3.29	-12.0±5.9	3.10
PMA	-93.7±2.1	-23.4±5.2	n.d.	-30.4±3.4	n.d.
Monocytes					
Peptide 174-185	-51.9±4.2	3.4±1.8	4.61	4.4±2.2	1.85
Peptide 201-206	-60.1±3.0	-1.3±9.2	4.71	-0.2±2.6	2.30
PAF	-67.9±5.0	1.0±4.2	5.04	0.6±3.0	2.20
LPS	-83.7±3.0	4.6±1.3	2.85	-0.6±3.3	4.80
fMLP	-71.9±4.1	2.5±0.9	4.50	1.5±1.1	3.90
PMA	-81.2±2.9	3.7±4.1	6.14	-7.7±2.4	6.88

Aliquots of human blood were preincubated with various concentrations of KD-IX-73-4, compound 3 or their vehicle for 10 min at 37°C, and then challenged with CRP-derived peptides 174-185 or 201-206 (200 μg/ml), PAF (1 μM), LPS (1 μg/ml), fMLP (1 μM) or PMA (0.16 μM) for 30 min at 37°C. L-selectin expression is presented as percentage changes of control (100% is the mean fluorescence intensity of neutrophils and monocytes, respectively, in whole blood incubated in medium only for 30 min). Values are expressed as mean±SEM of 3-5 experiments in duplicate using blood from different donors. n.d., not determined.

CHAPITRE III (Article III)

Filep JG, **Zouki C**, Petasis NA, Hachicha M, Serhan CN. Anti-inflammatory actions of Lipoxin A_4 stable analogs are demonstrable in human whole blood : modulation of leukocytes adhesion molecules and inhibition of neutrophil-endothelial interactions. *Blood*, 1999, 94(12) : 1-12. (sera publié en décembre)

Introduction au chapitre III

Contrairement à d'autres médiateurs lipidiques, qui sont surtout pro-inflammatoires, les lipoxines représentent une classe unique de médiateurs lipidiques avec des actions anti-inflammatoires puissantes (190,191), tels l'inhibition de la chimiotaxie des neutrophiles et des eosinophiles *in vitro* (206,207), ou le blocage de la transmigration des neutrophiles à travers des cellules épithéliales et endothéliales (195,209) en culture par exemple. Ces actions ont lieu sur des cellules isolées, des cellules épithéliales ou endothéliales en culture, ou même sur des vaisseaux isolés. Mais qu'elles seraient leurs actions dans un microenvironnement de sang total humain où l'on retrouve plusieurs composantes qui peuvent lier les composés lipophiliques?

Dans cet article, nous avons étudié l'effet d'analogues stables de la LXA_4 et des ATL dans du sang humains, et déterminé leur mécanisme d'action cellulaire sur les cellules isolées (leucocytes). Ces analogues de la LXA_4 ont préservé l'expression de la L-sélectine et diminué l'expression de CD11/CD18 à la surface des neutrophiles, des monocytes et à un moindres degré des lymphocytes au repos (non stimulés). Puis ils ont atténué les changements induit par une immunostimulation. De plus ils inhibent l'adhésion des neutrophiles aux cellules endothéliales d'artères coronariennes humaines en culture. Les analogues semblent agir différemment des glucocorticoïdes puisque leur action sur l'adhésion des neutrophiles aux cellules endothéliales n'est pas additive avec celle du dexaméthasone. Conséquemment les analogues de la LXA_4 native et de la 15-epi-LXA_4 induite par l'aspirine (ATL) représentent une nouvelle approche thérapeutique pour la régulation sélective du trafic des leucocytes dans la défense de l'hôte, l'inflammation et les dommages tissulaires.

SECTION HEADING

Anti-Inflammatory Actions of Lipoxin A_4 Stable Analogs Are Demonstrable in Human Whole Blood: Modulation of Leukocyte Adhesion Molecules and Inhibition of Neutrophil-Endothelial Interactions

By János G. Filep, Christine Zouki, Nicos A. Petasis, Mohamed Hachicha, and Charles N. Serhan

We have examined in whole blood the actions of 2 lipoxin A_4 (LXA_4) stable analogs, 15-R/S-methyl-LXA_4 and 16-phenoxy-LXA_4, for their impact on the expression of adhesion molecules on human leukocytes and coronary artery endothelial cells (HCAEC) and on neutrophil adhesion to HCAEC in vitro. Both LXA_4 analogs in nanomolar to micromolar concentrations prevented shedding of L-selectin and downregulated CD11/CD18 expression on resting neutrophils, monocytes, and lymphocytes. Changes in CD11/CD18 expression were blocked by the mitogen-activated protein kinase kinase inhibitor PD98059. The LXA_4 analogs also attenuated changes in L-selectin and CD11/CD18 expression evoked by platelet-activating factor (PAF), interleukin-8, or C-reactive protein-derived peptide 201-206 with IC_{50} values of 0.2 to 1.9 μmol/L, whereas they did not affect lipopolysaccharide (LPS)– or tumor necrosis factor-α–stimulated expression of E-selectin and intercellular adhesion molecule-1 on HCAEC. These LXA_4 analogs markedly diminished adhesion of neutrophils to LPS-activated HCAEC. Inhibition of adhesion was additive with function blocking anti–E-selectin and anti–L-selectin antibodies, but was not additive with anti-CD18 antibody. Combining LXA_4 analogs with dexamethasone (100 nmol/L) almost completely inhibited PAF-induced changes in adhesion molecule expression on leukocytes and gave additive inhibiton of neutrophil adhesion to HCAEC. Culture of HCAEC with dexamethasone, but not with LXA_4 analogs, also decreased neutrophil attachment. Together, these results indicate that LXA_4 stable analogs modulate expression of both L-selectin and CD11/CD18 on resting and immunostimulated leukocytes and inhibit neutrophil adhesion to HCAEC by attenuating CD11/CD18 expression. These actions are additive with those of glucocorticoids and may represent a novel and potent regulatory mechanism by which LXA_4 and aspirin-triggered 15-epi-LXA_4 modulate leukocyte trafficking.

LIPOXINS (trihydroxytetraene-containing eicosanoids) formed by leukocytes during cell-cell interactions represent a unique class of lipid mediators with potent anti-inflammatory actions.[1] In particular, lipoxin A_4 (LXA_4) was found to inhibit neutrophil and eosinophil chemotaxis in vitro,[2,3] to block transmigration of neutrophil granulocytes (PMNL) across epithelial cells[4] and endothelial monolayers,[5] and to reduce PMNL entry into inflamed renal tissues.[6] Recent results showed that stable analogs of LXA_4 resist rapid inactivation and retain biological activities of native LXA_4, including inhibition of PMNL adhesion and transmigration across epithelial and endothelial monolayers,[7] inhibition of interleukin-8 (IL-8) production with consequent impairment of the ability of bacteria-infected epithelia to direct PMNL movement,[8] and attenuation of P-selectin–dependent PMNL adhesion and rolling on the mesenteric microvasculature.[9] These studies described novel actions of LXA_4 and aspirin-triggered LXA_4 (15-epimer of LXA_4) on isolated cells, intestinal epithelial cells, or isolated vessels after their topical application, yet the question of whether these lipid mediators are active within the microenvironment of human whole blood that posses a wide array of components that can bind lipophilic compounds as well as the issue of the cellular mechanisms that account for their novel inhibitory actions in leukocyte trafficking have not been addressed.

Leukocyte extravasation into inflamed areas involves a multistep interaction of leukocytes and endothelial cells via regulated expression of surface adhesion molecules.[10,11] The initial capture and tethering of circulating neutrophils to endothelium is mediated by L-selectin. L-selectin is constitutively expressed by most leukocytes and is rapidly shed after cell activation with a concomitant upregulation of Mac-1 (CD11b/CD18).[12] The CD18 integrins, Mac-1 and LFA-1 (CD11a/CD18), are largely responsible for subsequent tightening of the adhesion and transendothelial migration of neutrophils via interactions with their endothelial counterreceptors, intercellular adhesion molecule-1 (ICAM-1) and ICAM-2.[10,11]

In the present experiments, we studied the impact of stable LXA_4 analogs in human whole blood and addressed their cellular mechanisms of action with isolated cells. We examined the expression of adhesion molecules on human leukocytes and human coronary artery endothelial cells (HCAEC) and on adhesion of PMNL to HCAEC. We used 16-phenoxy-LXA_4, an analog of the native LXA_4,[7] and 15-R/S-methyl-LXA_4, an analog of aspirin-triggered 15-epi-LXA_4.[13] Platelet-activating factor (PAF) and IL-8 were chosen to activate leukocytes, because these mediators can serve as signals for neutrophils to bind tightly to the endothelium.[14,15]

MATERIALS AND METHODS

Antibodies and reagents. In these studies, the monoclonal antibodies (MoAbs) used included fluorescein isothiocyanate (FITC)-

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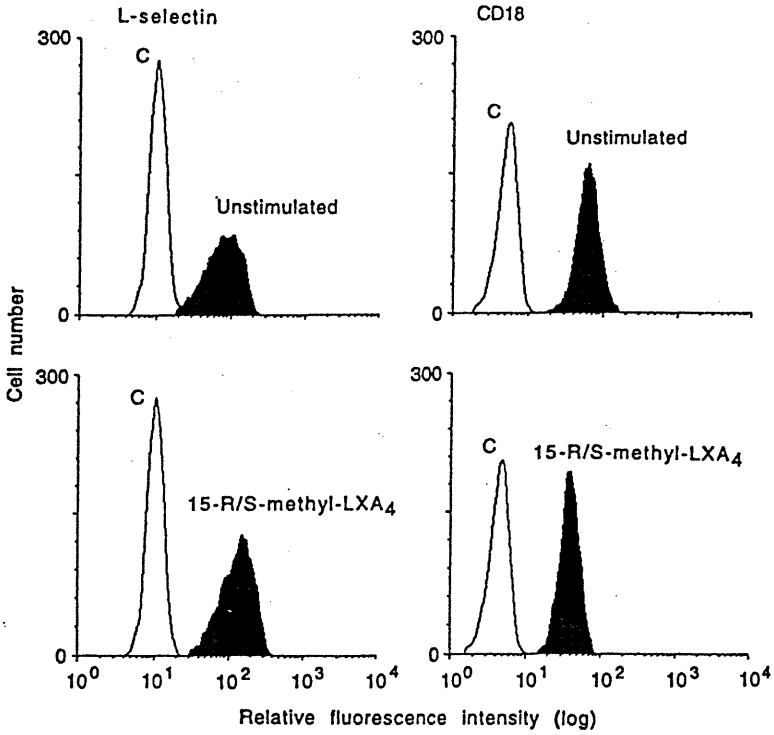

Fig 1. Whole blood actions of aspirin-triggered LXA_4 analog (15-R/S-methyl-LXA_4) on cell surface expression of L-selectin and CD18 by human neutrophils. Whole blood was incubated with 15-R/S-methyl-LXA_4 (5 μmol/L) for 10 miutes at 37°C. In each histogram is also displayed the negative control of immunostaining with class-matched irrelevant antibodies (C). Shown is a representative experiment of 5 experiments.

conjugated mouse antihuman L-selectin MoAb DREG-56 (PharMingen, San Diego, CA), R-phycoerythrin–conjugated mouse antihuman CD18 MoAb MEM-48 (Monosan, Uden, The Netherlands), FITC-labeled mouse antihuman CD11a MoAb G-25.2 (Becton Dickinson Immunocytometry Systems, Mountain View, CA), FITC-labeled mouse antihuman E-selectin MoAb 1.2B6 (Serotec, Kidlington, UK), and R-phycoerythrin–conjugated mouse antihuman ICAM-1 MoAb HA58 (PharMingen). Appropriately labeled, class-matched irrelevant mouse

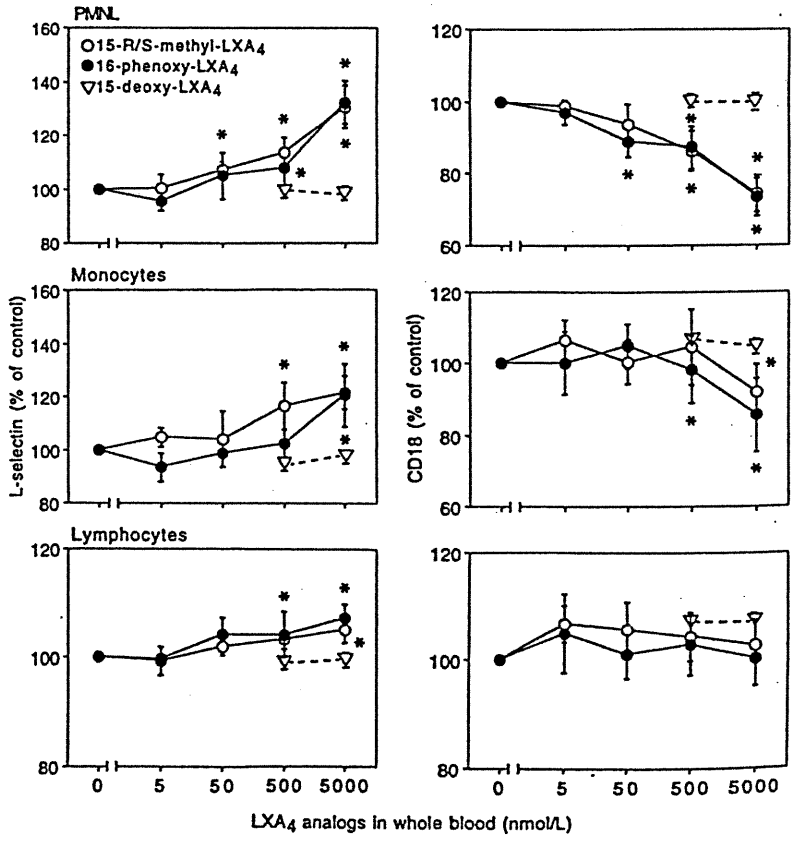

Fig 2. In whole blood, LXA_4 analogs modulate surface expression of L-selectin and CD11/CD18 on resting leukocytes. Blood aliquots were incubated with LXA_4 analogs for 30 minutes at 37°C. Adhesion molecule expression is presented as the percentage of control (unchallenged cells). Control mean fluorescence intensity for L-selectin: PMNL, 108 ± 7; monocytes, 30 ± 2; lymphocytes, 55 ± 3; for CD18: PMNL, 52 ± 4; monocytes, 113 ± 11; lymphocytes, 20 ± 1; n = 8. The results are the mean ± SEM of 4 to 8 experiments with different donor cell preparations. *$P < .05$ *v* control.

IgG_1 was used as a negative control for each staining. The following murine MoAbs were used in neutrophil-endothelial cell adhesion assays: anti–L-selectin MoAb DREG-56 (IgG_1; PharMingen) at 20 μg/mL[16]; anti–E-selectin MoAb ENA-2 [IgG_1, purified $F(ab')_2$ fragments; Monosan] at 10 μg/mL[17]; and anti-CD18 MoAb L130 (IgG_1; Becton Dickinson) at 10 μg/mL.[18] The irrelevant MoAb MOPC-21 (IgG_1; PharMingen) at 20 μg/mL was used as a negative control.

The LXA_4 analogs, 15-R/S-methyl-LXA_4, 16-phenoxy-LXA_4 and 15-deoxy-LXA_4, were prepared by total organic synthesis[7] and stored in ethanol at −70°C. An aliquot was removed and diluted in assay medium immediately before use. The highest concentration of ethanol (0.1%) had no detectable effects in any of the assays used.

Lipopolysaccharide (LPS; *Escherichia coli* O111:B4), C-reactive protein (CRP)-derived peptide 201-206 (Lys-Pro-Gln-Leu-Trp-Pro), dexamethasone 21-phosphate, wortmannin, and genistein were obtained from Sigma Chemical Co (St Louis, MO); PD98059, herbimycin A, and PAF were from Calbiochem (San Diego, CA); and human recombinant IL-8 and tumor necrosis factor-α (TNF-α) were purchased from R&D Systems (Minneapolis, MN).

Whole blood incubation. Venous blood (anticoagulated with 50 U/mL sodium heparin) was obtained from nonsmoking healthy volunteers (male and female, 24 to 55 years of age) who had not taken any drugs for at least 10 days before the experiments. Informed consent was obtained from each volunteer, and the protocol was approved by the Clinical Research Committee. White blood cell counts were between 4,500 and 9,500 cells/μL. Whole blood aliquots were incubated with one of the LXA_4 analogs for 30 minutes at 37°C, 95% air/5% CO_2. Preliminary experiments showed that the maximum effects of LXA_4 analogs could be achieved after 30 minutes of preincubation. Some blood samples were preincubated for 30 minutes with LXA_4 analogs or treated for 120 minutes with dexamethasone (100 nmol/L) with or without LXA_4 analogs and then challenged with PAF (1 μmol/L), IL-8 (10 nmol/L), or CRP peptide 201-206 (100 μg/mL), which downregulates L-selectin expression without inducing cell activation.[18] In some experiments, blood samples were challenged with Mg^{2+} (1 mmol/L) and EGTA (2 mmol/L) to induce a higher affinity form of β_2 integrins.[19]

Analysis of surface antigen expression. Direct immunofluorescence labeling of resting and treated leukocytes in whole blood was performed as described.[18,20] Leukocytes were stained with saturating concentration of fluorescence dye-conjugated antihuman L-selectin or antihuman CD18 MoAb. Nonspecific binding was evaluated by using appropriately labeled mouse IgG_1. Double- or single-color immunofluorescence staining was analyzed by a cytofluorometer (FACScan; Becton Dickinson) with Lysis II software. Antibody binding was determined as mean fluoresence intensity after gating for neutrophils, monocytes, and lymphocytes by their characteristic forward and side scatter properties. The results are presented as relative fluorescence units (RFU): $RFU = (Fu_{experimental} - Fu_{isotype}) \times 100/(FU_{control} - Fu_{isotype})$, where $FU_{experimental}$ and $FU_{control}$ are the L-selectin and CD18 fluorescence intensity of treated cells and cells cultured in medium only, respectively, and $FU_{isotype}$ is the fluorescence intensity of class-matched irrelevant antibody.

Isolation and treatment of neutrophils. PMNL were isolated from peripheral blood by centrifugation through Ficoll-Hypaque (Pharmacia Diagnostics, Uppsala, Sweden), sedimentation through dextran (3%, wt/vol), and hypotonic lysis of erythrocytes. Neutrophils (5×10^6 cells/mL; purity, >97%) were suspended in a modified Hanks' balanced salt solution; incubated with the MAPK kinase (MEK) inhibitor PD98059, the phosphatidylinositol 3-kinase inhibitor wortmannin, or the protein tyrosine kinase inhibitors genistein or herbimycin A for 30 minutes at 37°C; and then challenged with 15-R/S-methyl-LXA_4 for 30 minutes. Surface expression of L-selectin and CD18 was analyzed as just described.

Culture of endothelial cells. Normal HCAEC obtained from Clonetics Corp (San Diego, CA) were cultured as described.[18] HCAEC (passages 3 to 6) seeded into 24-well or 96-well microplates and grown to confluence were used in the experiments.

Neutrophil-endothelial cell adhesion assay. The adhesion assay was performed as in Zouki et al.[18] In brief, monolayers of HCAEC in 96-well microplates were stimulated with LPS (1 μg/mL) with or without LXA_4 analogs or dexamethasone (100 nmol/L) for 6 hours at 37°C in a 5% CO_2 atmosphere. The cells were then washed 3 times, and 2×10^5 ^{51}Cr-labeled neutrophils in 100 μL were added. In some experiments, neutrophils were preincubated with one of the LXA_4 analogs for 30 minutes or with dexamethasone (100 nmol/L) for 120 minutes with or without LXA_4 analogs for 30 minutes before the addition to HCAEC. In another set of experiments, LPS-activated HCAEC were incubated for 15 minutes with ENA-2 or MOPC-21 MoAb before the addition of neutrophils. Radiolabeled neutrophils were incubated with DREG-56, L130, or MOPC-21 MoAb for 15 minutes before the addition to HCAEC. After incubation of HCAEC with neutrophils for 30 minutes at 37°C on an orbital shaker at 90 rpm, loosely adherent or unattached neutrophils were washed 3 times and the endothelial monolayer plus the adherent neutrophils were lysed in 200 μL of 0.1% Triton X-100. The number of adhered neutrophils in each experiment was estimated from the radioactivity of a control sample. Treatment of HCAEC with any of the LXA_4 analogs did not affect the integrity of viable endothelial monolayers.

Expression of E-selectin and ICAM-1. After incubation for 4 hours at 37°C in a 5% CO_2 atmosphere with LPS (1 μg/mL) or TNF-α (2.5

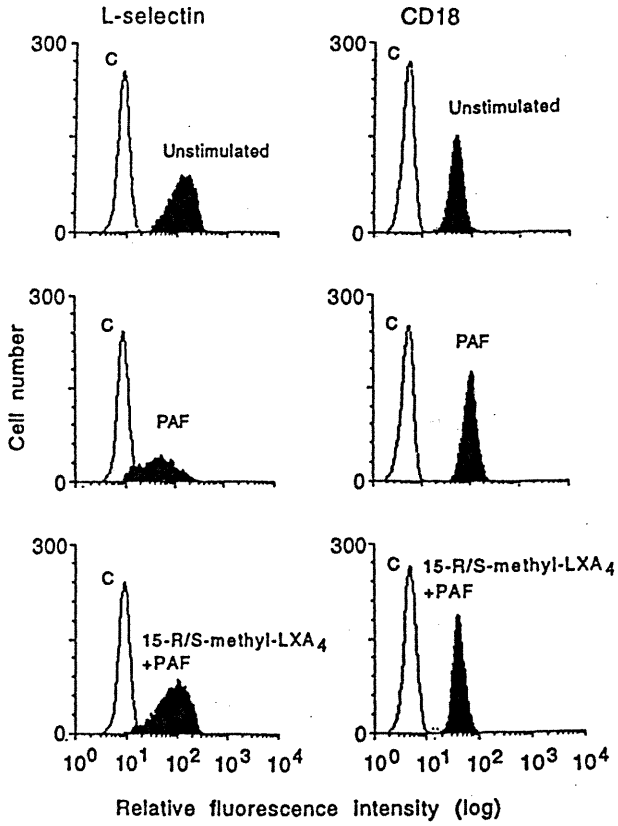

Fig 3. Aspirin-triggered LXA_4 analog (15-R/S-methyl-LXA_4) inhibits PAF-induced changes in cell surface expression of L-selectin and CD18 by human neutrophils. Whole blood was incubated in medium only (unstimulated) or with 15-R/S-methyl-LXA_4 (5 μmol/L) for 10 minutes and then with PAF (1 μmol/L) for 30 minutes at 37°C. In each histogram is also displayed the negative control of immunostaining with class-matched irrelevant antibodies (C). Shown is a representative experiment of 5 experiments.

ng/mL) in the absence or presence of various concentrations of 15-R/S-methyl-LXA_4, HCAEC were removed from the 24-well microplates by exposure to EDTA (0.01%) in phosphate-buffered saline (PBS) for 10 minutes at 37°C, followed by gentle trituration. Cells were resuspended in ice-cold saline containing sodium azide (0.02%), incubated with saturating concentration of fluorescein dye-conjugated anti–E-selectin or anti–ICAM-1 MoAb for 30 minutes at 4°C, washed, and fixed in formaldehyde (3.9% in PBS). Nonspecific binding was evaluated by using appropriately labeled mouse IgG_1. Immunofluorescence of HCAEC was then analyzed with a FACScan.

Data analysis. Results are expressed as the means ± SEM. Statistical comparisons were made by ANOVA using ranks (Kruskal-Wallis test), followed by Dunn's multiple contrast hypothesis test to identify differences between various treatments, or by the Wilcoxon signed rank test for paired observations. *P* values less than .05 were considered significant for all tests.

RESULTS

LXA_4 analogs in whole blood modulate expression of L-selectin and CD11/CD18 on resting and immunostimulated leukocytes. Incubation of heparinized whole blood with either 15-R/S-methyl-LXA_4 or 16-phenoxy-LXA_4 resulted in higher L-selectin expression on treated than control (untreated) cells and gave concentration-dependent downregulation of CD18 expression on PMNL. Figure 1 reports representative results illustrating the impact of 15-R/S-methyl-LXA_4 added to whole blood. Incubation of blood samples for 30 minutes at 37°C with 5 μmol/L of 15-R/S-methyl-LXA_4 or 16-phenoxy-LXA_4 resulted in 31% ± 8% and 32% ± 8% higher levels of expression for L-selectin than on cells from untreated whole blood and gave 26% ± 5% and 27% ± 5% decreases in CD18 expression, respectively (Fig 2). Similar changes were observed with monocytes and lymphocytes (Fig 2). Neither 15-deoxy-LXA_4 (Fig 2) nor structurally degraded (by thermal degradation) 15-R/S-methyl-LXA_4 or 16-phenoxy-LXA_4 affected expression of adhesion molecules (data not shown). When blood samples were incubated at 4°C, none of the LXA_4 analogs studied affected L-selectin expression. For instance, mean fluorescence intensity for L-selectin on PMNL was 129 ± 8 and 120 ± 11 ($n = 3$, $P > .5$) in control samples and in the presence of 15-R/S-methyl-LXA_4, respectively.

The addition of PAF (1 μmol/L) to whole blood gave a significant decrease in L-selectin and a marked upregulation of CD11/CD18 on leukocytes (Figs 3 and 4). Figure 3 shows a representative experiment on the effect of 15-R/S-methyl-LXA_4 on PAF-induced changes in the expression of these adhesion molecules. Preincubation of blood with either 15-R/S-methyl-LXA_4 or 16-phenoxy-LXA_4 attenuated PAF-induced upregulation of CD18 expression on PMNL, monocytes, and lymphocytes in a concentration-dependent fashion, with IC_{50} values of approximately 1.5 μmol/L (Fig 4). Essentially complete inhibition was achieved with 5 μmol/L of the LXA_4 analogs. PAF-induced decreases in L-selectin expression on PMNL and monocytes were also markedly, although not completely, inhibited by these LXA_4 analogs (Fig 4). Incubation of blood with IL-8 (10 nmol/L) downregulated L-selectin and upregulated CD18 expression on PMNL (Fig 5). As with PAF, both 15-R/S-methyl-LXA_4 and 16-phenoxy-LXA_4 at 5 μmol/L completely inhibited IL-8–induced upregulation of CD18, whereas they partially inhibited changes in L-selectin expression (Fig 5).

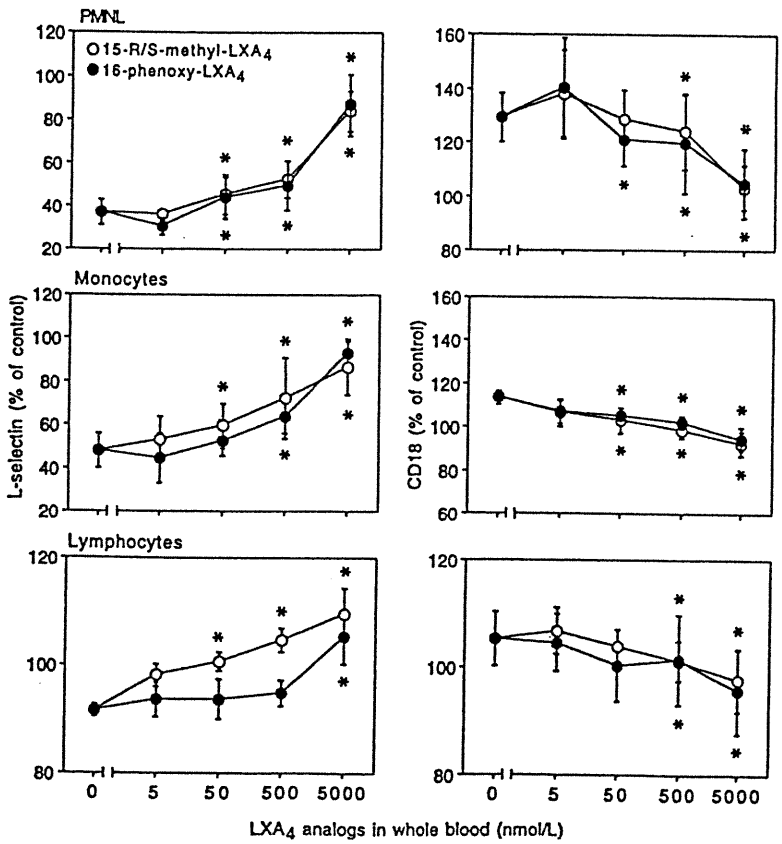

Fig 4. Inhibition of PAF-induced changes in L-selectin and CD18 expression on leukocytes. Whole blood aliquots were incubated with LXA_4 analogs for 30 minutes and then challenged with 1 μmol/L PAF for 30 minutes at 37°C. Adhesion molecule expression is presented as the percentage of control (unchallenged cells). Mean fluorescence intensity for L-selectin: PMNL, control, 103 ± 9; PAF, 43 ± 5; monocytes, control, 30 ± 3; PAF, 20 ± 2; lymphocytes, control, 53 ± 3; PAF, 50 ± 2, n = 5, all $P < .05$. Mean fluorescence intensity for CD18: PMNL, control, 48 ± 3; PAF, 61 ± 6; $P < .05$; monocytes, control, 107 ± 12; PAF, 123 ± 13; $P < .05$; lymphocytes, control, 19 ± 1; PAF, 21 ± 1. The results are the mean ± SEM of 4 to 5 experiments with different donor cell preparations. *$P < .05$ *v* PAF-stimulated cells.

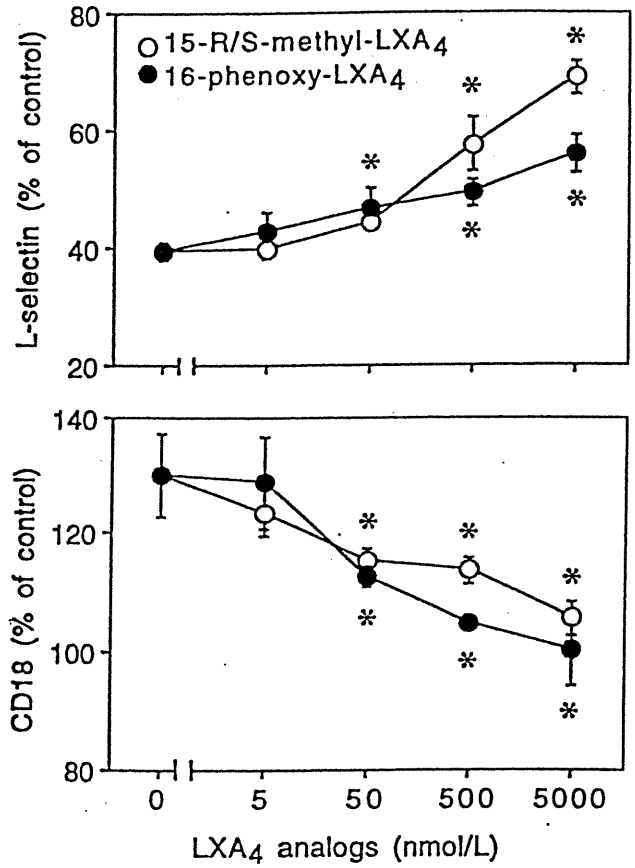

Fig 5. Inhibition of IL-8–induced changes in L-selectin and CD18 expression on human PMNL. Whole blood was preincubated with LXA_4 analogs for 30 minutes and then challenged with 10 nmol/L IL-8 for 30 minutes at 37°C. Adhesion molecule expression is presented as the percentage of control (unchallenged cells). Mean fluorescence intensity for L-selectin: control, 115 ± 8; IL-8, 61 ± 5; CD18: control, 45 ± 4; IL-8, 59 ± 9; n = 3; both $P < .05$. Values are the means ± SEM of 3 experiments with different donor cell preparations. *$P < .05$ *v* IL-8–stimulated cells.

Immunostaining of leukocytes with an anti-CD11b MoAb showed similar changes as those observed with the anti-CD18 MoAb (data not shown).

We also investigated whether LXA_4 analogs are able to modify the affinity of β_2 integrins. Formation of a higher affinity form of LFA-1 and Mac-1 is thought to involve conformational changes and association of the I domain with the β-propeller domain.[21,22] The MoAb G-25.2 recognizes this latter domain in LFA-1.[22] Incubation of whole blood with PAF or IL-8 in the absence or presence of 15-R/S-methyl-LXA_4 was not associated with a detectable increase in expression of MoAb G-25.2 (Fig 6A and B). On the other hand, activation of cells with Mg^{2+} and EGTA, which is known to induce the formation of a higher affinity form of LFA-1 without inducing clustering,[19] resulted in increases in MoAb G-25.2 expression (Fig 6C) that was not affected by 15-R/S-methyl-LXA_4 (Fig 6C and D) or 16-phenoxy-LXA_4 (data not shown).

Next, we examined whether LXA_4 analogs when added to whole blood could also affect the cell activation-independent downregulation of L-selectin expression. Incubation of blood with CRP peptide 201-206 (100 μg/mL) resulted in, on average, 49%, 42%, and 17% decreases in L-selectin expression on PMNL, monocytes, and lymphocytes, respectively (Fig 7). These changes were inhibited by 15-R/S-methyl-LXA_4 in a concentration-dependent manner, with apparent IC_{50} values of approximately 250 nmol/L (Fig 6). Similar inhibiton was observed with 16-phenoxy-LXA_4 (Fig 7).

Inhibition of MAPK kinase reverses LXA_4-induced changes in CD11/CD18 expression on neutrophils. Treatment of neutrophils with the MAPK kinase inhibitor PD98059 increased by approximately 27% L-selectin expression without altering CD11/CD18 expression (Fig 8). PD98059 prevented 15-R/S-methyl-LXA_4–induced downregulation of CD11/CD18 expression in a concentration-dependent fashion, whereas changes in L-selectin expression were not affected (Fig 8). Wortmannin at 0.2 μmol/L had no effect, whereas at 2 μmol/L, it prevented 15-R/S-methyl-LXA_4–induced downregulation of CD18 (Fig 8). Furthermore, wortmannin (2 μmol/L) alone downregulated L-selectin expression and inhibited the action of 15-R/S-methyl-LXA_4 (Fig 8). 15-R/S-methyl-LXA_4 induction was not affected by the tyrosine kinase inhibitor genistein (Fig 8) or herbimycin-A (data not shown).

LXA_4 analogs do not affect E-selectin or ICAM-1 expression on LPS- or TNF-α–stimulated endothelial cells. After stimulation by LPS, HCAEC increased 27- and 2.6-fold the expression of E-selectin and ICAM-1, respectively (n = 4, both $P <$ .05; Table 1). 15-R/S-methyl-LXA_4 did not affect basal expression of these adhesion molecules (data not shown) and produced only a slight inhibition of LPS-induced changes (Table 1). The maximum inhibition did not exceed 10%. Similarly, 15-R/S-methyl-LXA_4 failed to significantly inhibit TNF-α–induced E-selectin and ICAM-1 expression (Table 1).

LXA_4 analogs inhibit neutrophil adhesion to endothelial cells. Activation of HCAEC with LPS resulted in a 4.1-fold increase in the number of adherent neutrophils (Fig 9). When HCAEC were challenged with LPS in the presence of one of the stable LXA_4 analogs, only slight decreases in adhesion could be detected (Fig 9A). By contrast, addition of neutrophils preincubated with either 15-R/S-methyl-LXA_4 or 16-phenoxy-LXA_4 to LPS-activated HCAEC attenuated neutrophil attachment in a concentration-dependent fashion (Fig 9A). Significant inhibition of adhesion was detected with 15-R/S-methyl-LXA_4 at a concentration as low as 50 nmol/L. At 5 μmol/L, 15-R/S-methyl-LXA4 and 16-phenoxy-LXA_4 inhibited neutrophil adhesion by 38% ± 4% and 36% ± 3%, respectively (n = 5, both $P < .05$; Fig 9A). Neither 15-deoxy-LXA_4 nor decomposed 15-R/S-methyl-LXA_4 or 16-phenoxy-LXA_4 affected neutrophil adhesion (data not shown).

Because multiple receptors are involved in neutrophil adhesion to LPS-stimulated HCAEC[18] and LXA_4 analogs affected expression of both L-selectin and CD11/CD18 on PMNL, we assayed the contribution of L-selectin, E-selectin, and CD18 to the binding interaction. A significant proportion of neutrophil-HCAEC attachment was blocked by MoAbs binding to L-selectin (44% ± 4%, n = 5), CD18 (31% ± 4%), or E-selectin (38% ± 5%; Fig 9B). The combination of these MoAbs inhibited neutrophil adhesion by 87% to 98%. Treatment of neutrophils with 15-R/S-methyl-LXA_4 and anti-CD18 MoAb resulted in only a slightly greater inhibition of adhesion than

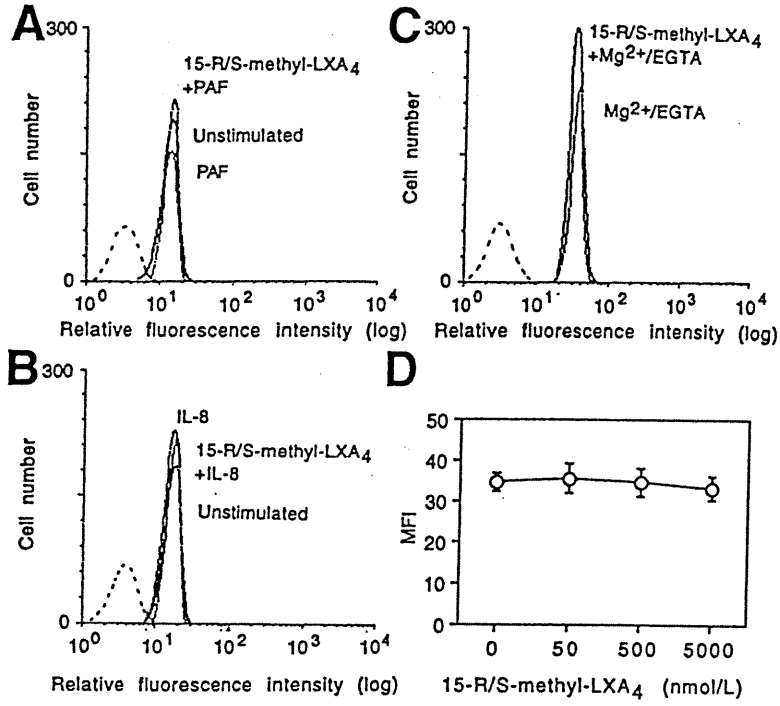

Fig 6. Effect of 15-R/S-methyl-LXA_4 on the expression of LFA-1 epitope expressed by the β-propeller domain of LFA-1 on neutrophils. Mean fluorescence of MoAb G-25.2 on PMNL in whole blood challenged with PAF (1 μmol/L; A) or IL-8 (10 nmol/L; B) for 30 minutes at 37°C in the presence of 15-R/S-methyl-LXA_4 (5 μmol/L). The dotted line represents MoAb G-25.2 binding at 4°C. Shown is a representative of 3 experiments. (C) The expression of MoAb G-25.2 on PMNL in whole blood challenged with Mg^{2+} (1 mmol/L) and EGTA (2 mmol/L) for 30 minutes at 4°C (dotted line) and at 37°C in the absence or presence of 15-R/S-methyl-LXA_4 (5 μmol/L). Shown is a representative of 3 experiments. (D) Mean fluorescence of MoAb G-25.2 on neutrophils challenged with Mg^{2+} and EGTA in the presence of 15-R/S-methyl-LXA_4. Values are the means ± SEM of 3 experiments.

those observed with neutrophils treated with either 15-R/S-methyl-LXA_4 or anti-CD18 MoAb (Fig 9B). The combination of 15-R/S-methyl-LXA_4 with either anti–L-selectin MoAb or anti–E-selectin MoAb resulted in additive inhibition, and the degree of inhibition was similar to those observed when anti–L-selectin MoAb or anti–E-selectin MoAb was combined with anti-CD18 MoAb, respectively (Fig 9B). Combining 15-R/S-methyl-LXA_4, anti–L-selectin MoAb, and anti–E-selectin MoAb blocked approximately 80% of adhesion. Similar results were obtained with 16-phenoxy-LXA_4 (data not shown).

Additive inhibition by LXA_4 analogs and dexamethasone of PAF-induced changes in neutrophil adhesion molecule expression and binding to endothelial cells. Dexamethasone was used at a concentration of 100 nmol/L, because our previous results showed that the maximum inhibitory effect of dexamethasone on neutrophil adhesion molecule expression can be achieved with this concentration.[20] As expected, dexamethasone added alone attenuated by 35% to 45% PAF-induced changes in L-selectin and CD11/CD18 expression on PMNL (Fig 10A). Combining dexamethasone with 15-R/S-methyl-LXA_4 resulted in an almost complete inhibition (Fig 10A). Similar inhibition was observed with both monocytes and lymphocytes (data not shown). Culture of HCAEC with dexamethasone diminished the number of adherent neutrophils (Fig 10B). However, no further decrements in neutrophil adhesion were detected in the presence of LXA_4 analogs. On the other hand, the inhibitory actions of dexamethasone and LXA_4 analogs were additive when neutrophils were preincubated with dexamethasone and LXA_4 analogs before exposure to LPS-activated HCAEC (Fig 10B).

DISCUSSION

In this report, we describe a novel mechanism(s) by which stable LXA_4 analogs can affect the inflammatory response, namely via the modulation of surface expression of adhesion molecules on resting and immunostimulated leukocytes, and inhibition of neutrophil adhesion to the activated endothelium. This inhibition is predominantly mediated via actions of LXA_4 analogs on leukocytes and is distinct from those of glucocorticoids. Moreover and of particular interest, these actions of stable LXA_4 analogs were monitored for the first time within the whole blood environment, where they clearly remain bioactive.

Because dehydrogenation of native lipoxins by human leukocytes results in their rapid inactivation,[23] we used LXA_4 stable analogs that carry substituents at the carbon 15 through ω 20 end of the native LXA_4 structure, resist conversion,[7,24] and retain the biological activities of native LXA_4 in leukocyte adhesion and migration assays.[5,24] 15-R/S-methyl-LXA_4 is an analog of aspirin-triggered 15-epi-LXA_4 and 16-phenoxy-LXA_4 is an analog of native LXA_4.[7,13] The effects of 15-R/S-methyl-LXA_4 and 16-phenoxy-LXA_4 observed in this study are attributed to the molecules themselves, because neither degraded LXA_4 analogs nor 15-deoxy-LXA_4 caused detectable changes in the assays used.

Our study documents the complex nature of actions of stable LXA_4 analogs on expression of leukocyte adhesion molecules. These analogs upregulated L-selectin and downregulated CD11/CD18 expression on human resting neutrophils, monocytes, and, to a lesser extent, lymphocytes in whole blood and markedly attenuated changes evoked by immunostimulation. These results are consistent with earlier reports, which showed that native LXA_4 inhibits formyl-Met-Leu-Phe–stimulated upregulation of CD11b/CD18 on human isolated neutrophils.[25] The present results indicated that this action is also demonstrable within whole blood with nanomolar to micromolar concentrations of LXA_4 analogs. As expected, higher concentrations were required in whole blood to inhibit the upregulation of CD18 expression than in isolated cells. This might reflect interactions of LXA_4 analogs with serum components such as

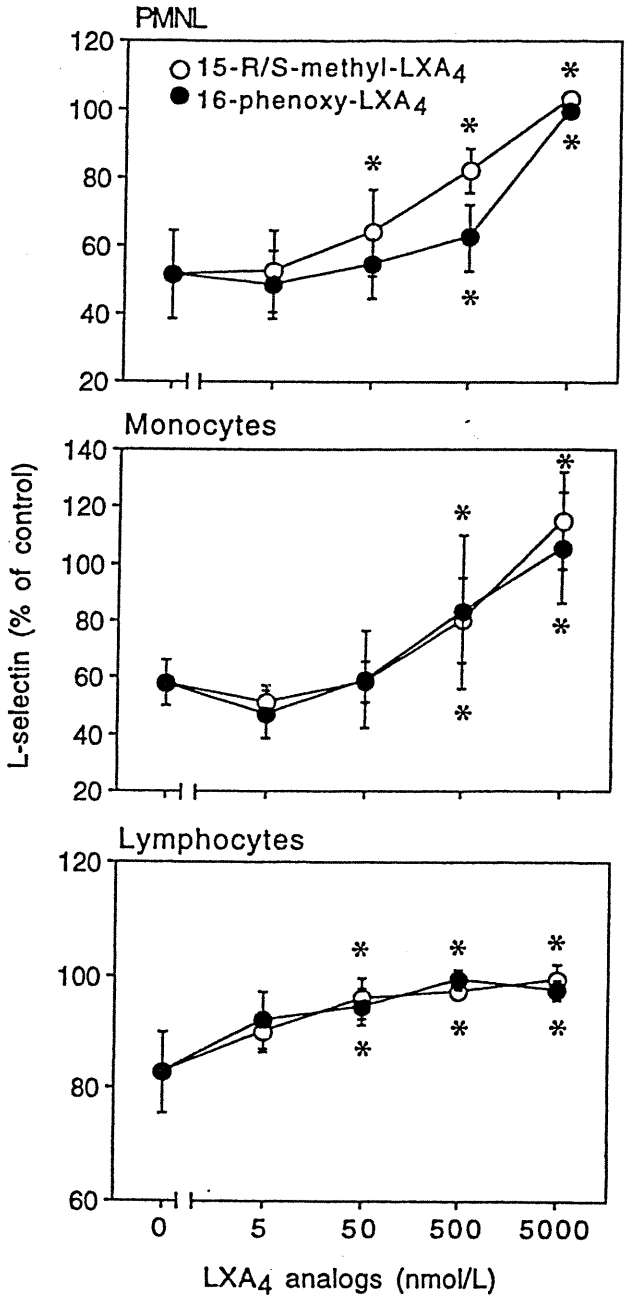

Fig 7. LXA_4 analogs prevent cell activation-independent downregulation of L-selectin expression on human PMNL, monocytes, and lymphocytes. Whole blood aliquots were incubated with LXA_4 analogs for 30 minutes at 37°C and then challenged with 100 μg/mL CRP peptide 201-206. Results are presented as the percentage of control (unchallenged cells). Mean fluorescence intensity for L-selectin: PMNL, control, 97 ± 6; peptide 201-206, 50 ± 14; monocytes, control, 35 ± 4; peptide 201-206, 22 ± 7; lymphocytes, control, 61 ± 4; peptide 201-206, 51 ± 4; n = 3; all $P < .05$. Values represent the mean ± SEM of 3 independent experiments. *$P < .05$ *v* peptide 201-206–stimulated cells.

albumin. Nevertheless, it is impressive that these lipophilic compounds are active in the microenvironment of whole blood and overcome interactions with blood components to specifically regulate leukocytes. Downregulation of CD11/CD18

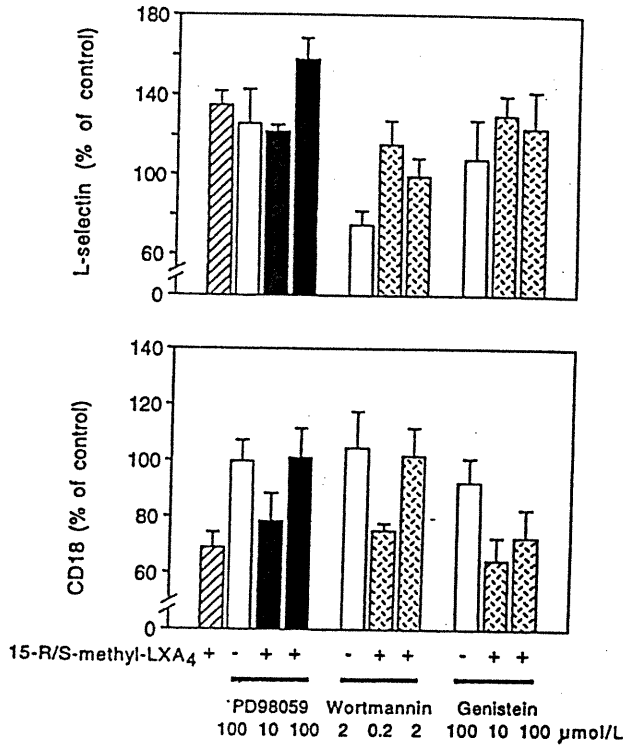

Fig 8. Effects of MAPK kinase (MEK) and tyrosine kinase inhibitors on neutrophil adhesion molecules. Isolated neutrophils (10^7 cells/mL) were preincubated with PD98059, wortmannin, or genistein for 30 minutes at 37°C and then challenged with 5 μmol/L 15-R/S-methyl-LXA_4 for 30 minutes. Adhesion molecule expression is presented as the percentage of control. Mean fluorescence intensity for control samples, L-selectin: 35 ± 1; CD18: 82 ± 14; n = 4. Values are the mean ± SEM of 4 independent experiments.

expression on resting neutrophils by LXA_4 analogs does not involve phosphatidylinositol 3-kinase or tyrosine kinases; rather, it appears to be mediated via activation of MAPK kinase, as suggested by the experiments with PD98059 and wortmannin, which, at a concentration of 2 μmol/L, also inhibits MAPK kinase.[26] The LXA_4 analogs did not initiate detectable changes in tyrosin phosphorylation of either Erk-2 or p38 MAPK in

Table 1. 15-R/S-methyl-LXA_4 Does Not Alter E-Selectin and ICAM-1 Expression on Human Coronary Artery Endothelial Cells

	15-R/S-methyl-LXA_4	E-Selectin (RFI)	ICAM-1 (RFI)
Control	0	2 ± 1	140 ± 31
LPS	0	50 ± 5	327 ± 41
LPS	5 nmol/L	51 ± 5	324 ± 31
LPS	50 nmol/L	49 ± 4	305 ± 21
LPS	500 nmol/L	45 ± 4	296 ± 27
LPS	5,000 nmol/L	45 ± 2	302 ± 21
TNF-α	0	24 ± 3	386 ± 27
TNF-α	500 nmol/L	19 ± 2	335 ± 32
TNF-α	5,000 nmol/L	20 ± 1	357 ± 12

Confluent HCAEC monolayers were left unstimulated (control) or challenged with 1 μg/mL LPS or 2.5 ng/mL TNF-α for 4 hours at 37°C in the presence of 15-R/S-methyl-LXA_4. Values are the mean ± SEM of 3 independent experiments.

Abbreviation: RFI, relative fluorescence intensity.

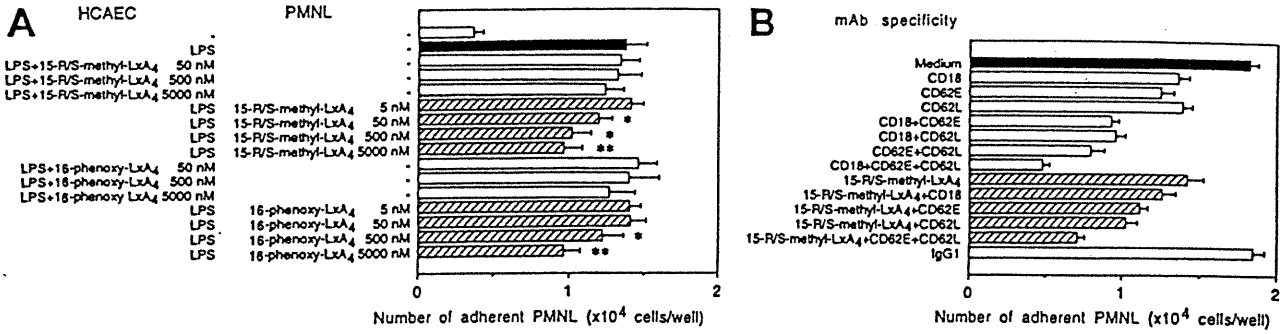

Fig 9. Inhibition of neutrophil binding to endothelial cells by LXA_4 analogs (A) or by anti–E-selectin, anti–L-selectin, and anti-CD18 MoAbs and 15-R/S-methyl-LXA_4 (B). Confluent HCAEC monolayers were cultured with 1 μg/mL LPS with or without LXA_4 analogs for 6 hours at 37°C. (A) Neutrophils were preincubated for 30 minutes with LXA_4 analogs or medium as indicated before addition to activated HCAEC. Values are the means ± SEM of 3 to 6 experiments using neutrophils from different donors. *$P < .05$ *v* LPS-treated HCAEC. (B) Neutrophils were treated with 15-R/S-methyl-LXA_4 (5 μmol/L) or the indicated MoAbs before and during the assay. Neutrophil adhesion to unstimulated HCAEC was 0.41 ± 0.03 × 10^4 cells per well. The irrelevant MoAb MOPC-21 (IgG_1) was used as a negative control. The results represent the mean ± SEM of 5 experiments using neutrophils from different donors.

resting human neutrophils as evaluated by immunoblotting using specific antibodies to the phosphorylated forms of these enzymes (Hachicha et al, unpublished observations). Recent results suggest that intracellular arachidonic acid induces integrin-dependent homotypic adhesion of neutrophils via the Raf-1/MAPK kinase/Erk pathway.[27] With respect of LXA_4 receptor activation, it is possible that other Erk or p38 MAPK-independent pathways may be involved in signaling neutrophil adhesion molecule expression.

Physiological stimuli regulate adhesion by either altering the affinity of the individual integrin molecule or by inducing clustering of β_2 integrins, thereby increasing the overall strength of binding.[19,28,29] Increasing intracellular Ca^{2+} concentration does not induce detectable changes in the affinity of LFA-1.[22] Because leukocyte activation evoked by PAF or IL-8 is mediated through increases in intracellular Ca^{2+} concentration, these agonists would not increase integrin affinity. Indeed, neither PAF nor IL-8 affected MoAb G-25.2 expression. Activation of leukocytes with Mg^{2+} and EGTA, which results in the formation of a higher affinity form of LFA-1,[19] increased the expression of MoAb G-25.2, which did not appear to be affected by the LXA_4 analogs studied. These results suggest that LXA_4 analogs do not alter the affinity of either LFA-1 and probably of Mac-1, but rather they interfere with activation-induced clustering of β_2 integrins. However, our results do not preclude the possibility that, in the presence of integrin ligands, LXA_4 analogs might affect a ligand-induced affinity increase secondary to integrin clustering.

Comparison of L-selectin expression at 4°C at 37°C indicated that, within whole blood, these LXA_4 analogs prevented temperature-induced L-selectin shedding on unstimulated leukocytes. For minutes of activation by either PAF or IL-8 or challenge with CRP peptide 201-206, which does not activate cells, leukocytes release L-selectin from their surface by a proteolytic enzyme. Because this enzyme appears to be constitutively active, formation of an appropriate 3-dimensional structure of L-selectin near the membrane is thought to regulate this proteolytic process.[30,31] Whereas it remains to be established whether the conformational changes induced by cell activation dependent or independent stimuli are identical, our results demonstrate that these events are effectively blocked by LXA_4 analogs. A recent study has reported that the intracellular tail of L-selectin is phosphorylated on serine upon activation with chemoattractants.[32] It is of interest that calmodulin inhibitors directly induce proteolytic shedding of L-selectin.[33] Whether this action of LXA_4 involves calmodulin or interference with phosphorylation step(s) remains a key area of inquiry.

Despite inhibition of L-selectin shedding from neutrophils within whole blood, which is thought to promote neutrophil-HCAEC attachment, LXA_4 analogs actually inhibited isolated neutrophil adhesion to HCAEC. This inhibition can primarily be attributed to their actions on neutrophils rather than on HCAEC in this interaction, because only slight decreases in the number of adherent neutrophils were observed after culture of HCAEC with LPS in the presence of LXA_4 analogs. Consistently, LXA_4 analogs have little effect on LPS- or TNF-α–stimulated expression of E-selectin and ICAM-1 on HCAEC. Our results indicate that inhibition of neutrophil-HCAEC adherence by LXA_4 analogs is predominantly attributable to inhibition of CD11/CD18 expression on neutrophils. The LXA_4 analogs or a function-blocking anti-CD18 MoAb resulted in similar decreases in neutrophil adhesion to HCAEC. The action of LXA_4 analogs and anti-CD18 MoAb were not additive, whereas the inhibition with LXA_4 analogs was additive with anti–E-selectin and anti–L-selectin MoAbs. These results suggest that LXA_4 analogs had little, if any, effect on E-selectin or L-selectin function and did not interfere with E-selectin ligand. Taken together, our data indicate that, in addition to inhibition of P-selectin expression on rat intestinal venular endothelial cells,[8] lipoxins are potent regulators of both leukocyte L-selectin and CD11/CD18 expression. Whereas inhibition of P-selectin–dependent capture of neutrophils may be a key mechanism by which LXA_4 analogs inhibit neutrophil-endothelial interactions in the mesenteric circulation,[8] functions of adhesion molecules may overlap, and even neutrophil recruitment into other vascular beds may not rely on a P-selectin–dependent adhesion.[34]

The present study and previous studies[20,35] indicate that the mechanisms of action of LXA_4 analogs differ from those of glucocorticoids. Adhesion molecule expression on resting neu-

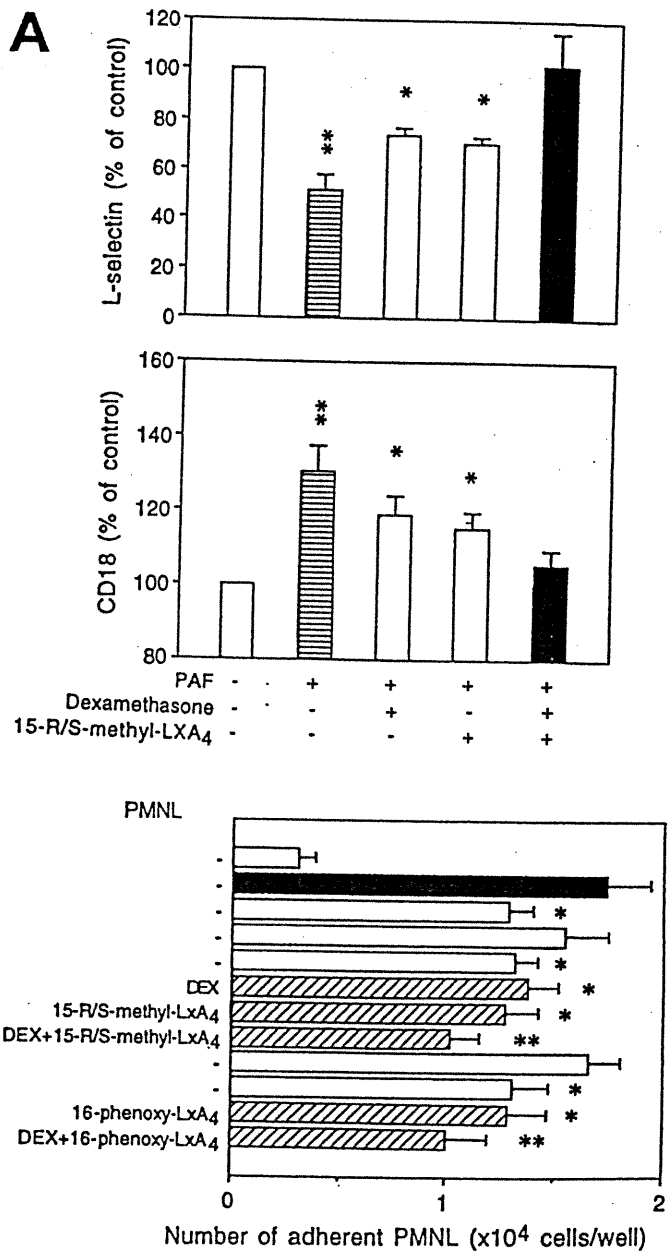

Fig 10. Cooperative actions of dexamethasone and LXA_4 analogs. (A) Human whole blood aliquots were incubated with 100 nmol/L dexamethasone for 120 minutes in the presence of 15-R/S-methyl-LXA_4 (5 μmol/L) for the last 30 minutes of incubation, as indicated, and then challenged with 1 μmol/L PAF for 30 minutes at 37°C. Adhesion molecule expression is presented as the percentage of control. Mean fluorescence intensity for L-selectin: control, 37 ± 3; PAF, 26 ± 4; CD18: control, 61 ± 4; PAF, 73 ± 7; n = 5; both $P < .05$. $^*P < .05$; $^{**}P < .01$ *v* control (untreated). L-selectin and CD18 expression on dexamethasone plus 15-R/S-methyl-LXA_4–treated cells did not differ significantly from those of control. (B) Confluent HCAEC monolayers were cultured for 6 hours with LPS (1 μg/mL) in the presence of dexamethasone (DEX; 100 nmol/L), 15-R/S-methyl-LXA_4, or 16-phenoxy-LXA_4 (5 μmol/L). Neutrophils pretreated with dexamethasone and/or LXA_4 analogs as described for whole blood were added to activated HCAEC and then challenged with PAF (1 μmol/L). The results are expressed as the mean ± SEM of 5 experiments using neutrophils from different donors. $^*P < .05$ *v* LPS-treated.

trophils can be modulated by LXA_4 analogs, but not by dexamethasone.[20] Both dexamethasone and LXA_4 analogs alone inhibited by approximately 20% to 45% PAF-induced changes in L-selectin and CD11/CD18 expression on neutrophils, representing the near maximum inhibition that can be achieved with these compounds (Filep et al[20] and the present study). Interestingly, the inhibitory actions of dexamethasone and LXA_4 analogs were additive, resulting in almost complete inhibition of PAF-stimulated changes. The inhibitory actions of dexamethasone on PMNL requires de novo protein synthesis.[20] Preincubation of neutrophils with either dexamethasone or LXA_4 analogs before addition to LPS-activated HCAEC resulted in partial inhibition of adhesion, and the inhibitory actions of dexamethasone and LXA_4 analogs were additive. Culture of HCAEC with LPS and dexamethasone, but not with LXA_4 analogs, resulted in marked decreases in the number of adherent neutrophils. These findings are consistent with the glucocorticoid inhibition of LPS-induced expression of ICAM-1 and E-selectin on endothelial cells.[35] By contrast, LXA_4 analogs had little, if any, effect on expression of these adhesion molecules. Interestingly, the mechanism by which LXA_4 analogs inhibit neutrophil-endothelial adhesion also differs from that of several nonsteroidal anti-inflammatory drugs, which induce shedding of L-selectin from neutrophils and inhibit L-selectin–mediated attachment.[36] The actions of LXA_4 analogs on β_2 integrins appear to be similar to those reported for piroxicam,[37] tepoxalin, a dual cyclooxygenase/lipoxygenase inhibitor,[38] and the anti-inflammatory agent leumedin NPC 15669.[39] Piroxicam prevents expression of a CD11b activation neo-epitope,[37] whereas tepoxalin and leumedin NPC 15669

inhibit upregulation of Mac-1.[38,39] However, unlike the LXA_4 analogs, none of these agents affected β_2 integrin expression on resting leukocytes.

In conclusion, this study demonstrates that stable LXA_4 analogs modulate adhesion molecule expression on leukocytes monitored within the microenvironment of human whole blood and inhibit neutrophil adhesion to HCAEC via downregulation of CD11/CD18 expression. These effects are distinct from those of either glucocorticoids or nonsteroidal anti-inflammatory drugs. Therefore, stable analogs of native LXA_4 and aspirin-triggered 15-epi-LXA_4 may represent a novel therapeutic approach for selective regulation of leukocyte trafficking in host defense, inflammation, and reperfusion injury.

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CHAPITRE IV (Article IV)

Zouki C, Ouellet S, Filep JG. The anti-inflammatory peptides, antiflammins, regulate adhesion molecules on human leukocytes and prevent neutrophil adhesion to coronary artery endothelial cells. *FASEB J.* 1999, accepté pour publication.

Introduction au chapitre IV

Il est bien connu que les glucocorticoïdes inhibent l'accumulation des leucocytes dans les tissus (221). Certains des effets des glucocorticoïdes sont médiés par une protéine inductible nommée la lipocortine-1 (229,230). Des études récentes ont démontré que certains fragments de la lipocortine 1 peuvent mimer les actions de la protéine native (243).

Nous avons étudié l'effet des antiflammines (AF), des nonapeptides synthétiques correspondant à une région de haute similarité de séquence entre la lipocortine-1 et l'utéroglobine (244), une autre protéine induite par les stéroides, sur l'expression des molécules d'adhésion à la surface des leucocytes et des cellules endothéliales d'artères coronariennes. Tout comme la lipocortine-1, les antiflammines diminuent les changements d'expression de la L-sélectine et de CD18 à la surface de leucocytes stimulés avec du PAF ou de l'IL-8, bien qu'ils n'aient aucun effet sur l'expression de la E-sélectine et d'ICAM-1 à la surface des cellules endothéliales. De plus les AFs inhibent l'adhésion des neutrophiles aux cellules endothéliales en inhibant l'adhésion CD18-dépendante.

Ceci suggère donc que les antiflammines pourraient représenter une nouvelle approche thérapeutique en bloquant le trafic des leucocytes dans l'inflammation qui accompagne différentes maladies, et pourraient alors prévenir le dommage tissulaire causé par les neutrophiles.

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The Anti-Inflammatory Peptides, Antiflammins, Regulate the Expression of Adhesion Molecules on Human Leukocytes and Prevent Neutrophil Adhesion to Endothelial Cells

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Running title: Antiflammins Prevent Neutrophil Adhesion

Abbreviations used in this paper: HCAEC, human coronary artery endothelial cells; IL-8, interleukin-8; LPS, lipopolysaccharide; mAb, monoclonal antibody; PAF, platelet-activating factor; PMNs, neutrophil granolocytes

Abstract

Antiflammin-1 and antiflammin-2 are nonapeptides corresponding to the region of highest similarity between glucocorticoid-inducible proteins lipocortin-1 and uteroglobin. We have studied whether antiflammins could affect expression of adhesion molecules on human leukocytes and coronary artery endothelial cells (HCAEC) and binding of neutrophils (PMNs) to HCAEC. While neither antiflammin-1 nor antiflammin-2 affected expression of adhesion molecules on resting PMNs, monocytes and lymphocytes in whole blood, they attenuated changes in L-selectin and CD11/CD18 expression evoked by platelet-activating factor (PAF) or interleukin-8 (IL-8) with IC_{50} values of 4-20 μmol/l. The maximum inhibition was similar to those seen with human recombinant lipocortin-1 (100 μg/ml). Unlike dexamethasone (100 nmol/l), the antiflammins had little effect on LPS-stimulated expression of E-selectin and ICAM-1 on HCAEC. Consistently, culture of HCAEC with dexamethasone, but not with antiflammins, decreased PMN binding to endothelial cells. Preincubation of PMNs with antiflammins markedly decreased their adhesion to LPS-activated HCAEC. Inhibition of adhesion was additive with function blocking anti-E-selectin and anti-L-selectin antibodies, but was not additive with anti-CD18 antibody. These results show that antiflammins inhibit PMN adhesion to HCAEC by attenuating activation-induced upregulation of CD11/CD18 expression on leukocytes, and suggests that antiflammins may represent a novel therapeutic approach in blocking leukocyte trafficking in host defense and inflammation.

Key words: lipocortin-1-derived peptides, L-selectin, integrin, neutrophil recruitment, inflammation

Introduction

Glucocorticoids are powerful inhibitors of leukocyte trafficking in inflammation. Multiple mechanisms have been proposed to account for this action, including inhibition of adhesion molecule expression on human endothelial cells (1) and neutrophil granulocytes (PMNs) (2). Glucocorticoid injection in humans induce synthesis and surface expression of lipocortin-1 on PMNs and monocytic cells (3,4). Lipocortin-1 has been suggested to act as the "second messenger" for the glucocorticoid inhibition of leukocyte migration (5). Recent evidence indicates that several regions of the lipocortin-1 molecule may be associated with biological activity.

Antiflammins, discovered as inhibitors of phospholipase A_2, are synthetic nonapeptides corresponding to a region of high amino-acid sequence similarity between lipocortin-1 and uteroglobin (6). Antiflammin-1 (MQMKKVLDS) is equivalent to the C-terminal part of α-helix three in uteroglobin (7), whereas antiflammin-2 (HDMNKVLDL) corresponds to residues 246-254 of lipocortin-1 (8). Antiflammins show potent anti-inflammatory effects. Although an apparent controversy exists whether antiflammins can or cannot affect phospholipase A_2 activity (6,9-11), in vitro, they inhibit synthesis of platelet-activating factor (PAF) (12), PMN and mononuclear cell chemotaxis and aggregation (12,13) and platelet aggregation (14). Antiflammins also reduce the increase in vascular permeability and leukocyte infiltration induced in rats by an Arthus reaction or by intradermal injection of C5a (12), and inhibit murine ear edema (15) and endotoxin-induced uveitis in rats (16). These studies raised the possibility that antiflammins might be potent regulators of leukocyte trafficking, but provided little insight into the underlying mechanisms.

Leukocyte extravasation into inflamed areas is a multistep process which is regulated by several adhesion molecules (17,18). The initial capture and tethering of circulating PMNs to endothelium is mediated by L-selectin (CD62L) constitutively expressed by most leukocytes (19,20) and by P- and E-selectins expressed by activated edothelium (17,18). L-selectin is rapidly shed after cell activation with a concomitant upregulation of Mac-1 (CD11b/CD18) (21). The β_2 integrins, Mac-1 and LFA-1 (CD11a/CD18), are largely responsible for subsequent tightening of the adhesion and transendothelial migration of PMNLs via interactions with their endothelial counterreceptors, ICAM-1 and ICAM-2 (17,18). PAF and interleukin-8 (IL-8) are thought to serve as signals for PMNs to promote activation of β_2 integrins leading to firm adhesion (22,23).

In the present experiments we studied the impact and the cellular mechanisms of action of antiflammins on expression of adhesion molecules on human leukocytes and human coronary artery endothelial cells (HCAEC) and on binding of PMNs to HCAEC.

MATERIALS AND METHODS

Antibodies and reagents

In these studies, the monoclonal antibodies (mAbs) used included FITC-conjugated mouse anti-human L-selectin mAb DREG-56 (PharMingen, San Diego, CA), R-phycoerythrin-conjugated mouse anti-human CD18 mAb MEM-48 (Monosan, Uden, The Netherlands), FITC-labeled mouse anti-human E-selectin mAb 1.2B6 (Serotec, Kidlington, England) and R-phycoerythrin-conjugated mouse anti-human ICAM-1 mAb HA58 (PharMingen). Appropriately labeled, class-matched irrelevant mouse IgG_1 was used as a negative control for each staining. The following murine mAbs were used in neutrophil-endothelial cell adhesion assays: anti-L-selectin mAb DREG-56 (IgG_1, PharMingen) at 20 μg/ml (24); anti-E-selectin mAb ENA-2 (IgG_1, purified $F(ab')_2$ fragments, Monosan) at 10 μg/ml (25), and anti-CD18 mAb L130 (IgG_1, Becton-Dickinson Immunocytometry Systems, Mountain View, CA) at 10 μg/ml (26). The irrelevant mAb MOPC-21 (IgG_1, PharMingen) at 20 μg/ml was used as a negative control.

Synthetic antiflammin-1 and antiflammin-2 were obtained from Bachem Bioscience (King of Prussia, PA). A scrambled sequence of antiflammin-2 (MLNHKLDVD, synthesized by Biosynthesis Inc., Lewisville, TX) and the unrelated synthetic peptide VPVEAVNPM corresponding to residues 24-32 of the rat cholecystokinin prepro-sequence (Bachem) were used as controls. Purity of the peptides, as analyzed by the manufacturer, was >98%, amino acid composition and molecular mass were confirmed by mass spectrometry. Lipopolysaccharide (*Escherichia coli* O111:B4) and dexamethasone 21-phosphate were obtained from Sigma Chemical Co. (St. Louis, MO), PAF was from Calbiochem (La Jolla, CA), human recombinant IL-8 was purchased from R&D Systems (Minneapolis, MN). Recombinant human lipocortin-1

(8) was a gift from Dr. R.B. Pepinsky (Biogen, Cambridge, MA).

Whole blood incubation

Venous blood (anticoagulated with sodium heparin 50 U/ml) was obtained from non-smoking healthy volunteers (male and female, 24-45 years of age) who had not taken any drugs for at least 10 days before the experiments. Informed consent was obtained from each volunteer, and the protocol was approved by the Clinical Research Committee. White blood cell counts were between 4,500 and 9,000 cells/μl. Whole blood aliquots were incubated with various concentrations of antiflammin-1, antiflammin-2 or lipocortin-1 for 30 min at 37°C, 95% air/5% CO_2 and then challenged with PAF (1 μmol/l), or IL-8 (10 nmol/l) for 30 min.

Analysis of surface antigen expression

Direct immunofluorescence labeling of resting and treated leukocytes in whole blood were performed as described (2,26). Leukocytes were stained with saturating concentration of fluorescence dye-conjugated anti-human L-selectin or anti-human CD18 mAb. Nonspecific binding was evaluated by using appropriately labeled mouse IgG_1. Double or single-color immunofluorescence staining was analyzed by a cytofluorometer (FACScan, Becton Dickinson) with Lysis II software. Antibody binding was determined as mean fluorescence intensity after gating for PMNs, monocytes and lymphocytes by their characteristic forward and side scatter properties.

7

Culture of endothelial cells

Normal human coronary artery endothelial cells (HCAEC) obtained from Clonetics Corp. (San Diego, CA) were cultured as described (26). HCAEC (passages 3 to 6) seeded into 24-well or 96-well microplates and grown to confluence were used in the experiments. Two days prior to the experiments, the cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum only.

Expression of E-selectin and ICAM-1

After incubation for 4 h at 37°C in a 5% CO_2 atmosphere with LPS (1 μg/ml) in the absence or presence of antiflammin-1 or antiflammin-2 (100 μmol/l) or dexamethasone (100 nmol/l), HCAEC were removed from the 24-well microplates by exposure to EDTA (0.01%) in PBS for 10 min at 37°C followed by gentle trituration. Cells were resuspended in ice-cold saline containing sodium azide (0.02%), incubated with saturating concentration of fluorescein dye-conjugated anti-E-selectin or anti-ICAM-1 mAb for 30 min at 4°C, washed, fixed in formaldehyde (3.7% in phosphate buffered saline), and immunofluorescence was analyzed with a cytofluorometer. Nonspecific binding was evaluated by using appropriately labeled class-matched, irrelevant mouse IgG_1.

Neutrophil-endothelial cell adhesion assay

The adhesion assay was performed as described previously (26). In brief, monolayers of HCAEC in 96-well microplates were stimulated with LPS (1 μg/ml) with or without antiflammin-1, antiflammin-2 (100 μmol/l) or dexamethasone (100 nmol/l) for 6h at 37°C in a

5% CO_2 atmosphere. The cells were then washed three times, and $2x10^5$ ^{51}Cr-labeled PMNs in 100 μl were added. In some experiments, PMNs were preincubated with antiflammin-1, antiflammin-2 for 30 min or with dexamethasone (100 nmol/l) for 120 min before addition to HCAEC. In another set of experiments, LPS-activated HCAEC were incubated for 15 min with ENA-2 or MOPC-21 mAb before addition of PMNs. Radiolabeled PMNs were incubated with DREG-56, L130 or MOPC-21 mAb for 15 min before addition to HCAEC. After incubation of HCAEC with PMNs for 30 min at 37°C on an orbital shaker at 90 rpm, losely adherent or unattached leukocytes were washed three times, and the endothelial monolayer plus the adherent PMNs were lysed in 200 μl of 0.1% Triton X-100. The number of adhered PMNs in each experiment was estimated from the radioactivity of a control sample. Treatment of HCAEC with antiflammins did not affect the integrity of viable endothelial monolayers.

Statistical analysis

Results are expressed as means$\pm$SEM. Statistical comparisons were made by ANOVA using ranks (Kruskal-Wallis test) followed by Dunn's multiple contrast hypothesis test to identify differences between various treatments or by the Mann-Whitney U test for unpaired observations. P values <0.05 were considered significant for all tests.

RESULTS

Antiflammins attenuate activation-induced changes in L-selectin and CD11/CD18 expression on leukocytes in whole blood

Neither antiflammin-1 nor antiflammin-2 had significant effect on expression of L-selectin and CD18 on resting PMNs, monocytes and lymphocytes. For instance, L-selectin and CD18 expression by PMNs was 91±4 and 109±4% of control in the presence of 100 μmol/l antiflammin-1, and 97±6 and 109±10% of control in the presence of 100 μmol/l antiflammin-2, respectively. Addition of PAF (1 μmol/l) to whole blood gave a significant decrease in L-selectin and a marked upregulation of CD18 on leukocytes. Fig. 1 reports representative results illustrating the effects of antiflammin-2 on PAF-activated neutrophils. Addition of "scrambled" antiflammin-2 (MLNHKLDVD) (Fig. 1) or the nonapeptide corresponding to residues 24-32 of the rat prepro-cholecystokinin (data not shown) had no effect on PAF-induced changes. Preincubation of blood with antiflammin-1 or antiflammin-2 inhibited PAF-induced downregulation of L-selectin and upregulation of CD18 expression on PMNs, monocytes and lymphocytes in a concentration-dependent fashion (Fig. 2). The apparent IC_{50} values for antiflammin-1 and antiflammin-2 were similar for PMNs (6.3 and 4.7 μmol/l, respectively), monocytes (9.5 and 4.8 μmol/l, respectively) and lymphocytes (5.2 and 7.8 μmol/l, respectively). Addition of lipocortin-1 to whole blood gave a concentration-dependent attenuation of PAF-induced changes in leukocyte adhesion molecule expression (Fig. 3). The apparent maximum inhibition that can be achieved with antiflammins was similar to that detected with lipocortin-1 (Fig. 3) and to that we have previously found with dexamethasone (2). Denaturation of lipocortin-1 resulted in a complete loss of its inhibitory actions (data not shown). As with

PAF, both antiflammin-1 and antiflammin-2 partially blocked IL-8-induced changes in L-selectin and CD18 expression by PMNs with apparent IC_{50} values of 9 and 18 μmol/l, respectively (Fig. 4). Immunostaining of leukocytes with an anti-CD11b mAb revealed similar changes as those observed with the anti-CD18 mAb (data not shown).

Effect of antiflammins on expression of E-selectin and ICAM-1 on LPS-stimulated endothelial cells

After stimulation by LPS, HCAEC increased on average 25.8-fold and 2.2-fold the expression of E-selectin and ICAM-1, respectively ($n=3$, both $P<0.05$) (Fig. 5). Neither antiflammin-1 nor antiflammin-2 affected expression of these adhesion molecules on unstimulated HCAEC (data not shown). Furthermore, antiflammin-2 produced only a slight inhibition of LPS-induced changes (Fig. 5). The maximum inhibition did not exceed 7%. Similar results were obtained with antiflammin-1 (data not shown). Treatment of HCAEC with dexamethasone (100 nmol/l) inhibited ~60% of the LPS-stimulated expression of E-selectin and ICAM-1 (Fig. 5) without altering basal expression of these molecules (data not shown).

Antiflammins inhibit PMN adhesion to endothelial cells

Activation of HCAEC with LPS resulted in a 3.7-fold increase in the number of adherent PMNs (Fig. 6A). Culture of HCAEC with LPS in the presence of antiflammin-1 or antiflammin-2 produced only slight decreases in adhesion, whereas culture of HCAEC with LPS in the presence of dexamethasone (100 nmol/l) resulted in on average a 40% decrease in the number of adherent PMNs (Fig. 6A). Pretreatment of PMNs with either antiflammin-1 or antiflammin-2 before

addition to LPS-activated HCAEC attenuated their attachment to endothelial cells in a concentration-dependent fashion with apparent IC_{50} values of 1.2 and 3.0 μmol/l, respectively (Fig. 6B). At 100 μmol/l, antiflammin-1 and antiflammin-2 inhibited PMN adhesion by 54 ± 4 and $43\pm4\%$, respectively ($n=3$, both $P<0.05$) (Fig. 6B). No adhesion experiments were done where PAF or IL-8-activated neutrophils were added to HCAEC stimulated in the presence of antiflammins since both PAF and IL-8 provoke neutrophil aggregation, therefore making interpretation of the results difficult. "Scrambled" antiflammin-2 or the rat prepre-cholecystokinin nonapeptide (1-100 μmol/l) had no detectable effect on the number of adherent neutrophils (data not shown).

Since PMN adhesion to LPS-stimulated HCAEC is mediated by multiple adhesion receptors (26), we assayed the contribution of L-selectin, E-selectin and CD18 to the binding interaction. A significant proportion of PMN-HCAEC attachment was blocked by mAbs binding to L-selectin ($24\pm4\%$, $n=3$), CD18 ($27\pm4\%$) or E-selectin ($35\pm5\%$) (Fig. 7). The combination of these mAbs inhibited PMN adhesion by $\sim90\%$. Treatment of PMNs with antiflammin-1 and anti-CD18 mAb resulted in only a slightly greater inhibition of adhesion than those observed with PMNs treated with either antiflammin-1 or anti-CD18 mAb (Fig. 7). Combination of antiflammin-1 with either anti-L-selectin mAb or anti-E-selectin mAb resulted in additive inhibition, and the degree of inhibition was similar to those observed when anti-L-selectin mAb or anti-E-selectin mAb was combined with anti-CD18 mAb, respectively (Fig. 7). Combining antiflammin-1, anti-L-selectin mAb and anti-E-selectin mAb blocked $\sim82\%$ of adhesion. Similar results were obtained with antiflammin-2 (data not shown).

DISCUSSION

In this report, we propose a novel mechanism by which antiflammins can affect the inflammatory response, namely through modulation of surface expression of adhesion molecules on activated leukocytes, and inhibition of neutrophil-endothelial cell adhesion.

The glucocorticoid-inducible protein, lipocortin-1 appears to be an important negative effector of leukocyte emigration in various experimental models (27-30). Three regions of the lipocortin-1 molecule, a 188-amino acid N-terminal fragment (5), peptide N-acetyl-2-26 (5) and peptide 246-254 (termed antiflammin-2) (6) have been reported to inhibit leukocyte accumulation. In our experiments, antiflammin-2 and antiflammin-1 (the C-terminal part of α-helix three in uteroglobin) appeared to be equally potent inhibitors. The first two residues (which differ in antiflammin-1 and antiflammin-2) can be replaced, but not delated, without loss of activity, indicating that the length of antiflammins is critical for the biological activity (6). Oxidation of the Met residue in position 1 in antiflammin-1 has been suggested to account for the rapid (within 5 min) decline in the inhibitory action of antiflammin-1 incubated with human isolated PMNs (12). However, this Met residue is not a prerequisite for activity, since it is substituted with His in antiflammin-2 (6). We could not detect any significant loss of inhibitory action of antiflammin-1 in our experiments. Besides differences in the experimental conditions (e.g. presence of serum in our experiments), the reasons for this apparent discrepancy are not known at present. The inhibitory action of antiflammins observed in this study are specific for these peptide sequences, because no inhibitory effects were detected with the peptide MLNHKLDVD (a "scrambled" sequence of antiflammin-2) or the unrelated nonapeptide VPVEAVNPH in the assays used.

Our study documents that while antiflammins had no effect on expression of adhesion molecules on human resting leukocytes in whole blood in vitro, they markedly attenuated changes in L-selectin and CD11/CD18 expression evoked by PAF or IL-8. Thus, antiflammins can fully duplicate the actions of lipocortin-1 on leukocyte adhesion molecule expression. Assuming that no lipocortin-1 molecule has been denaturated during the experiments (8), on a molar concentration basis, 30-fold more antiflammins than native lipocortin-1 was needed to achieve a similar degree of inhibition. It is likely that higher concentrations of antiflammins may be needed to occupy the putative lipocortin-1 receptor. Newertheless, it is impressive that these nonapeptides are active in the whole blood and overcame interactions with blood components to specifically regulate leukocytes. Human PMNs and monocytic cells possess specific and saturable binding sites for lipocortin-1 (30), that mediate the action of lipocortin-1 on these cells. Our results demonstrate that in addition to consolidating binding to the target cells (31), the antiflammin-2 sequence (residues 246-254 in lipocortin-1) exerts actions similar to those of the parent protein. These findings are also consistent with our previous study, which showed that dexamethasone attenuates by $\sim 60\%$ the PAF and formyl-Met-Leu-Phe-induced changes in L-selectin and CD11b/CD18 expression by human neutrophils through induction of protein synthesis (2), most likely lipocortin-1 (5).

Within minutes of activation with PAF or IL-8, leukocytes release L-selectin from their surface by a proteolytic enzyme. Inasmuch as this enzyme appears to be constitutively active, formation of an appropriate three-dimensional structure of L-selectin near the membrane is thought to regulate this proteolytic process (32,33). Although the nature of conformational changes required for the cleavage is not understood at present, our study suggests that this can

partially be prevented by antiflammins. Phosphorylation of serine in the intracellular tail of L-selectin (34) and calmodulin inhibitors (35) were reported to induce proteolytic shedding of L-selectin. Whether the action of antiflammins involves activation of calmodulin or interference with a phosphorylation step remains to be investigated. Leukocyte integrins change their conformation during cell activation with characteristics of the active molecule (i.e. increase in the binding avidity or affinity) depending on the method of stimulation (36,37). Activation of leukocytes with stimuli which increase intracellular Ca^{2+} concentration, such as PAF or IL-8, induce clustering of β_2 integrins, thereby increasing the overall strength of binding without affecting affinity (37). However, it seems unlikely that antiflammins might interfere with Ca^{2+} signaling, because recent results suggest that antiflammin-2 does not inhibit calcium-dependent mobilization of arachidonic acid (13). Activation of leukocytes from the outside of the cell with Mg^{2+} in the presence of EGTA results in the formation of a higher affinity form of the integrins by either unmasking the ligand binding site or by tertiary changes within the ligand binding domain (36,38). Although both mechanisms may operate in integrins, it is not known at present how these mechanisms are activated.

Despite inhibition of L-selectin shedding from PMNs, which would be expected to promote their adhesion to HCAEC, antiflammins markedly reduced the number of adherent neutrophils. This inhibition can primarily be attributed to their effect on PMNs rather than on HCAEC, because antiflammins had little effect on LPS-stimulated expression of E-selectin and ICAM-1 on HCAEC. The antiflammins or a function-blocking anti-CD18 mAb resulted in similar decreases in PMN adhesion to HCAEC. Furthermore, the actions of antiflammin-1 or antiflammin-2 and anti-CD18 mAb were not additive, indicating that inhibition of PMN-HCAEC

adherence by antiflammins is predominantly attributable to attenuation of upregulation of CD11/CD18 expression on PMNs. Since the inhibition with antiflammins was additive with anti-E-selectin and anti-L-selectin mAbs, it is unlikely that antiflammins interfered with E-selectin or L-selectin function or ligands. Interestingly, inhibition of neutrophil adhesion by antiflammins resembles to that of peptide N-acetyl-2-26 of lipocortin-1 (5). This peptide can also inhibit PMN adhesion when using a leukocyte stimulus, but not when using edothelial cell stimulation, although the underlying mechanisms of action are not known at present.

The present and previous studies (1,2) point to similarities and striking differences between the actions of antiflammins and glucocorticoids. Antiflammins exert similar inhibitory actions as glucocorticoids on activation-induced changes in adhesion molecule expression by PMNs, resulting in attenuation of PMN adhesion to HCAEC. By contrast, dexamethasone, but not antiflammins, inhibits LPS-induced expression of ICAM-1 and E-selectin on human umbilical vein endothelial cells (1) as well as on HCAEC (present study), leading to decreases in PMN adherence. While most of the actions of glucocorticoids are mediated by translocation of the glucocorticoid-glucocorticoid-receptor complex to the nucleus and its binding to genes containing glucocorticoid-responsive elements (1,39), the absence of a glucocorticoid-responsive element in the gene for E-selectin (40) suggests that glucocorticoids either interfere directly with a transcriptional regulator or induce the synthesis of a secondary regulatory element (1). The lack of effect of antiflammins on E-selectin and ICAM-1 expression on HCAEC would suggest that lipocortin-1 may not be this regulatory element.

Our results may have relevance to inhibition of excessive trafficking of leukocytes both in acute and chronic inflammation. By inhibiting upregulation of CD11/CD18 expression,

antiflammins attenuate firm adhesion of PMNs to the endothelium and consequently their transendothelial migration, key events for leukocyte accumulation in tissues. Therefore, antiflammins may be useful therapeutic agents to prevent and/or attenuate neutrophil-mediated tissue injury that accompanies myocardial reperfusion injury (41,42) as well as other chronic disease states such as rheumatoid arthritis (43). The mechanisms of action of antiflammins differ from those of non-steroid antiinflammatory drugs or the acute-phase reactant C-reactive protein, which inhibit PMN binding to endothelial cells by inducing shedding of L-selectin from the leukocyte surface without affecting CD11/CD18 expression (26,44).

In conclusion, this study demonstrates that antiflammins mimic the actions of glucocorticoids on adhesion molecule expression on human leukocytes, but not on endothelial cells, and attenuate PMN adhesion to HCAEC via inhibition of cell-activation-iduced changes in CD11/CD18 expression. Therefore, antiflammins may represent a novel therapeutic approach in blocking leukocyte trafficking in host defense and inflammation.

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Figure legends

Figure 1. Whole blood actions of antiflammin-2 on cell surface expression of L-selectin and CD18 by human neutrophils. Whole blood aliquots were incubated with antiflammin-2 (AF-2, 100 μmol/l) or the peptide MLNHKLDVD (scrambled antiflammin-2, sAF-2, 100 μmol/l) for 30 min and then challenged with PAF (1 μmol/l) for 30 min at 37°C. In each histogram is also displayed the negative control of immunostaining with class-matched irrelevant antibodies (C). Shown are representative results of 5 experiments.

Figure 2. Antiflammins inhibit PAF-induced changes in expression of L-selectin and CD18 on human leukocytes. Whole blood aliquots were incubated with antiflammins for 30 min and then challenged with PAF (1 μmol/l) for 30 min at 37°C. Adhesion molecule expression is presented as percentage of control (unchallenged cells). Mean fluorescence intensity for L-selectin: PMNs, control, 91$\pm$14, PAF, 41$\pm$6; monocytes, control, 43$\pm$6, PAF, 21$\pm$5; lymphocytes, control, 50$\pm$9, PAF, 45$\pm$9, n=6, all $P<0.05$. Mean fluorescence intensity for CD18: PMNs, control, 52$\pm$5, PAF, 83$\pm$6; monocytes, control, 74$\pm$9, PAF, 105$\pm$10; lymphocytes, control, 16$\pm$2, PAF, 18$\pm$2, n=6, all $P<0.05$. The results are the mean$\pm$SEM of three to six experiments with different donor cell preparation. * $P<0.05$, ** $P<0.01$ vs PAF-stimulated cells.

Figure 3. Lipocortin-1 blocks PAF-induced changes in L-selectin and CD18 expression on human leukocytes. Whole blood aliquots were incubated with lipocortin-1 for 30 min and then challenged with PAF (1 μmol/l) for 30 min at 37°C. Adhesion molecule expression is presented

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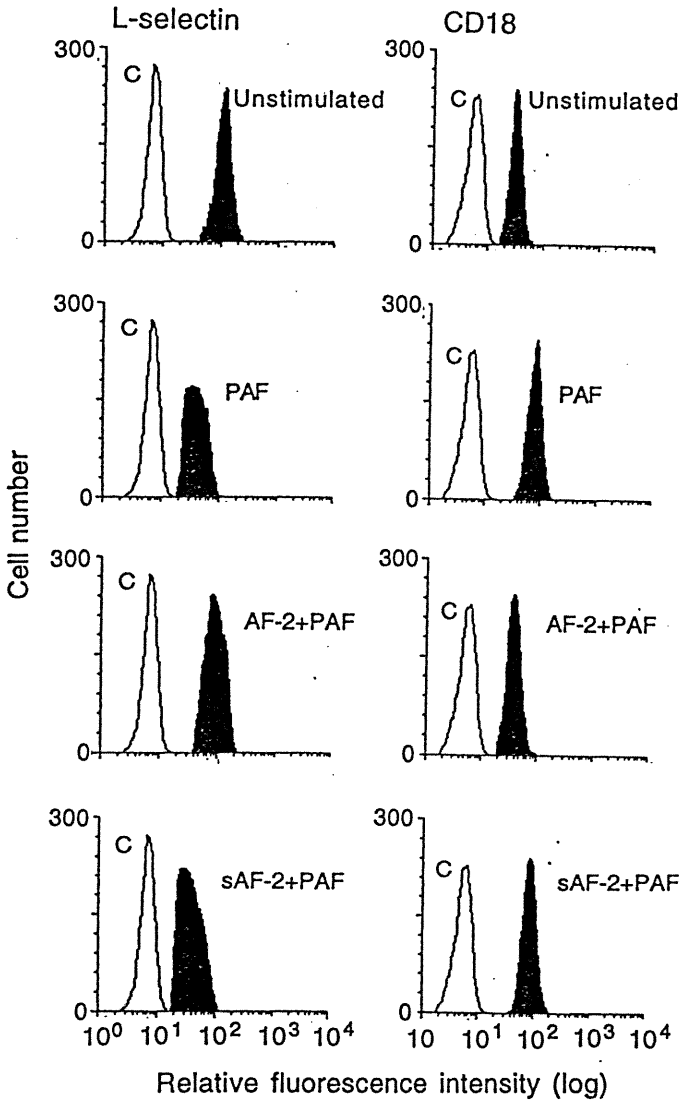
Figure 4. Antiflammins inhibit IL-8-induced changes in L-selectin and CD18 expression on human PMNs. Whole blood was incubated with antiflammins for 30 min at 37°C and then challenged with 10 nmol/L IL-8 for 30 min. Adhesion molecule expression is presented as percentage of control (unchallenged cells). Mean fluorescence intensity for L-selectin: control, 89±10, IL-8, 43±5; CD18: control, 42±4, IL-8, 58±5, n=4, both $P<0.05$. Values are means±SEM of four experiments with different donor cell preparations. * $P<0.05$ vs IL-8-stimulated cells.

Figure 5. Effects of antiflammin-2 and dexamethasone on E-selectin and ICAM-1 expression on human coronary artery endothelial cells (HCAEC). Confluent monolayers of HCAEC were left unstimulated (control, C) or were challenged with 1 μg/mL LPS in the presence of antiflammin-2 (100 μmol/l) or dexamethasone (100 nmol/l) for 4 h at 37°C. Values represent the mean±SEM of three independent experiments. * $P<0.05$ vs LPS-treated.

Figure 6. Inhibition of PMN binding to endothelial cells by antiflammins and dexamethasone. **(A)** Confluent HCAEC monolayers were cultured with LPS (1 μg/ml) in the presence of antiflammin-1 (100 μmol/l), antiflammin-2 (100 μmol/l) or dexamethasone (100 nmol/l) for 6 h at 37°C before addition of radiolabeled PMNs. **(B)** PMNs were preincubated for 30 min with

medium or antiflammins; or with dexamethasone (100 nmol/l) for 120 min as indicated prior to addition to LPS-activated HCAEC. PMN adhesion to unstimulated HCAEC was $0.35 \pm 0.04 \times 10^4$ cells per well. Values are means±SEM of three experiments using PMNs from different donors. * $P < 0.05$ vs. LPS-treated HCAEC.

Figure 7. Inhibition of PMN binding to endothelial cells by anti-E-selectin, anti-L-selectin and anti-CD18 mAbs and antiflammin-1. PMNs were treated with antiflammin-1 (100 μmol/l) or the indicated mAbs before and during the assay. PMN adhesion to unstimulated HCAEC was $0.37 \pm 0.02 \times 10^4$ cells per well. The irrelevant mAb MOPC-21 (IgG_1) was used as a negative control. The results are means±SEM of three experiments using PMNs from different donors.



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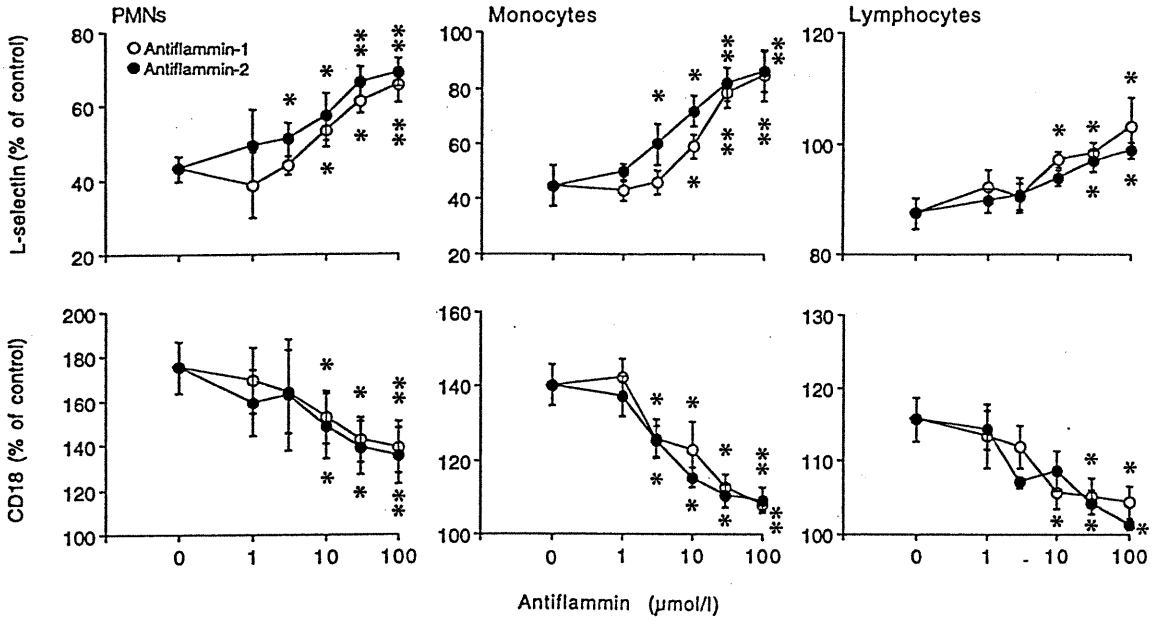

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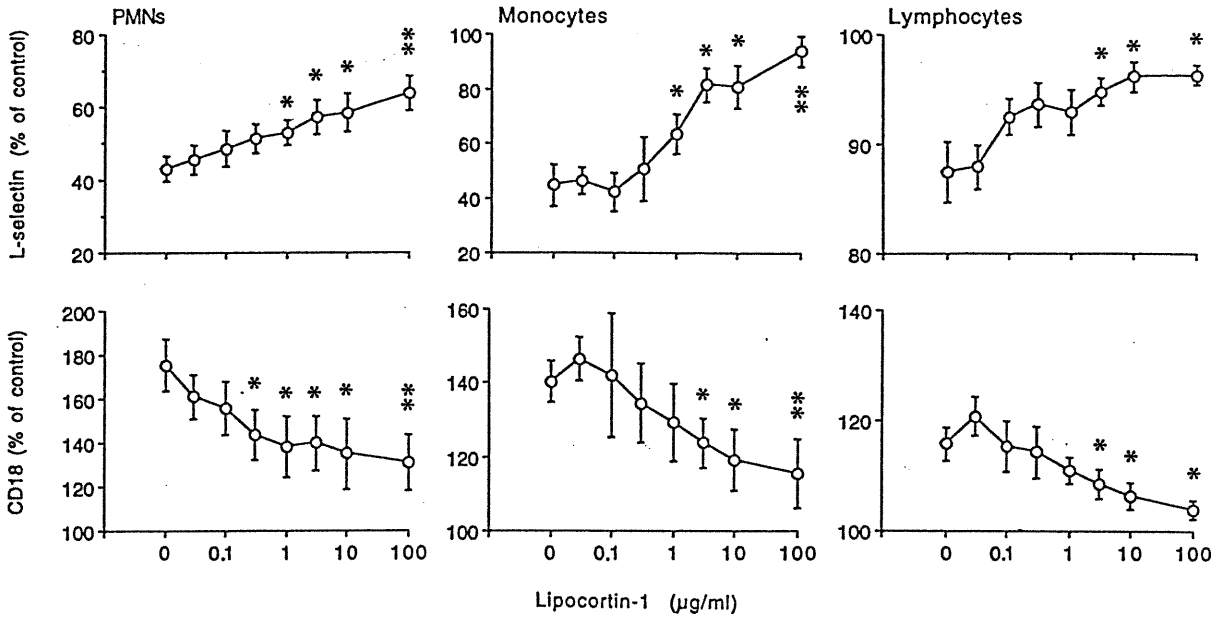

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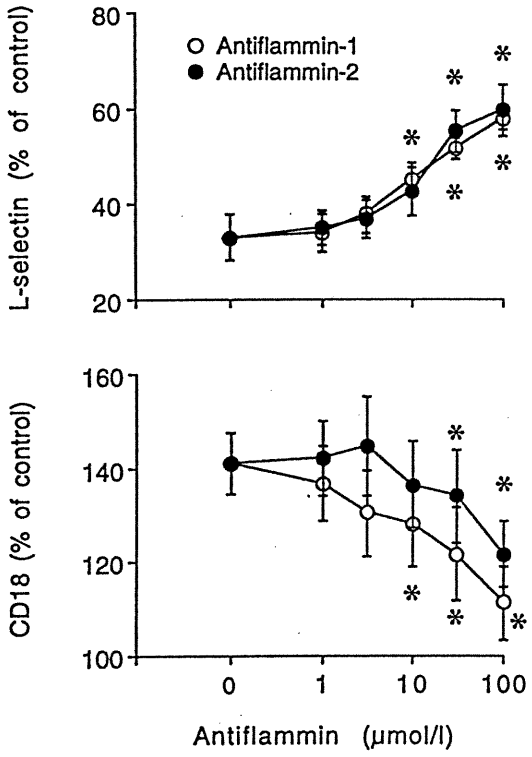

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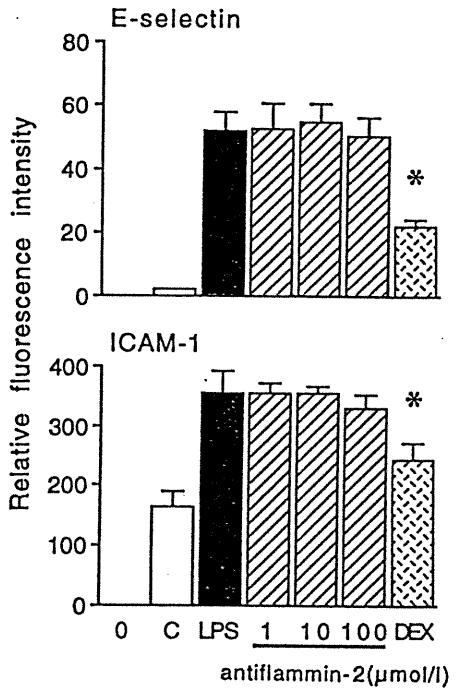

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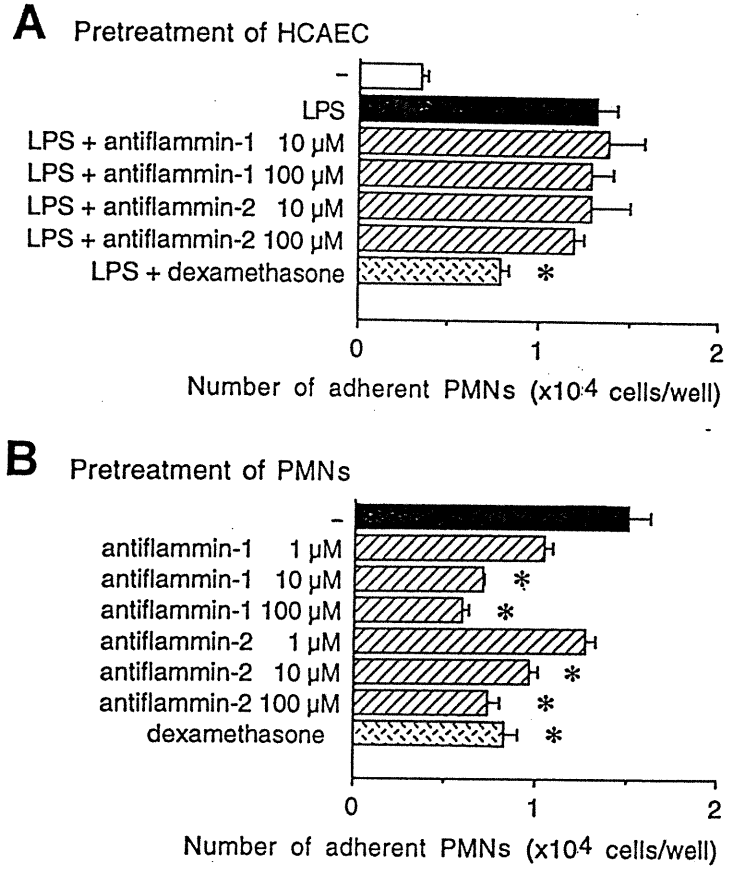

Figure 6. Inhibition of PMN binding to endothelial cells by antiflammins and dexamethasone. **(A)** Confluent HCAEC monolayers were cultured with LPS (1 µg/ml) in the presence of antiflammin-1 (100 µmol/l), antiflammin-2 (100 µmol/l) or dexamethasone (100 nmol/l) for 6 h at 37°C before addition of radiolabeled PMNs. (B) PMNs were preincubated for 30 min with

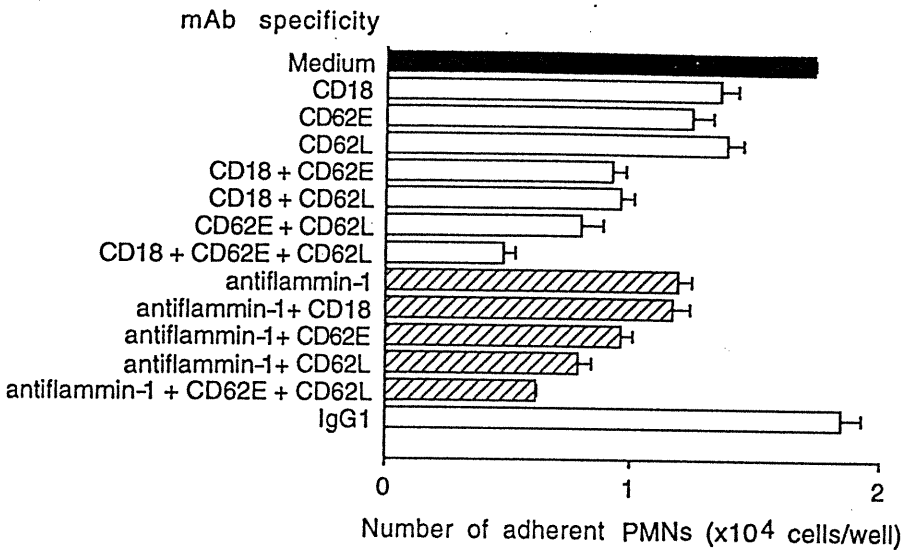

Figure 7. Inhibition of PMN binding to endothelial cells by anti-E-selectin, anti-L-selectin and anti-CD18 mAbs and antiflammin-1. PMNs were treated with antiflammin-1 (100 μmol/l) or the indicated mAbs before and during the assay. PMN adhesion to unstimulated HCAEC was $0.37\pm0.02\text{x}10^4$ cells per well. The irrelevant mAb MOPC-21 (IgG_1) was used as a negative control. The results are means$\pm$SEM of three experiments using PMNs from different donors. medium or antiflammins; or with dexamethasone (100 nmol/l) for 120 min as indicated prior to addition to LPS-activated HCAEC. PMN adhesion to unstimulated HCAEC was $0.35\pm0.04\text{x}10^4$ cells per well. Values are means$\pm$SEM of three experiments using PMNs from different donors.

* $P<0.05$ vs. LPS-treated HCAEC.

IV. DISCUSSION GÉNÉRALE

Ces études nous permettent de proposer de nouveaux mécanismes par lesquels des médiateurs endogènes peuvent affecter la réponse inflammatoire. Plus particulièrement en modulant l'expression des molécules d'adhésion à la surface des leucocytes et des cellules endothéliales, puis en agissant sur l'adhésion des neutrophiles aux cellules endothéliales.

Par des études de liaison et en utilisant des antagonistes sélectifs compétitifs pour les récepteurs ET_A et ET_B, nous avons caractérisé les sous-types de récepteurs qui médient l'action de l'endothéline-1 (ET-1) sur l'expression de la L-sélectine et de CD11/CD18 à la surface des neutrophiles, sur l'expression de la E-sélectine et d'ICAM-1 à la surface des cellules endothéliales d'artères coronariennes humaines, ainsi que sur l'adhésion des neutrophiles aux cellules endothéliales. Cette étude démontre que l'ET-1 est capable de diminuer l'expression de la L-sélectine, et d'augmenter l'expression de CD11b/CD18 et de CD45 à la surface des neutrophiles. Ceci indique une activation cellulaire, mais elle est partielle car bien qu'il y a relâche de gélatinase des granules tertiaires, il n'y a pas induction de β-glucuronidase ni relâche de lysozymes. Ces actions de l'ET-1 sont médiées par le récepteur ET_A car d'après nos résultats les neutrophiles expriment de façon prédominante ce type de récepteur. D'une part, nous pouvions inhiber les actions de l'ET-1 avec l'antagoniste sélectif pour le récepteur ET_A FR 139317 (136,137), mais pas avec le BQ 788 (138), l'antagoniste ET_B-sélectif. D'autres part, un agoniste sélectif pour ET_B, IRL-1620 (141), ne pouvait mimer ces actions. En effet, les études de liaison au récepteur indiquent la présence d'une seule classe de site de liaison de haute affinité pour l'ET-1 sur les neutrophiles. De plus les observations que la liaison de l'ET-1 est inhibée en présence de FR 139317, tandis qu'elle était insensible au BQ 788, supporte l'expression prédominante de récepteurs ET_A par les neutrophiles humains.

De la même façon, nous avons confirmé l'expression constitutive de récepteurs ET_B sur les cellules endothéliales. En effet, la liaison de l'ET-1 marquée à l'I^{125} est inhibée sélectivement par le BQ 788, mais non par le FR 139317. De plus l'induction de l'expression de la E-sélectine et d'ICAM-1 est médiée par l'activation du récepteur ET_B puisqu'on peut l'inhiber sélectivement par le BQ 788 (138).

D'autres part, l'ET-1 augmente considérablement l'adhésion des neutrophiles aux cellules endothéliales d'artères coronariennes humaines stimulées avec du LPS ou de l'ET-1. Par contre, il a été démontré dans une étude que l'ET-1 n'augmente que de peu le nombre de neutrophiles adhérents à des cellules endothéliales d'artères coronariennes humaines (155). De plus Lopez-Farré et al. (148) ont rapporté que 30 min d'incubation de l'ET-1 seule avec des cellules endothéliales bovines n'avait aucun effet stimulateur sur l'adhésion. Dans notre étude, nous avons cultivé des cellules seules avec l'ET-1 pour 4-6 h et ceci résulte en l'augmentation de l'expression de la E-sélectine et d'ICAM-1, ainsi qu'en une augmentation significative de l'adhésion des neutrophiles. Puis étant donnée que dans les essais d'adhésion les neutrophiles furent incubés avec les cellules endothéliales activées, en présence d'ET-1 pour 30 min, la stimulation de l'adhésion par l'ET-1 pourrait en premier lieu être attribuée aux effets de ce peptide sur les neutrophiles.

Pour caractériser les molécules d'adhésion impliquées dans cette interaction entre les leucocytes et les cellules endothéliales, nous avons utilisé des anticorps monoclonaux dirigés contre différentes molécules d'adhésion. Ces expériences démontre que les intégrines CD18, la E-sélectine et la L-sélectine médient l'adhésion des neutrophiles aux cellules endothéliales par l'ET-1. Par contre, cette adhésion est CD18-dépendante puisqu'elle peut être grandement inhibée par un anticorps anti-CD18. Les mécanismes intracellulaires sont encore inconnus.

D'autres informations au sujet des caractéristiques de l'effet de l'ET-1 furent obtenues en étudiant l'implication du PAF. En effet, l'ET-1 stimule la synthèse et la relâche de PAF par les neutrophiles humains (154) et les microvaisseaux de cerveau de rat (252). Par conséquent, l'augmentation de l'adhésion des neutrophiles aux cellules endothéliales induite par l'ET-1 peut être inhibée par le BN 52021 et le WEB 2086, deux antagonistes puissants et spécifiques au récepteur du PAF

(253,254), comparativement à ce qu'on observe avec l'inhibition du récepteur ET_A. De plus, l'ajout de PAF produit des changements semblables à ceux observés avec l'ET-1. Ce qui implique que le PAF semble jouer un rôle important pour médier les actions pro-adhésives de l'ET-1. Par contre, des études récentes ont démontré que l'ET-1 pourrait aussi démontrer des actions anti-adhésives. Des périodes courtes d'incubation (10 min) de segments d'artères coronariennes félines avec de l'ET-1 mènent à l'inhibition de l'adhésion des neutrophiles à l'endothélium stimulé avec de la thrombine (155). Ceci est le résultat de l'inhibition des interactions leucocytes-cellules endothéliales P-sélectine-dépendantes par la relâche de NO suite à l'activation des récepteurs ET_B sur les cellules endothéliales. Dans nos essais d'adhésion des neutrophiles aux cellules endothéliales nous n'avons pas étudié l'adhésion P-sélectine-dépendante car l'expression de la P-sélectine se produit dans les 10-20 min suite à la stimulation et elle est soutenue pour environ 60 min.

Donc en se basant sur ces résultats, nous proposons le modèle suivant (fig. 1) dans lequel l'ET-1 exerce une action double sur l'adhésion des neutrophiles. On observe alors une action anti-adhésive transitoire, qui est médiée par le récepteur ET_B couplée à la production de NO, et une action pro-adhésive qui est médiée de façon prédominante par les changements d'expression des molécules d'adhésion à la surface des neutrophiles suite à l'activation des récepteurs ET_A. De plus, l'ET-1 peut aussi augmenter l'expression de la E-sélectine et d'ICAM-1 par les cellules endothéliales par l'activation des récepteurs ET_B.

Dans la deuxième étude (chapitre II), nous démontrons que le mécanisme par lequel la protéine C-réactive et les peptides 174-185 et 201-206 dérivés de la protéine C-réactive pourraient affecter la réponse inflammatoire, est la modulation de l'adhésion des neutrophiles aux cellules endothéliales. La protéine C-réactive et ses peptides (174-185 et 201-206) agissent sur les neutrophiles en diminuant l'attachement aux cellules endothéliales stimulées avec du LPS. Cette action est attribuée à la diminution de l'expression de la L-sélectine de la surface des neutrophiles, comme nous l'avons mesuré par cytométrie en flux. En fait, la diminution de l'expression de la L-sélectine résulte du clivage de celle-ci de la surface des neutrophiles. En effet, par ELISA nous observons des augmentations de

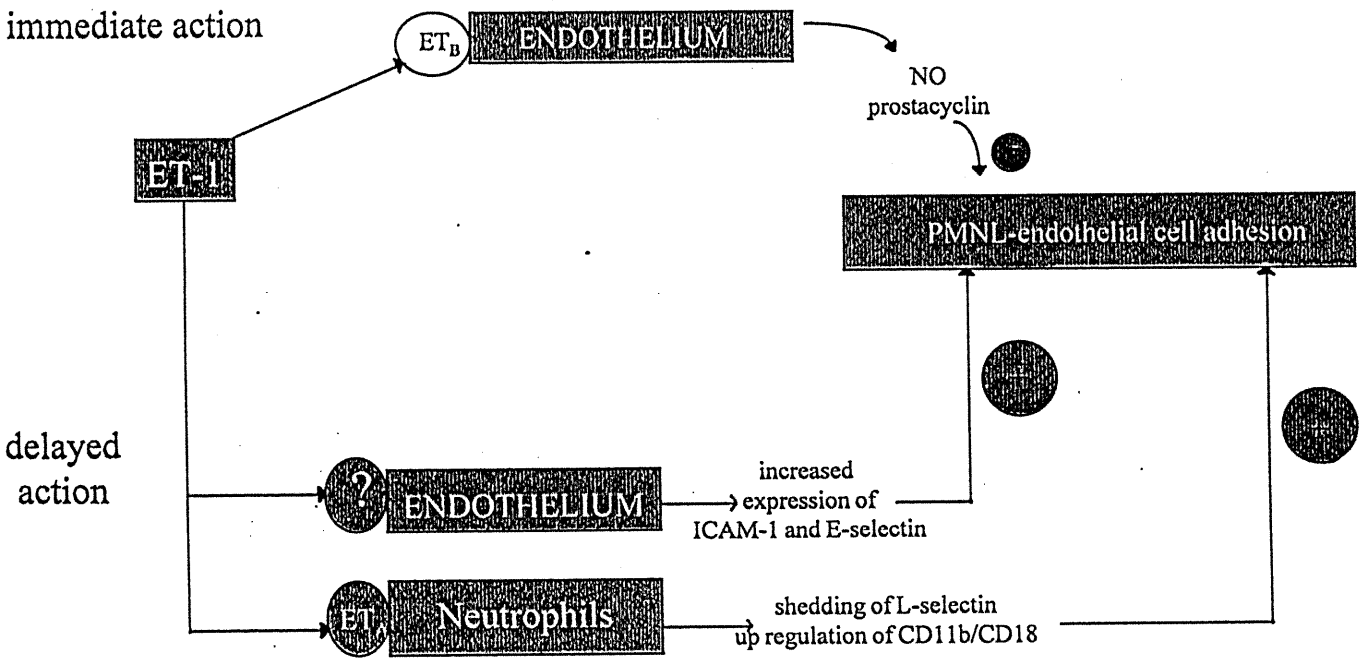

Figure 1: Modèle pour l'action double exercée par l'endothéline-1 sur l'adhésion des neutrophiles.

L-sélecine solubles dans le milieu de culture suite au traitement avec la protéine C-réactive ou les peptides dérivés de la protéine C-réactive. Pour supporter encore mieux ces observations, nous avons vérifié la contribution de différentes molécules d'adhésion dans l'interaction entre les neutrophiles et les cellules endothéliales. Des anticorps monoclonaux dirigés contre la L-sélectine, la E-sélectine et le CD18 inhibent chacun partiellement l'adhésion aux cellules endothéliales d'artères coronariennes, mais ensemble ils peuvent inhiber jusqu'à 85% de l'adhésion. Ces résultats suggèrent que chacune de ces molécules d'adhésion fonctionne indépendamment et interagit comparativement pour promouvoir de façon optimale l'adhésion des neutrophiles aux cellules endothéliales. La protéine C-réactive et l'anticorps contre la L-sélectine réduisent similairement l'adhésion des neutrophiles, ce qui effectivement indiquent que l'inhibition de l'adhésion par la protéine C-réactive est surtout attribuée au clivage de la L-sélectine de la surface des neutrophiles. De plus, le KD-IX-73-4 un inhibiteur du clivage de la L-sélectine peut inhiber complètement la diminution de l'expression de la L-sélectine induite par la protéine C-réactive et aussi prévenir les effets de la protéine C-réactive sur l'adhésion des neutrophiles.

Généralement l'activation des neutrophiles résulte en une diminution de l'expression de la L-sélectine et une augmentation de l'expression de CD11b/CD18 et de CD45 (255,256). Quelques minutes suite à cette activation, la L-sélectine est clivée de la surface par un clivage protéolytique. Par contre ni la protéine C-réactive, ni les peptides dérivés (174-185 et 201-206) de la protéine C-réactive affectent l'expression de CD11b/CD18 et CD45, indiquant que les neutrophiles ne sont pas activés. En effet, la protéine C-réactive et ses peptides n'induisent pas la relâche d'enzymes. La nature biochimique et moléculaire de l'enzyme protéolytique n'a pas encore été identifiée, mais elle semble être constitutivement active (53,54). La formation d'une structure tridimensionnelle appropriée de la L-sélectine semblerait être impliquée dans le clivage, et il est tentant de spéculer que la protéine C-réactive et les peptides 174-185 et 201-206 pourraient activer ce processus. Par contre, le/les enzyme(s) et les mécanismes de signalisation qui régulent la conformation de la L-sélectine en réponse à la protéine C-réactive ou aux peptides ne sont pas encore bien

compris. Tout ce qui est connu, c'est que la protéine C-réactive peut augmenter l'activité de Erk2 (177) et de la PI-3 kinase (177), par contre elle inhibe l'activité de p38 MAPK (178). De plus elle augmente les niveaux d'AMPc intracellulaires (179), mais inhibent la mobilisation de Ca^{2+} (165). De plus, dans les résultats non publiés, nous avons démontré que les peptides 174-185 et 201-206 ont des effets similaires à ceux de la protéine C-réactive. Donc ils peuvent mimer les actions de la protéine native sur les interactions entre les neutrophiles et les cellules endothéliales.

Dans la prochaine étude (chapitre III), nous avons décrit un nouveau mécanisme par lequel des analogues stables de la LXA_4, un médiateur lipidique (191), et les ATL (191,194) peuvent affecter la réponse inflammatoire, et ce en modulant l'expression de molécules d'adhésion à la surface des leucocytes immunostimulés ou non dans le sang total, et en inhibant l'adhésion des neutrophiles à l'endothélium activé. Ces analogues augmentent l'expression de la L-sélectine, et diminuent l'expression de CD11b/CD18 à la surface des neutrophiles, des monocytes et à un moindre degré des lymphocytes non stimulés. Ils atténuent aussi les changements induit par l'immunostimulation avec du PAF ou del'IL-8. Précédemment, d'autres études avaient démontré que la LXA_4 pouvait inhiber l'augmentation de CD11b/CD18 suite à une stimulation avec le FMLP, mais sur des neutrophiles humains isolés (211). Dans cette étude, nous démontrons cette action dans le sang total, avec des concentrations nanomolaires à micromolaires d'analogues de la LXA_4. Bien que nous devions utiliser des concentrations plus élevées dans le sang total que sur des cellules isolées, il est tout de même impressionnant que ces agents lipophiliques puissent être actifs dans un microenvironnement du sang.

Pour explorer le mécanisme responsable de la diminution de l'expression de CD11b/CD18 par les analogues stables de la LXA_4, nous avons utilisé des inhibiteurs de différentes kinases. La diminution de l'expression de CD11b/CD18 par les analogues stables sur les neutrophiles non stimulés n'implique pas la phosphatidylinositol-3 kinase ni la tyrosine kinase, mais semblerait plutôt être médiée par l'activation de la MAPK kinase, tel que suggéré par les expériences avec

le PD 98059 et le wortmannin, qui à une concentration de 2 μmol/L, peut aussi inhiber la MAPK kinase (257).

Les analogues stables de la LXA_4 peuvent non seulement empêcher les changements d'expression dépendant de l'activation, mais ils peuvent aussi empêcher le clivage de la L-sélectine induit par le peptide dérivé de la CRP, le 201-206, qui comme je l'ai mentionné plus tôt n'active pas les cellules.

Bien qu'ils inhibent le clivage de la L-sélectine, ce qui devrait promouvoir l'attachement des neutrophiles aux cellules endothéliales, les analogues de la LXA_4 inhibent l'adhésion des neutrophiles aux cellules endothéliales d'artères coronariennes. Cette inhibition est surtout attribuée à leur action sur les neutrophiles plutôt que sur les cellules endothéliales, puisque l'on observe que de faibles diminutions du nombre de neutrophiles adhérents lorsqu'on traite les cellules endothéliales avec du LPS en présence d'analogues de la LXA_4. De plus les analogues de la LXA_4 ont très peu d'effet sur l'expression de la E-sélectine et d'ICAM-1 suite à une stimulation avec du LPS ou du TNF-α. L'activation régule l'adhésion en altérant soit l'affinité de chaque molécule de β_2-intégrine par un changement de conformation ou en induisant l'agrégation (clustering) des β_2-intégrines, ce qui augmente alors l'avidité (77-79). Lorsqu'il y a augmentation de Ca^{2+} intracellulaire, on ne peut détecter de changement dans l'affinité de LFA-1 (258),en mesurant l'expression avec un anticorps (MoAb G-25.2) contre l'épitope sur le domaine β-propeller de LFA-1. Pour cette raison ni le PAF, ni l'IL-8 n'affectent l'expression de MoAb G-25.2. L'activation des leucocytes avec du Mg^{2+} et de l'EDTA, qui résulte en la formation d'une forme de haute affinité de LFA-1 (77), augmente l'expression de MoAb G-25.2, qui ne semble pas être affectée par les analogues de la LXA_4. Ces résultats suggèrent que les analogues de LXA_4 n'altèrent pas l'affinité, mais pourraient probablement interférer avec le "clustering" des β_2-intégrines. Nous avons aussi caractérisé les molécules d'adhésion impliquées dans l'adhésion des neutrophiles aux cellules endothéliales, en utilisant des anticorps monoclonaux contre les différentes molécules d'adhésion. Les analogues de la LXA_4 et l'anticorps contre CD18 diminuent le nombre de neutrophiles adhérents de façon similaire, et leurs effets ne sont pas additifs, contrairement à ce qu'on observe avec

les anticorps anti-L-sélectine ou anti-E-sélectine. Conséquemment, les analogues inhibent l'adhésion CD18-dépendante.

De plus, nous avons comparer l'action des analogues de la LXA_4 sur l'adhésion à celle des glucocorticoïdes. Nos résultats indiquent que les mécanismes d'action sont différents puisque les analogues de la LXA_4 et le dexaméthasone sont tous les deux capables d'inhiber l'adhésion, et que leurs actions sont additives. En effet le dexaméthasone agit sur les cellules endothéliales tandis que la LXA_4 peuvent agir sur les leucocytes. Donc les analogues de la LXA_4 représentent un nouveau mécanisme dans l'inhibition de l'adhésion des neutrophiles.

Finalement, nous proposons un nouveau mécanisme par lequel les antiflammines peuvent affecter la réponse inflammatoire, particulièrement en modulant l'expression des molécules d'adhésion sur les leucocytes activés, et en inhibant l'adhésion des neutrophiles aux cellules endothéliales. La lipocortine-1, la protéine inductible par les glucocorticoïdes (229,230), semble être un inhibiteur de l'émigration des leucocytes dans différents modèles expérimentaux (231). Plusieurs fragments de la lipocortine-1, dont l'antiflammine-2 (AF-2), peuvent inhiber l'accumulation des leucocytes (243). Dans nos expériences, l'AF-2 et l'AF-1 semblaient agir avec la même efficacité. De plus les actions inhibitrices des AFs observés dans cette étude sont spécifiques à ces peptides, puisque aucun effet inhibiteur n'a pu être détecté avec le "scrambled peptide" MLNHKLDVD (une séquence modifiée de l'AF-2) ou avec un nonapeptide non relié VPVEAVNPH. Ces peptides furent utilisés comme contrôles négatifs dans nos expériences.

Notre étude démontre que les AFs, bien que n'ayant aucun effet sur l'expression des molécules d'adhésion à la surface des leucocytes non stimulés, elles pouvaient diminuer de façon marquée les changements d'expression de la L-sélectine et de CD11b/CD18 induit par le PAF ou l'IL-8. Alors les AFs peuvent mimer l'action de la lipocortine-1 sur l'expression des molécules d'adhésion à la surface des leucocytes. Les AFs inhibent l'adhésion des neutrophiles aux cellules endothéliales d'artères coronariennes. Cette diminution est surtout attribuée à son effet sur les neutrophiles plutôt que sur les cellules endothéliales, puisque les AFs

avaient presque aucun effet sur l'expression de la E-sélectin et d'ICAM-1 à la surface des cellules endothéliales. De plus les actions de l'AF-1 ou de l'AF-2 et de l'anticorps anti-CD18 n'était pas additives, indiquant que l'inhibition de l'adhésion des neutrophiles aux cellules endothéliales par les AFs est surtout attribuable à la diminution de l'expression de CD11b/CD18 sur les neutrophiles.

D'après ces résultats ainsi que d'autres études faites auparavant (221), nous pouvont démontrer des différences et des similarités entre les actions des antiflammines et des glucocorticoïdes. Les AFs exercent des actions inhibitrices similaires à celles des glucocorticoïdes quant aux changement de l'expression des molécules d'adhésion induit par l'activation, et résultant en une diminution de l'adhésion des neutrophiles aux cellules endothéliales. Contrairement au dexaméthasone (221), les antiflammines n'ont aucun effet sur l'expression des molécules d'adhésion à la surface des cellules endothéliales. Alors les mécanismes d'action des antiflammines diffèrent de ceux des anti-inflammatoires non-stéroidiens.

Ces études nous permettent de décrire différents mécanismes régulateurs de la fonction des leucocytes et de leur adhésion aux cellules endothéliales. D'une par nous avons défini un mécanisme pro-adhésif, et d'autres part nous avons comparer différents mécanismes anti-adhésifs qui agissent chacun à des niveaux spécifiques des interactions entre les leucocytes et les cellules endothéliales.

V. CONCLUSIONS

Ces études révèlent des mécanismes importants qui peuvent affecter l'adhésion des neutrophiles aux cellules endothéliales, l'étape clé de l'accumulation des leucocytes dans les tissus. Alors nous avons caractérisé un mécanisme pro-adhésif, et trois mécanismes anti-adhésifs, mais dont les actions diffèrent soit au niveau de l'expression des molécules d'adhésion, soit au niveau de l'adhésion des neutrophiles aux cellules endothéliales.

1. Nos résultats indiquent que l'ET-1 promouvoit l'adhésion des neutrophiles aux cellules endothéliales et que celle-ci est médiée prédominément par l'activation des récepteurs ET_A sur les neutrophiles, et subséquemment par la génération de PAF. De plus nous avons démontré que la protéine C-réactive et les peptides dérivés de la

2. La protéine C-réactive peuvent agir sur les cellules non stimulées pour induire le clivage de la L-sélectine sans induire d'activation cellulaire. La protéine C-réactive agit donc en prévenant la capture des neutrophiles, ce qui conséquemment inhibe l'adhésion des neutrophiles aux cellules endothéliales et donc prévient l'accumulation dans les tissus.

3. L'antiflammine miment l'action des glucocorticoïdes sur l'expression des molécules d'adhésion à la surface des leucocytes, mais pas à la surface des cellules endothéliales.

4. Les antiflammines et les analogues de la LXA_4, quant à eux inhibent tous les deux l'adhésion des neutrophiles CD18-dépendante, par contre il semble que leurs mécanismes diffèrent puisque, bien que nous n'ayons pas comparé leurs actions, nous avons démontré que les actions du dexaméthasone et des analogues de la LXA_4 ne sont pas additives. De plus les analogues de la LXA_4 peuvent agir aussi bien sur les cellules stimulées que non.

5. Donc, par différents mécanismes, en inhibant l'accumulation des leucocytes, nous pouvons limiter dans l'espace et dans le temps la réponse inflammatoire et donc limiter les dommages tissulaires médiés par les neutrophiles qui accompagnent les maladies cardio-vasculaires ischémiques aiguës ainsi que d'autres maladies inflammatoires (aiguës ou chroniques) tel l'arthrite.

VI. DIRECTIONS FUTURES

Nos études démontrent que plusieurs médiateurs endogènes régulent les fonctions des leucocytes et les interactions des neutrophiles aux cellules endothéliales. Mais les mécanismes de signalisation intracellulaire ne sont pas encore bien compris. Il serait important d'identifier les étapes menant à l'expression des molécules d'adhésion en étudiant les kinases impliquées, et donc de voir comment ces différents agents modulent ces mécanismes.

De plus, il serait intéressant de vérifier si ces mécanismes sont fonctionnels sous différents débits sanguins, car il est bien connu que le dommage à la paroi vasculaire altère le "sheer rate", changeant alors le flux de laminaire à turbulent, et conséquemment pourrait affecter les interactions entre les leucocytes et les cellules endothéliales. Il serait alors bien de voir si la lipocortine-1, l'antiflammine, la protéine C-réactive, et la lipoxine sont fonctionnelles dans de telles conditions.

Il serait aussi bien de vérifier in vivo, à l'aide de modèles animaux, si les quantités de lipoxines induites par l'aspirine (ATL) seraient présentes en quantités suffisantes pour produire des effets biologiques.

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