Endotoxin, platelet-activating factor, and sepsis-related cytokines: Effects on lung pericyte growth.

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To my parents, my brother, and my grandmother for encouraging me to strive higher.
Without you, I am nothing.

Abstract

Gram-negative sepsis is an important cause of the acute respiratory distress syndrome (ARDS). Pulmonary hypertension (PH), which often develops in ARDS, results in part from the remodelling of the pulmonary microvasculature. Vascular pericytes (PC), dedifferentiated smooth muscle-like cells, are thought to play a role in the remodelling and neomuscularization of pulmonary microvessels. Endotoxin, lipopolysaccharide (LPS) from the outer membrane of gram-negative bacteria, activates macrophages, endothelial cells, and other cells to release potent cytokine and phospholipid mediators such as platelet-activating factor (PAF). The effects of these mediators on inflammation, vascular permeabilization, and progression of injury have been previously described. However, the role of these mediators in stimulating PC growth during the remodelling process is unknown. We show that compared with control growth, PAF (10^{-9}M, semisynthetic) stimulates the 7-day mean growth of proliferating PC by 31% in medium with serum, and 29% without serum. Furthermore, PAF stimulates the growth of quiescent PC by 12% with serum and 23% without serum. These proliferative effects are blocked by the addition of the PAF-receptor antagonist CV-3988 (10^{-7}M). The addition of the individual cytokines does not affect PC proliferation *in vitro*. A mixture of these cytokines, simulating *in vivo* conditions, does not alter PC proliferation in the

presence of serum, but reduces it in its absence. LPS from E.coli increases proliferation by 72%, compared with control. The proliferative effect of endotoxin requires the presence of serum. Thus, LPS and PAF, but not inflammatory cytokines, are direct mitogens of lung pericytes *in vitro*. This is the first demonstration that these molecules have direct effects on cells found in substantial areas of the vessel wall, possibly contributing to the neomuscularization and vascular remodelling of the pulmonary vvasculature in acute lung injury.

Résumé

La septicémie à bactéries gram-négatives est une cause importante du syndrome de détresse respiratoire de l'adulte (SDRA). L'hypertension pulmonaire, qui se développe souvent durant le SDRA, se manifeste en partie par des modifications des micro-vaisseaux pulmonaires. Les péricytes (PC), qui sont des cellules précurseures des muscles lisses, sont impliqués dans la reconfiguration et la néovascularisation des micro-vaisseaux pulmonaires. L'endotoxine, qui est une lipopolysaccharide (LPS) provenant de la membrane extérieure des bactéries gram-négatives, peut activer les cellules macrophagiques, endothéliales, et d'autres cellules de façon à libérer des médiateurs puissants, comprenant aussi les cytokines et le facteur activateur des plaquettes (FAP). Les effets de ces médiateurs sur l'inflammation, la perméabilité vasculaire, et la progression du dommage furent déjà décrits. Mais le rôle de ces médiateurs en stimulant la prolifération des PC durant le processus de la modification vasculaire est inconnu. Nous démontrons que, par rapport au contrôle, FAP (10^{-9}M, semi-synthétique) stimule la croissance moyenne au septième jour des PC "proliférants" de 31% et 29% respectivement avec et sans sérum. En plus, PAF stimule la croissance des PC "dormants" par 12% et 23% respectivement avec et sans sérum. Ces effets prolifératifs sont bloqués en ajoutant l'antagoniste du récepteur de PAF CV-3988 (10^{-7}M). L'addition

subséquente et séquentielle des cytokines n'affecte pas la croissance des PC *in vitro.* Ajoutées simultanement pour reproduire les conditions *in vivo*, l'addition des cytokines n'affecte pas la croissance des PC en présence de sérum, mais la réduit en son absence. LPS provenant de E.coli augmente de 72% la prolifération par rapport au contrôle. Les effets prolifératifs de l'endotoxine exigent la présence de sérum. LPS et FAP, mais pas les cytokines, peuvent jouer un rôle direct sur les PC *in vitro.* Ceci est la première démonstration que ces molécules ont un effet direct sur des cellules situés à des endroits importants dans les parois vasculaires, contribuant à la néovascularisation et la reconfiguration des vaisseaux pulmonaire dans le SDRA.

Preface

Candidates have the option of including, as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly-duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

If this option is chosen, **connecting texts that provide logical bridges between the different papers are mandatory.** The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". **The thesis must include:** A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional materials must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors of the co-authored papers. **Under no circumstances can a co-author of any component of such a thesis serve as an examiner for that thesis.**

This thesis is based on two papers co-authored by the candidate:

1. **Khoury, J. and D. Langleben**. Platelet-activating factor stimulates lung pericyte growth in vitro. *Am J Physiol* 270 (*Lung Cell Mol Physiol* 14): L298-L304, 1996.

2. **Khoury, J. and D. Langleben**. Effects of endotoxin and inflammatory cytokines on lung pericyte proliferation in-vitro. Submitted to *Am J Physiol* (*Lung Cell Mol Physiol*), 1996

Contribution by the candidate:

In the first paper, the candidate's contribution include all the laboratory work, including, but not limited to, pericyte culture, growth experiments elicited by PAF, and studies of apoptosis. The candidate assisted in the preparation of the manuscript, mainly done by the co-author, Dr. David Langleben.

In the second paper, the candidate's contribution include all the laboratory work, including, but not limited to, pericyte culture, growth experiments elicited by LPS, cytokines and PAF, and studies of apoptosis. The candidate assisted in writing the paper including writing sections of *Materials and Methods* and parts of *Introduction* and *Discussion*.

The candidate wishes to acknowledge the great help supplied by Drs. Langleben, Michel, and Brisson in the editing process and helpful advice in the preparation of the thesis manuscript.

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Last, but not least, a special thanks to Wenlin Shao, for always being with me, helping me, teaching me, encouraging me, and caring about me, "Wo ai ni".

Table of Contents

List of Abbreviations

AGEPC	acetyl glycerol ether phosphoryl choline
ARDS	adult (acute) respiratory distress syndrome
BSA	bovine serum albumin
mCD14	membrane CD14 receptor
sCD14	soluble CD14 receptor
FBS	fetal bovine serum
FIO_2	fraction of inspired oxygen
Gal	galactose
Glu	glucose
GluNAc	N-acetylglucosamine
Hep	heptose
HBSS	Hanks balanced salt solution
IRDS	infantile respiratory distress syndrome
IFNγ	interferon gamma
IL-1β	interleukin-1 beta
IL-6	interleukin-6
IL-8	interleukin-8
IV	intravenous
Kdo	ketodeoxyoctonate
LBP	lipopolysaccharide (lipid) binding protein
LPS	lipopolysaccharide (endotoxin)
MHC	major histocompatability complex
NK	natural killer cells
PC	pericytes
PAF	platelet-activating factor
rac-PAF	racemic platelet-activating factor
PH	pulmonary hypertension
SMC	smooth muscle cells

Chapter 1

Review of literature and general introduction

1.1 **Introduction**

Endotoxin, lipopolysaccharide (LPS) is an integral part of the outer membrane of gram-negative bacteria. LPS aids the bacteria in nutrient transport and defence against toxic compounds (94,100). However, when gram-negative bacteria multiply, die, or lyse, LPS is released from their surface and into the blood stream (93,100).

LPS is thought to act as a trigger initiating a generalized inflammatory response. Various activated cells contribute to the inflammation by releasing potent protein and phospholipid mediators (94,100,121), which in turn, cause decreased barrier function of the endothelial layer of capillary and pre-capillary segments (61,93,94).

Gram-negative sepsis is an important cause of the adult respiratory distress syndrome and pulmonary hypertension. Pulmonary vascular remodelling reduces the cross sectional area of the lung bed, increasing vascular resistance (70,130). Pericytes, located abluminally on the wall of pre-capillary arteries, are thought to play a role in the remodelling process (49,70,130).

Although the effects of LPS and the sepsis-related mediators have been well studied in the onset and progression of disease, their direct effects on the growth of lung pericytes has not been studied.

1.2 **SEPSIS and Septic Shock**

1.2.1 Definition

Sepsis is defined as the presence of various pathogenic organisms, or their toxins, in the blood or tissues, resulting in a systemic response (3). Septic shock is defined as sepsis with hypotension (ie a systolic blood pressure of less than 90 mmHg) and perfusion abnormalities (16). Sepsis and septic shock are two clinical syndromes that can be triggered by many microorganisms, including gram-negative and gram-positive bacteria, and fungi (61,75). In essence, the septic process can result from any type of infectious process. The only feature which differentiates a septic patient from an infected or bacteremic patient is the systemic response (3). Sepsis may lead to multiple organ dysfunction syndrome, adult respiratory distress syndrome (ARDS), and death (23,61,73,86,95,112).

1.2.2 Epidemiology

The incidence of sepsis has increased in recent years due to changes in the practice of medicine, including the use of aggressive chemotherapy and of corticosteroids and immunosuppressants for organ transplantations or treatment of inflammation, and the increase in invasive procedures (3,23). According to a 1990 publication from the Centre for Disease Control, the incidence of sepsis diagnosed in the United States of America increased from 164,000 in 1979 to 425,000

cases in 1987 (23,61). It is estimated that approximately 40% of these cases were due to gram-negative bacteria (17,25,61,113). Therefore, approximately 170,000 cases of gram-negative sepsis were reported in 1987 in the USA. Since 10 - 40% of gram-negative septic patients develop ARDS (3,17,31,61,62), a conservative 25% estimate would yield approximately 42,500 patients who developed ARDS from gram-negative sepsis that year. Moreover, the mortality rate of ARDS is estimated to be as high as 60% (3,17,31,61,62), yielding an estimated 38,000 deaths in 1987 from gram-negative sepsis-induced ARDS in the USA alone.

1.2.3 Pathogenesis of Sepsis and Septic Shock

Many mediators cause the progression of septic shock, however, it is believed that endotoxin, lipopolysaccharide (LPS), mediates the first stages of the cascade in septic shock (24,44,61,93). LPS infection starts the systemic inflammatory response which involves the release of several cytokines and phospholipid mediators. These mediators will be discussed later in this chapter in greater detail.

The release of these mediators, and the LPS itself, are capable of causing endothelial cell injury, increased platelet adhesion, and vasodilation and vasoconstriction (8,44,54,61,112,118). These vascular events, in turn, lead to septic shock. Septic shock may lead to multiple organ dysfunction syndrome affecting the heart, brain, kidneys, lung, the gastro-intestinal tract, and other organs. The pulmonary entity of

multiple organ dysfunction syndrome involves the adult respiratory distress syndrome (ARDS), which may lead to pulmonary hypertension.

1.3 **Adult Respiratory Distress Syndrome**

1.3.1 History

Many names were once applied to this syndrome, making categorization of the disease and obtaining pertinent statistics almost impossible. In 1967, a group led by Ashbaugh and Petty coined the term adult respiratory distress syndrome (ARDS) (1) because of its similarities to the infantile respiratory distress syndrome (IRDS). However, the term "adult" may not have been ideal since they found that children also developed the same clinical syndrome (86). Many have now replaced the word "adult" with the word "acute" (55,85-87,99). Since 1967, the term ARDS has been universally adopted and has replaced past terminology including congestive atelectasis, shock lung, traumatic wet lung, Da Nang lung, and other terms (24,85,86).

1.3.2 Identification and Clinical Description of ARDS

ARDS is a clinical syndrome and the diagnosis is still based on the clinical observations made by Ashbaugh et al. in 1967 (1): marked respiratory distress, diffuse pulmonary infiltration on chest radiographs, decreased pulmonary compliance, marked impairment in oxygen transport despite ventilatory assistance, pulmonary congestion. Hyaline

membrane formation and other findings of diffuse alveolar damage are the light microscope findings.

Ashbaugh and Petty (1,88) described that patients with ARDS "suddenly develop marked tachypnea, dyspnea and cyanosis which is refractory not only to nasal oxygen but also to intermittent positive pressure breathing". The general early clinical picture includes the requirement of a high inspired oxygen fraction (FIO_2 ~1.0) (86), and sudden onset of intrapulmonary shunt (20,61,84,86). Acute respiratory distress usually develops between 1 hour and 96 hours after the initial insult, and usually within 12-48 hours of the predisposing event (61,86,88); rarely, it can resolve within a few days (84). In many cases, ARDS may take months to clear and a year before lung function tests become once again normal (84). Progressive ARDS can lead to pulmonary hypertension, which may develop within six to ten days after the onset of ARDS (49).

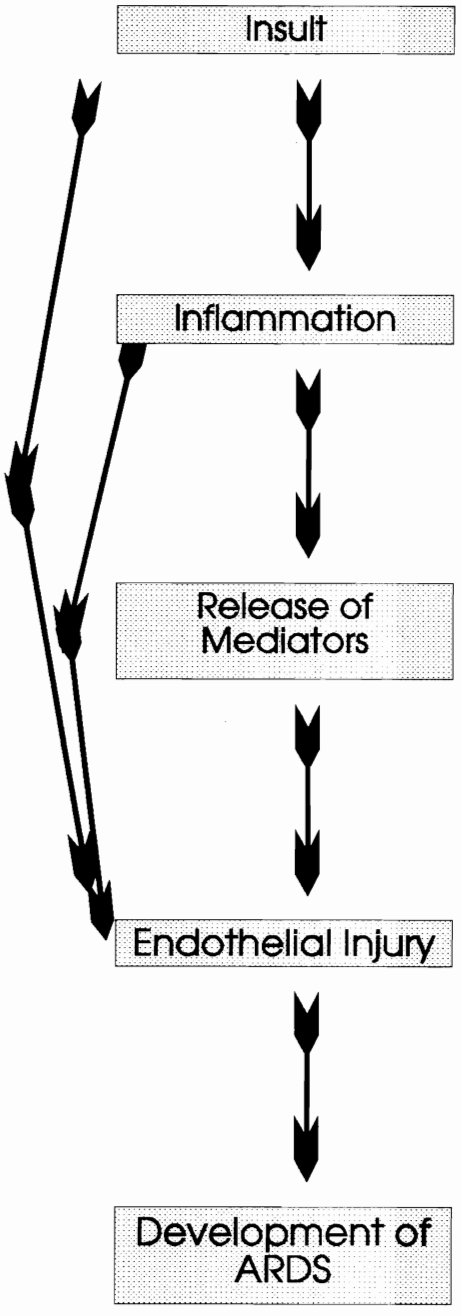
1.3.3 Epidemiology

Many types of injury can produce ARDS (see fig.1), the risk of developing ARDS increases with the number of clinical disorders that develop in the patient. The major triggers for ARDS are: 1) sepsis syndrome, 2) aspiration of gastric contents, 3) pulmonary trauma, 4) multiple emergency transfusions, 5) multiple major fractures, 6) near drowning, 7) acute pancreatitis, and 8) prolonged hypotension

Figure 1.

Many factors can cause ARDS, including sepsis. The progression of disease can be partially explained by this flow chart. Insult, such as sepsis, can lead not only to inflammation, but also directly to endothelial injury. The same is true for inflammation. Conversely, mediators can also cause further inflammation in the host.

ARDS



: Trauma, Surgery, Sepsis, Burns, Hyperoxia

: Neutrophils, Macrophages, and other cells

: Cytokines, Lipid Mediators, Oxygen Free Radicals

: Loss of barrier function, adhesion molecule upregulation, amplification of inflammation

: Acute severe hypoxemia, diffuse pulmonary infiltrates on chest radiographs, no evidence of heart failure

(24,61,84,86). Sepsis is the most common clinical disorder leading to the development of ARDS. Among patients with sepsis, the incidence of ARDS is 18% to 38% (24,61,86). ARDS may develop in septic patients as early as six hours after the onset of sepsis (24,86). In addition to being a trigger for ARDS, bacterial infections may be a secondary complication in established ARDS. Bacterial infection is found in 98% of patients with ARDS post-mortem (7). Ongoing sepsis is detectable in 73% of patients who died of ARDS after more than 72 hours of onset (73). It has been suggested that sepsis, rather than respiratory failure, is the leading cause of death among patients with infection-induced ARDS (7,61,73,88).

Aspiration of gastric contents (Mendelson's syndrome) is a potentially preventable problem occurring mainly in debilitated patients, and much less frequently after general anesthesia (24). ARDS associated with trauma, severe hypotension, and multiple transfusions may develop within 24-48 hours after initial injury, but is accelerated when two or more of these clinical disorders occur (24,84). However, the incidence, severity, and mortality is greatly increased in patients with increased levels of tumor necrosis factor alpha (TNFα) in plasma as compared to patients with no detectable TNFα (61).

1.4 **Pulmonary Hypertension**

Acute and subacute lung injury often causes pulmonary hypertension (49). Pulmonary hypertension (PH) is defined as an

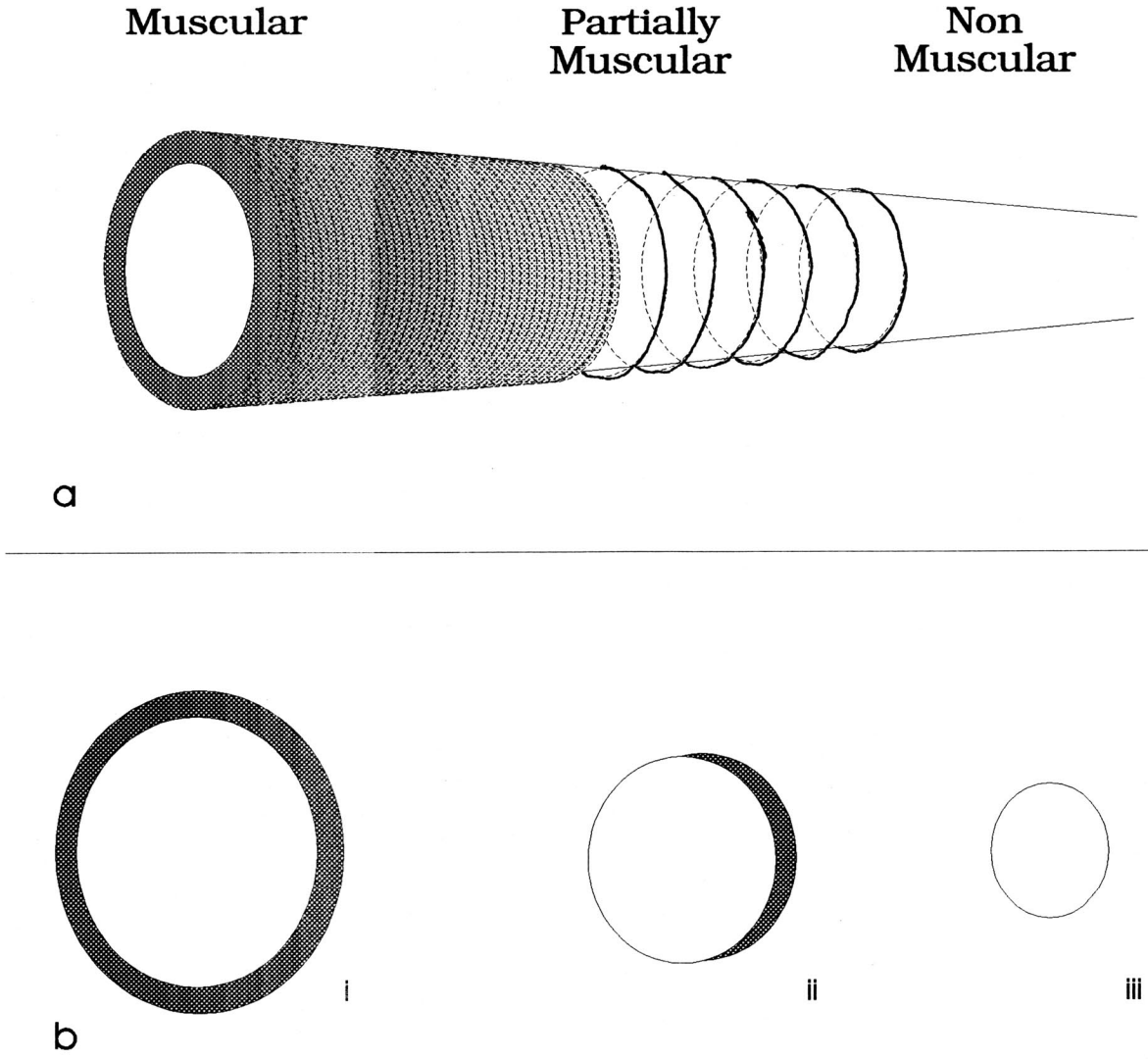
elevated pulmonary artery pressure, usually from an increase in vascular tone or thickening of the walls of small pulmonary arteries (37). In sepsis and ARDS, injury to the pulmonary microcirculation causes endothelial injury, disruption of the vessel wall matrix, vascular smooth muscle cell proliferation and structural remodelling of precapillary units (49,115). This remodelling causes narrowing of the lumen of the vessels throughout the lung leading to increased pulmonary vascular resistance and pulmonary artery pressure (48,49,70,130). In clinical ARDS, pulmonary hypertension increases mortality (12,130).

1.4.1 Vascular Remodelling

Vascular cell proliferation, leading to structural remodelling and neomuscularization of precapillary segments, is seen in many types of PH. In the rat, the normal precapillary segments of the pulmonary artery may be divided into three types (see fig 2): muscular, partially muscular, and nonmuscular, the latter generally more distal than the former (70). In the pre-acinar segment of the vessel, differentiated smooth muscle cells (SMC) form a complete layer in the pulmonary artery, and these vessels are termed "muscular". This smooth muscle is the principal contributor to tone in the pulmonary artery (48,49,70). Within the acinus, the medial layer of SMC gradually become incomplete and envelops the intima in a "partially muscular" spiral manner leading to crescentic appearance of muscle in cross section (70). As the diameter of

Figure 2.

Precapillary segments can be divided into three types, muscular, partially muscular which spiral around the artery, and non-muscular (a). The middle part of the figure (b) shows cross sectional features of the walls of muscular, crescentic partially muscular, and thin walled non-muscular. Bottom (c) electron microscopy reveals the presence of pericytes and intermediate cells in the non-muscular and partially muscular segments of the artery. Modification of reference 78.


Muscular
Partially Muscular
Non Muscular
a
i
ii
iii
b
M
M
M
M
M
P
Lumen
M
M
M
I
c

the artery decreases further and approaches the capillary, there is an absence of mature SMC in the wall giving a "nonmuscular" appearance by light microscopy (70).

Further examination of the rat peripheral lung circulation reveals the presence of two cell types in the nonmuscular segment of the artery that are thought to represent precursor smooth muscle cells (see figure 2) (69,70). In the nonmuscular regions of the partially muscular arteries, intermediate cells are found (termed as such due to their intermediate positioning between pericytes and mature SMC), and in the nonmuscular arteries, pericytes are found (70). When stimulated appropriately, in pulmonary hypertensive states, these cells are believed to be able to differentiate morphologically to obtain characteristics of mature SMC, and to contribute to muscularization of the microvasculature. In all, there are at least five cell types in the vessel wall from the hilum to the lung periphery: endothelial cells, SMC and its precursors, intermediate cells and pericytes, and the adventitial fibroblasts (48). Endothelial cells are found throughout the vascular bed whereas precursor SMC are found only in the pre-capillary microvessels (48,70). However, heterogeneity of SMC, in terms of phenotypic maturity, has been detected in the media of large fully muscular arteries from proximal to distal segments of the arterial wall, suggesting that immature cells may also be present in more proximal vessels (109). Immunohistochemistry reveals that all the SMC demonstrate specific markers for smooth muscle

(smooth muscle-specific actin, vimentin, and desmin) but not all hybridize with extracellular matrix proteins elastin or collagen (109).

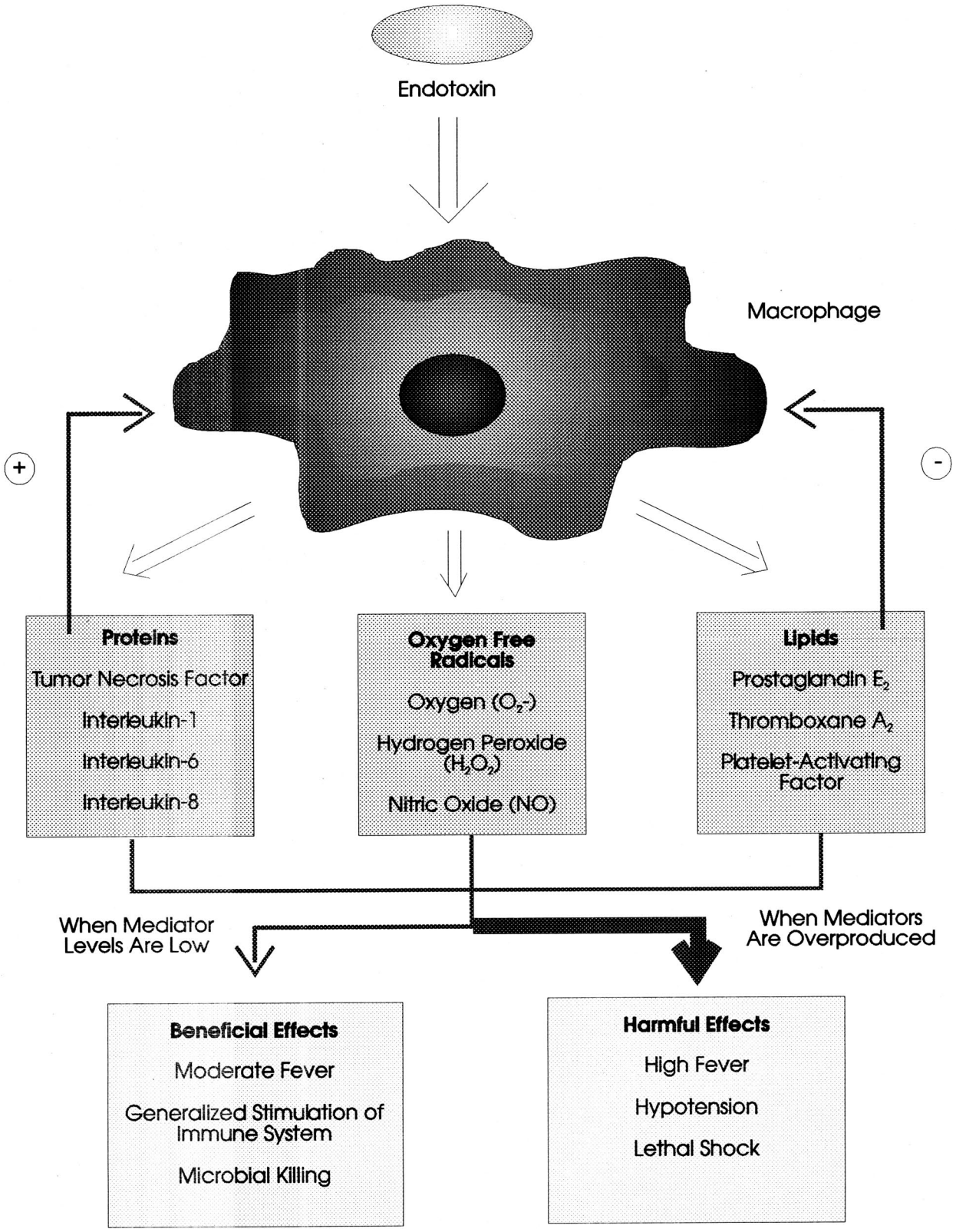
1.4.2 Pathogenesis of Arterial Wall Remodelling

The endothelium's luminal position in the wall of the microvessels makes it most susceptible to the action of toxic agents carried in the blood, and endothelial cell damage is a hallmark of acute lung injury (49,56). Endothelial injury increases the release of factor VIII, and plasminogen activator inhibitor, and decreases prostacyclin production, allowing for platelet aggregation, and thrombosis (49).

Nitric oxide produced by endothelial cells is a potent vasodilator which has antiproliferative effects on cultured SMC (36). In endothelial injury, loss of normal nitric oxide production may contribute to vasoconstriction and vascular narrowing. Endothelin is a peptide produced by endothelial cells which causes vasoconstriction (129) and stimulates the proliferation of SMC, pericytes and fibroblasts (29). Endothelin, therefore, may also contribute to the vascular narrowing. Platelet-derived growth factor released from platelets, macrophages, and endothelial cells, stimulates the proliferation of SMC and pericytes (28,98,102). Cytokines, including IL-1, IL-6, IL-8, TNFα, and IFNγ have also been implicated as possible mediators in the pathogenesis of PH in ARDS (8,54).

Figure 3.

Endotoxin stimulate macrophages and other cells to produce various potent mediators, which may be sub-divided into three groups: proteins, oxygen free radicals, and lipids. When produced in low levels, these mediators have beneficial effects, but when overproduced, these mediators lead to the systematic inflammatory response, possibly leading to ARDS and pulmonary hypertension. In addition to its other functions, TNF amplifies mediator synthesis, while prostaglandin E_2 inhibits this synthesis. Modification from reference 93.


Endotoxin
Macrophage
+
-
Proteins
Tumor Necrosis Factor
Interleukin-1
Interleukin-6
Interleukin-8
Oxygen Free Radicals
Oxygen (O_2-)
Hydrogen Peroxide (H_2O_2)
Nitric Oxide (NO)
Lipids
Prostaglandin E_2
Thromboxane A_2
Platelet-Activating Factor
When Mediator Levels Are Low
When Mediators Are Overproduced
Beneficial Effects
Moderate Fever
Generalized Stimulation of Immune System
Microbial Killing
Harmful Effects
High Fever
Hypotension
Lethal Shock

1.5 **Mechanisms and Mediators**

1.5.1 Cellular mechanisms

1.5.1.1 *Macrophages, Neutrophils and Platelets*

Bacterial endotoxin causes the release of many inflammatory mediators, including monokines, lymphokines, complement, platelet activating factor, and others. These activate alveolar macrophages which, in turn, produce a variety of bioactive lipids including thromboxane A_2, lipooxygenase products, and platelet-activating factor (see figure 3). The lipid mediators may have direct vasoconstrictor or vasodilator activity and are chemoattractants that recruit PMNs into the alveolar space (45). PMNs marginate endothelial cells in the capillary, further producing factors that contribute to injury of the microvascular endothelial cells and result in increased permeability of pulmonary capillaries (20,24,33,40,45,61,96,122). Platelets are thought to be recruited by macrophage- and neutrophil-derived platelet-activating factor. These platelets then release serotonin and cyclooxygenase products such as thromboxane A_2, which contribute to the increased vascular tone and airway resistance, characteristic of gram-negative endotoxemia (31,33,40,45,59)

1.5.1.2 *Pericytes*

Pericytes (PC), literally meaning from the Greek origins, *peri*,

around; *cyto*, cell or vessel, are found around pre-capillary arteries, capillaries, and post-capillary venuoles. PC are found in virtually all vertebrate vessels (106), and were first described in lungs by Weibel (123). Thus, PC are polymorphic, elongated, multibranched cells wrapped around endothelial cells (for review, see (101,106)). In culture, PC are large, spreading cells with irregular edges, and when PC reach confluence, they form multilayered bundles or nodules. The morphology of PC differentiates them from the cobblestone morphology of endothelial cells, the long spindle-shape of fibroblasts, and the compact and fusiform shape of SMCs.

Morphology, however, is not the only way to distinguish PC from endothelial cells or fibroblasts. PC show staining for smooth muscle actin and non-smooth muscle actin (79,107), desmin and vimentin (34), tropomyosin (51,52), and cyclic GMP (cGMP) - dependent protein kinase for contractility (50). PC do not stain for the endothelial marker factor VIII (106). Distinguishing PC from dedifferentiated SMC remains problematic since there is no known specific marker for PC.

Pericytes have several important roles in 1) regulating endothelial proliferation and differentiation; 2) contraction and regulation of capillary blood flow; and 3) as progenitor cells (101).

Microvessel development is characterized by endothelial cell proliferation and migration, which is modulated by the appearance of PC (2). Orlidge and D'Amore showed that endothelial cells co-cultured with

fibroblasts, epithelial cells, or 3T3 cells stimulated endothelial cell growth (83). Furthermore, close proximity between PC and SMC was required for these inhibitory effects. In an experimental culture system which separated PC or SMC from physical contact with endothelial cells, but which allowed for diffusion of soluble factors, endothelial cell proliferation was not inhibited (83). Such studies support a role for PC in the regulation of endothelial cell growth.

Pericytes contain cGMP-dependent protein kinase, an enzyme important in SMC contraction (50). Pericytes demonstrate tension development and contractility *in vitro* (53).

Pericytes are thought to be capable of transforming into SMC (68,106), and other cell types (106). PC lack the dense bodies of SMC on electron microscopy, but contain the SMC markers. In cases of prolonged hypoxia, PC can transform to SMC (26,53,101).

Together, all three features of PC can contribute to the vascular remodelling and increased vascular resistance seen in pulmonary hypertension.

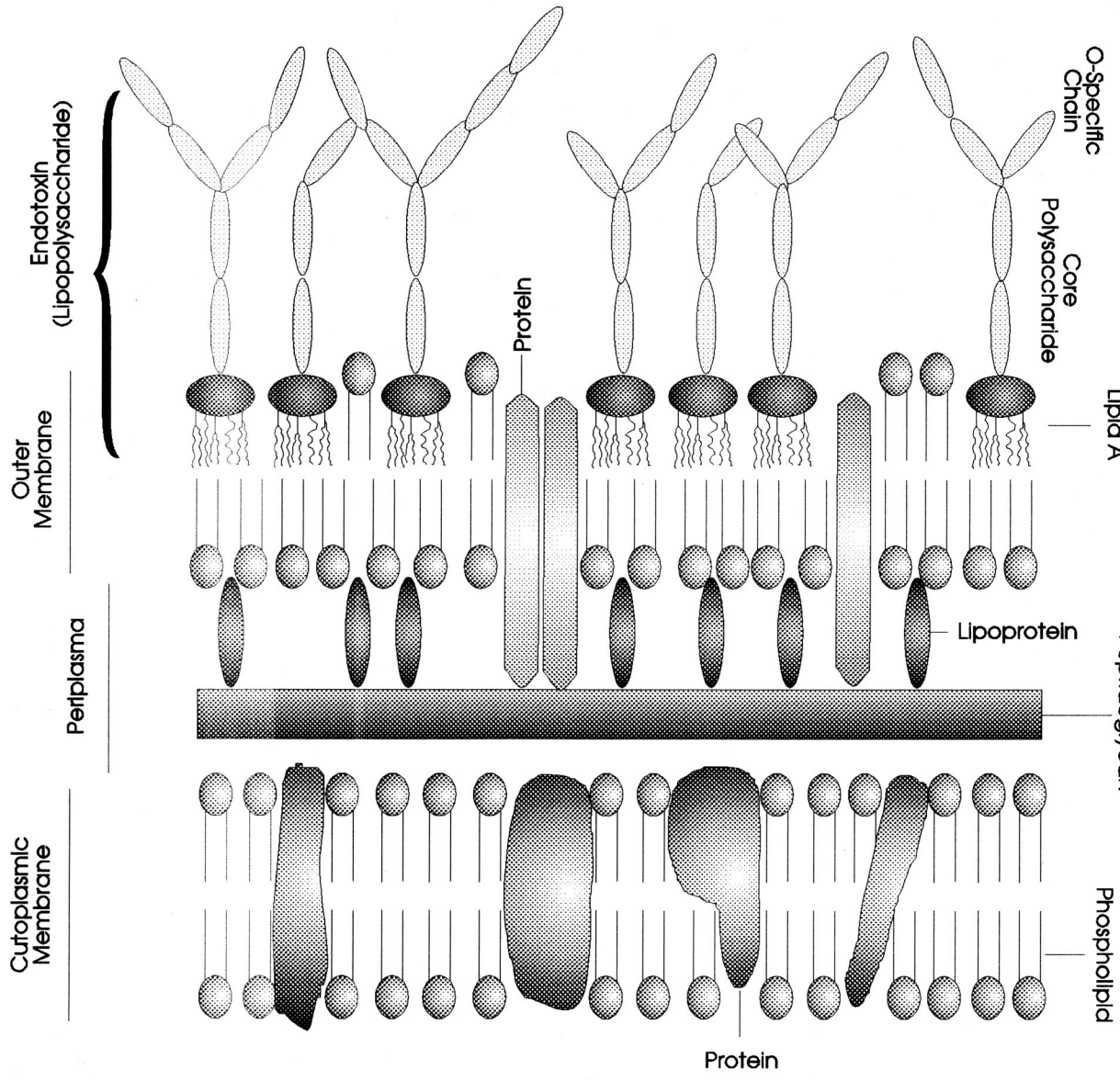
1.5.2 Mediators involved in Sepsis and Septic Shock

1.5.2.1 *Endotoxin*

Bacteria are surrounded by an envelope that retains the shape and integrity of the microbial body. In the case of the gram-negative bacteria, this envelope represents an asymmetrical bilayered membrane called the

Figure 4.

Endotoxins are located in the outer membrane of the gram-negative bacteria. This outer membrane is unique to gram-negative bacteria, it surrounds the cytoplasmic membrane found on gram-positive bacteria, and other cells. Modification of reference 19.


Endotoxin
(Lipopolysaccharide)
Outer
Membrane
Periplasma
Cytoplasmic
Membrane
O-Specific
Chain
Core
Polysaccharide
Lipid A
Protein
Lipoprotein
Peptidoglycan
Phospholipid
Protein
Cell Interior

outer membrane, which is found surrounding the cytoplasmic membrane present in gram-positive and other cells. The outer membrane is constructed not only of phospholipids, as is the plasma membrane, but also contains lipids, polysaccharides, and proteins (see fig 4). The lipid and polysaccharide are linked in the outer membrane to form lipopolysaccharide (LPS). The outer membrane restricts entrance of toxic compounds such as antibiotics, and plays an important role in nutrient transport and pathophysiological interactions of the bacteria with the host. Therefore, the integrity of the membrane, especially the LPS component, is essential for bacterial viability. In fact, mutants that are unable to produce LPS on their surface are not viable (94,100). Due to its important role, and its exposed position, LPS is an ideal target for attack by antibodies and other immunological or pharmacological agents (reviewed in (93,94,100)).

When gram-negative bacteria multiply, die, or lyse, LPS is released from their surface. Small amounts of endotoxin are regularly absorbed into the bloodstream from the intestine and detoxified in the liver (89). Release of large amounts of LPS initiates the toxic manifestations of severe gram-negative infections and generalized inflammation (93).

1.5.2.1.1 *Structure and Effects*

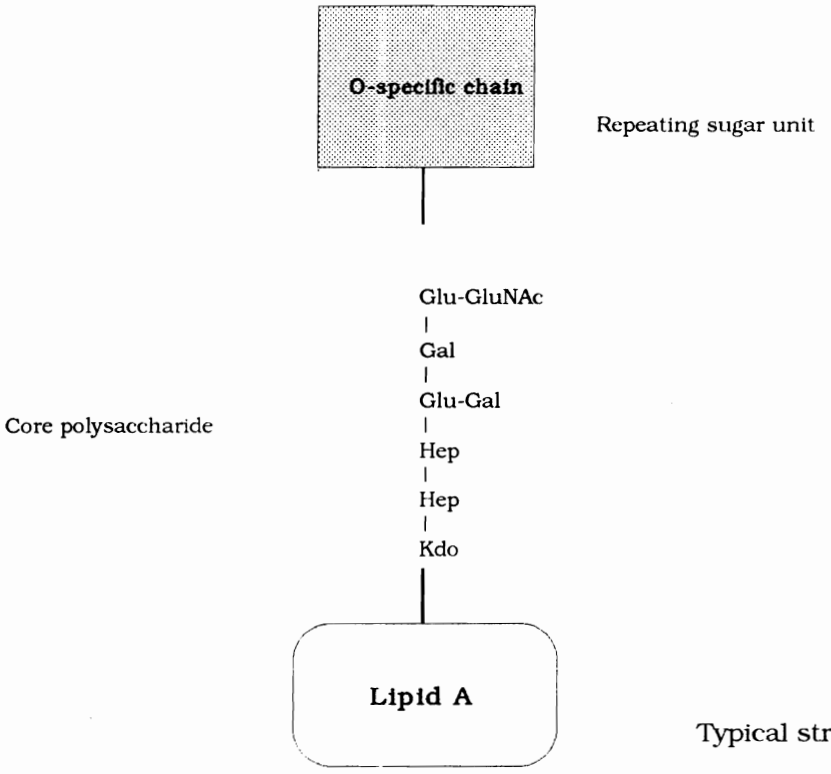
LPS consists of a hydrophillic heteropolysaccharide, and a covalently bound lipid component termed lipid A (94). Because of its

profound pharmacological effects on animal cells, especially those of the immune system, lipid A was originally called, and is still referred to, as endotoxin (100). The heteropolysaccharide may be subdivided into the O-specific chain and the core oligosaccharide.

The O-specific chain, the longer of the sugar chains, projects from the core and is the outermost part of endotoxin (93). The O-specific chain is the most variable segment of the LPS. It consists of six-carbon sugars including glucose (Glu), galactose (Gal), rhamnose, and mannose, connected in repeating four- or five-sugar sequences. The core polysaccharide links the lipid A to the O-specific chain and has a minimally varying structure. It consists of six-carbon sugars and N-acetylglucosamine (GluNAc) linked to heptose (Hep, seven-carbon sugar) and ketodeoxyoctonate (Kdo, 3-deoxy-D-manno-2-octulosonic acid) an eight-carbon sugar which has not been detected elsewhere in nature except in certain plants and algae (93).

Lipid A does not have the structure of a usual glycerol lipid, but instead the fatty acids, including β-hydroxymyristic, lauric, myristic, and palmitic acid are connected by ester links to N-acetylglucosamine (19). Lipid A possesses all the biologic activities of "endotoxin" (19,93). Synthetic and natural E.coli lipid A produces the same effects of the whole LPS molecule including lethal toxicity, pyrogenicity, induction of prostaglandins synthesis in macrophages, and antigenic specificity (35). Furthermore, lipid A has been shown to be the portion of LPS mainly

responsible for induction of IL-1 activity (58).



Typical structure of LPS

1.5.2.1.2 *Mechanism of Toxic Action*

Endotoxins are not thought to produce their toxic effects by killing host cells or by inhibiting cellular functions. Rather, LPS is thought to act as a trigger, requiring the active response of the host cells. LPS, through lipid A, interacts with macrophages and monocytes, endothelial cells, SMC, polymorphonuclear granulocytes, and thrombocytes (94,100,121). Of importance is the effect of endotoxin on macrophages,

resulting in production of cytokines IL-1, IL-6, IL-8, and TNFα, oxygen free radicals, lipids prostaglandin, thromboxane, and platelet-activating factor. These secondary mediators are then capable of eliciting high fever, hypotension, and lethal shock. Conversely, the same endotoxins that threaten human health can enhance the body's overall immune resistance to bacterial and viral infections as well as cancer (see figure 3) (93). If the mediators are produced in low levels, beneficial effects such as moderate fever, stimulation of immune system, and microbial killing are observed (93,94,100).

In order for LPS to elicit the production of secondary mediators by macrophages, LPS binding protein (LBP) and either the macrophage membrane bound CD14 (mCD14) receptor or the serum bound soluble CD14 (sCD14) receptor must be present (22,126-128). LPS binds the circulating LBP molecule which can then link to the CD14 receptor. This finding makes endotoxin one of the few known substances that interact with a receptor only after first forming a complex with a circulating protein (93).

1.5.2.1.3 *Effects of Endotoxin*

A single infusion of endotoxin into animals has been used as a model of the response in lungs of patients with sepsis-induced adult respiratory distress syndrome (ARDS). Hypoxemia and pulmonary infiltrates develop after the infusion (66). High doses of endotoxin

usually result in death from shock, but doses of 0.05μg/kg intravenously (IV) almost always allow the animal to recover so that the effects may be analyzed (18). Fifteen minutes following a single IV endotoxin infusion, there is already evidence of accumulation and margination of neutrophils and accumulation of activated lymphocytes; by 60 minutes, endothelial and vessel wall damage is observable by microscopy (67). Endothelial cells exposed to endotoxin undergo contraction, become pyknotic, and finally die (66). Although endotoxin may cause direct injury to the pulmonary endothelium (61,93,94), its other major role is to activate the inflammatory cascade, which includes production of cytokines. Early, the pulmonary endothelial barrier is destroyed, increased capillary permeability develops, and neutrophils adhere to the endothelium with subsequent migration into the tissues (20,24,44,61,71,86,88,112). The clinical picture then includes acute respiratory distress with decreased lung compliance, hypoxemia, and noncardiogenic pulmonary edema (20,44,49,61,88,112). The release or activation of inflammatory mediators, free radicals and cytokines intensifies the vascular injury (49,61,115). As endothelial injury becomes widespread, multiple organ failure including the kidneys, brain, intestine, and liver develops (for review, see (49,55,61,95)). These findings support the conclusion that, in patients with septicemia, endotoxin is a key factor in the development of ARDS.

1.5.2.2 *Surfactant*

The primary function of the lung is the delivery of oxygen and the removal of carbon dioxide across a very thin alveolar-capillary membrane. Surfactant lines the alveoli and decreases the surface tension, facilitating alveolar expansion. Decreased surfactant has been detected in ARDS (1,20,86,88). Surfactant deficiency could cause alveolar instability and collapse, resulting in hydrostatic forces which could aggravate and increase pulmonary edema (1,86). However, the reduction in surfactant levels in ARDS is secondary rather than the primary problem (20,86).

1.5.2.3 *Complement system, Cytokines, Oxygen Radicals and Other Mediators*

Complement activation contributes to the development of ARDS. C3a and C5a, which are potent neutrophil chemoattractants, are increased in bronchoalveolar lavage fluid in patients with ARDS (97). Complement is activated during gram-negative sepsis, and in patients at risk for developing ARDS, and others who have established ARDS (66,97). However, C5a can not be used as a predictor for the progression of ARDS (62,97,124).

A number of cytokines have been implicated in the development of ARDS. Tumor necrosis factor alpha (TNFα), interleukin (IL) 1β, IL-2, IL-

6, IL-8 and interferon gamma (IFNγ) have all been identified as mediators present in ARDS and as contributors to the pulmonary hypertension (8,54,114). The principal cell involved in mediating the effects of endotoxin is the macrophage, and its release of TNFα may be an important event in sepsis (13,47,105). Each of the above mentioned cytokines will be discussed in greater detail later in this chapter.

Arachidonic acid metabolites also contribute to the ARDS. Cyclo-oxygenase products thromboxane A_2 and prostaglandin F_2 have been implicated as initial mediators of pulmonary hypertension and prostaglandin F_2 for bronchoconstriction following gram-negative sepsis (59,108,122). Oxygen radicals derived from neutrophils, platelets, and endogenous catalytic reactions, including superoxide, hydrogen peroxide and peroxynitrite are also considered as mediators of ARDS (24,33). Anti-oxidants such as catalase, glutathione peroxidase, and superoxide dismutase may have a beneficial role in therapy of experimental ARDS as they may reduce the concentrations of toxic oxygen free radicals (24).

1.5.2.4 *Platelet-Activating Factor*

Platelet-activating factor (PAF) is a biologically active phospholipid with a broad spectrum of inflammatory and pathophysiologic effects. Benveniste et al., in 1972, first described this phospholipid as a leukocyte-derived activator of platelets, hence the name "platelet-activating factor" (10). PAF is now known to have biologic effects on a

wide range of cells and tissues including macrophages, monocytes, neutrophils, T lymphocytes, vascular endothelial and SMC, and platelets, as well as in blood, heart, and lung. It is derived from an equally broad variety of cells and tissues including leukocytes, macrophages, platelets, and endothelial cells. PAF can be detected in blood, bronchoalveolar lavage fluid, and lung tissues (for review, see (41,64)). The structure of PAF was first determined in 1979 by Demopoulos et al. as 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, also termed acetyl glycerol ether phosphoryl choline (AcGEPC) (27). The length of the alkyl chain varies (39). Two molecular species, differing only in the length of their alkyl chains, were isolated from rabbit basophils (39), as 1-O-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine and 1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine. In terms of their length of alkyl chain and degree of unsaturation, they are also identified as 16:0 PAF and 18:0 PAF, respectively. It is now known that the heterogenous molecular structure of PAF may vary from 15:0 to 22:2, although 16:0 PAF, with lesser amounts of 18:0 PAF comprise the predominant active species (11,27,41,64).

PAF is recognized to be an important mediator in a variety of inflammatory processes (9), acting on many cell types, including pulmonary vascular endothelial cells (30,41). Experimental animals injected with either 16:0- or 18:0-PAF display rapid systemic hypotension, pulmonary hypertension, increased vascular permeability,

decreased lung compliance, and increased pulmonary resistance when injected with either 16:0- or 18:0-PAF (41,63,64). PAF is also implicated in endotoxic shock, and macrophages activated by LPS secrete PAF (57,63,93). PAF is a very potent mediator, cells release PAF in small amounts, and its extreme potency makes these small amounts significant in the cell and tissue response (41).

1.5.2.5 *Cytokines*

"Cytokine" is a term used for extracellular signalling molecules secreted by various effector cells. These effector cells include macrophages, neutrophils, T-lymphocytes, B-lymphocytes, fibroblasts, and endothelial cells. The primary function of cytokines is the ability to modify the behaviour of closely adjacent cells, or the effector cell itself, working in a paracrine or autocrine manner respectively (8,38,43,54). The following is a mini-review of the cytokines thought to be involved following lipopolysaccharide (LPS) release into the circulation during sepsis and during lung injury (43,44,47,61).

Levels of interleukin (IL) 1β, IL-6, IL-8, TNFα, and interferon gamma (IFNγ), as well as the phospholipid platelet-activating factor (8,15,32,54,78,125) increase during sepsis. Tumor necrosis factor alpha (TNFα) is also an important mediator of lung injury during sepsis, contributing to the effects of endotoxin in animal models (8,74,105,110,116).

1.5.2.5.1 *Interleukin 1*

Interleukin 1 (IL-1) is classified into two different proteins, IL-1α and IL-1β, which are the products of different genes (60). IL-1 is not produced by cells of healthy individuals (91). However, IL-1β is the predominant active protein species secreted by monocytes in response to stimulation by LPS (60), and macrophages and other cells greatly increase IL-1β production in response to stimuli such as those produced by infections or bacterial endotoxin (91). IL-1 is known to activate thymocyte proliferation, induce endothelial cell activation, chemotaxis for PMNs, macrophages, and lymphocytes, and it acts on macrophages inducing its own synthesis as well as the production of TNFα and IL-6 (reviewed in (8,54,82,91,104)). It also increases vascular permeability (103). If IL-1 is produced in appropriate low concentrations, it may enhance the immune defence, but if it is produced in elevated quantities, IL-1 alone, or in combination with TNFα may induce many of the features of septic shock (5,90,92). Experimental infusion of IL-1β (5μg per kg) induces hypotension in rabbits, and the combination of IL-1β (1μg/kg) and TNFα (1μg/kg) results in a shock-like state with pulmonary edema and hemorrhage, leukopenia, and thrombocytopenia (81). Although TNFα appears to induce more damage in the lung than IL-1β, the

combination of both produces much greater injury (81). This suggests that these cytokines may act synergistically in the disruption of the pulmonary vascular endothelium. IL-1 concentrations are increased in ARDS patients in response to LPS, and are further increased by LPS and IFNγ (46). IL-1 may contribute to the development of pulmonary hypertension: an IL-1 receptor antagonist inhibits pulmonary hypertension in the rat monocrotaline model (120).

1.5.2.5.2 *Interleukin 6*

Interleukin 6 (IL-6) is a glycoprotein produced by activated T and B cells, macrophages, endothelial cells, fibroblasts, various carcinomas, and a wide variety of other cells (91,119). IL-6 production in fibroblasts is constitutive *in vitro*, but it may be increased by viral infections, LPS, IL-1, TNFα, IFNγ and platelet-derived growth factor (reviewed in (8,54,91,119)).

Plasma levels of IL-6 are increased during septic shock and may cause fever (8) Il-6 produces hypertension in rats (72). IL-6 is produced by fibroblasts in the lung and its release can be increased greatly by IL-1 and TNFα (8,54,104). This in turn stimulates thymocyte proliferation in the same manner as IL-1 (8,54,80,104). Thus, IL-6 produced by fibroblasts may mediate or amplify the effects of IL-1.

1.5.2.5.3 *Interleukin 8*

Inteleukin 8 (IL-8) is a peptide produced by macrophages, T cells, neutrophils, endothelial cells, epithelial cells, and fibroblasts in response to stimulation by IL-1, TNF, LPS, and viral infections (8,91,104). This effect is not seen if the cells are pre-treated with IL-2, IL-6, or IFNγ (reviewed in (8,91,104)). IL-8 is a very potent and selective chemotactant for neutrophils, with little chemotactic effects on eosinophils or basophils, and no effect on platelets, macrophages and monocytes. IL-8 also induces neutrophils to release proteolytic enzymes, produce oxygen radicals such as superoxide, and increase surface adhesion molecules, enhancing adherence of neutrophils to endothelial cells (8,104). Production of IL-8 is increased following hypoxia-reoxygenation (65) or following administration of endotoxin (6). Elevated levels of IL-8 have been found in bronchoalveolar lavage fluid of patients with ARDS as well as in pulmonary fibrosis and septic shock (8,91,104). Thus, IL-8 may play a role in the development of ARDS.

1.5.2.5.4 *Interferon gamma*

Interferon (IFN) is a group of proteins which is subdivided into three major types; alpha (α), produced by leukocytes; beta (β), from fibroblasts; and gamma (γ), produced by T lymphocytes and natural killer (NK) cells. Type I interferons, including IFNα and IFNβ, share a common receptor and have similar functions, while type II interferons or IFNγ has

a distinct receptor and serves a different function from type I interferon (reviewed in (4,8,91,104)).

IFNγ interacts closely with other cytokines, enhancing the immune response by inducing major histocompatibility complex (MHC) class I and II proteins and increasing expression of cytokine receptors (4). IFNγ also increases production of IL-1, platelet-activating factor, and hydrogen peroxide, as well as down regulating IL-8 induced by IL-2 (91). When added to LPS, IFNγ is capable of inducing TNFα in endotoxin-resistant C3H/HeJ mouse macrophages *in vitro* (15); and along with IL-2, IFNγ produces both TNFα and TNFβ in human peripheral blood mononuclear cells (78). IFNγ also increases survival and reduces sepsis in mice with surgical wound infections (42).

1.5.2.5.5 *Tumor Necrosis Factor alpha*

Tumor necrosis factor (TNF) consists of two proteins that bind to the same receptors and produce a vast range of effects. TNFα is also known as cachectin, and TNFβ is also known as lymphotoxin. TNFα is produced by neutrophils, activated lymphocytes, macrophages, endothelial cells, smooth muscle cells, and other cells, while TNFβ is produced by lymphocytes (reviewed in (8,54,104,105)). TNF was originally noted for its ability to kill certain tumor cells directly, but its main action now consists of initiating the cytokine cascade and

production of other factors involved with the inflammatory response, such as IL-1, and platelet-activating factor (8,54,105).

TNFα plays a major role in sepsis and the development of ARDS. Infusion of purified recombinant TNFα into rats reproduces many deleterious effects of sepsis, including shock, hypotension, multiple organ failure, and death as a result of respiratory arrest (74). The strongest evidence supporting an important role for TNFα in septic shock and ARDS is that passive immunization against TNFα with a highly specific polyclonal antiserum protects mice from the lethal effects of LPS (14). Moreover, a monoclonal anti-TNF antibody prevents septic shock in baboons given lethal doses of LPS (117). Guinea pigs which were injected with recombinant human TNFα have increased pulmonary microvascular permeability and edema similar to that seen with gram-negative sepsis (110).

TNFα increases the expression of endothelial cell adhesion molecules thereby enhancing neutrophil adherence to pulmonary endothelium, an important early event in ARDS (111). Cachectin directly stimulates the endothelial biosynthesis of IL-1 (77), and the release of platelet-activating factor from macrophages, PMNs, and vascular endothelial cells (21). TNFα also generates superoxide and cyclooxygenase products from neutrophils (76).

1.6 **Rationale and Research Objectives**

Gram-negative sepsis is a well recognized cause of the acute respiratory distress syndrome (ARDS) and subsequent pulmonary hypertension. Pulmonary vascular remodelling, through neomuscularization of microvessel, is also well established in the course of the disease. Pericytes, as precursor smooth muscle cells, are thought to play a role in the process of muscularization.

The lung as an internal organ, is extremely difficult to study when examination is directed to the microvasculature. Acute lung injury involves many mediators and many cell types. To understand the events relative to the contribution of each cell and mediator, and their importance, they must be studied individually. *In vitro* experiments, while not a complete simulation of *in vivo* events, allow clear definition of effects of mediators on cells.

Gram-negative lipopolysaccharide (LPS) acts as a trigger causing the generalized inflammatory response. Macrophages, endothelial cells, and other activated cells release mediators, such as cytokines and phospholipids, in response to the sepsis. The roles of these mediators are well documented in inflammation and progression of injury. However, the direct effects of LPS, and the sepsis derived cytokines and platelet-activating factor (PAF) on the growth of pulmonary vascular pericytes *in vitro* is not known.

Knowing that LPS can directly cause increased permeability and

damage of endothelial cells, and due to the close proximity of PC to endothelial cells, we hypothesized that:

- LPS diffusing into the vessel wall might itself have a proliferative effect on PC.
- LPS elicits its effects in both serum and serum free media.
- A combination of LPS and PAF will result in increased proliferation compared to each individually.
- A combination of LPS and the mixture of cytokines will result in increased proliferation compared to each individually.

Knowing that LPS induces the release of PAF and several cytokines including interleukin 1 beta (IL-1β), IL-6, IL-8, tumor necrosis factor alpha, and interferon gamma, the hypotheses we have attempted to explore are:

- PAF has direct effects on PC growth and it acts via the PAF receptor.
- PAF elicits its effects in both serum and serum free media.
- The individual cytokines have a proliferative effect on PC.
- A mixture of cytokines similar to those found *in vivo* has a proliferative effect on PC.

Finally, hypothesizing that these mediators and LPS would induce a proliferative effect, we would not expect a harmful effect from their

addition to PC. Hence, we hypothesize:

- The addition of LPS or the mediators will not cause cell death or apoptosis of the PC.

Our specific goal was to study the direct effect of these putative mitogens on pericytes, with the overall goal being a better understanding of the vascular remodelling process following endotoxic sepsis. This work brings new insights into the pathophysiology of the acute respiratory distress syndrome and pulmonary hypertension, two disorders that cause high morbidity and mortality in septic patients.

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Chapter 2

Platelet-activating factor stimulates lung pericyte growth in vitro

2.1 **Preface**

Platelet-activating factor (PAF) is released from a variety of cells in response to sepsis. The role of PAF in inflammation and progression of injury have been previously described, but its direct effects on the growth of lung PC is not known.

In this article, we show that PAF may act as a direct mitogen on PC growth and proliferation *in vitro*. This may give insights as to how circulating PAF, during sepsis, may directly have a role in the vascular remodelling process in pulmonary hypertension.

Furthermore, we show that PAF acts via a PAF receptor as the proliferative effects were inhibited by the addition of the receptor antagonist CV-3988.

2.2 **ABSTRACT**

Platelet-activating factor (PAF) is released from activated leukocytes and endothelial cells in sepsis, lung injury and the Adult Respiratory Distress Syndrome. With these disorders, pulmonary hypertension develops, partly due to muscularization of the microvasculature by proliferation of pericytes. PAF may be a mediator of this process. Therefore, we examined the effects of PAF on in-vitro growth of rat lung pericytes. As compared to control growth, semi-synthetic PAF (10^{-9} M) stimulated the 7-day mean growth of proliferating pericytes by 31% in medium with serum and 29% without serum, and of previously growth-arrested pericytes by 12% with serum and 23% without serum. These effects were blocked by the PAF-receptor blocker CV-3988. PAF also increased ^{3}H-thymidine incorporation into pericytes by 79%. Synthetic 16:0-PAF stimulated pericyte growth, but 18:0 PAF did not. PAF exposure did not induce apoptosis in pericytes. Thus, PAF compounds, similar to those found in-vivo, stimulate lung pericyte growth in-vitro. PAF may act as a direct cytokine on cells involved in muscularization of the pulmonary vessel walls.

2.3 INTRODUCTION

Platelet-activating factor (PAF) encompasses a group of structurally related ether phospholipids that have been implicated as mediators in sepsis and acute lung injury (4,5,8-10,15,26,38). PAF, upon release from leukocytes (2,36) and activated endothelial cells (7,24,42), increases vascular permeability and alters vessel tone (9,13,16,18,26,28,31). Infusions of PAF cause pulmonary hypertension in animals (25,30).

The pulmonary hypertension in humans which can occur with sepsis, lung injury and the Adult Respiratory Distress Syndrome (ARDS) (43), results in part from structural remodelling of the pulmonary vasculature (21,41), developing over days to weeks. This includes muscularization and narrowing of the arterial bed, with, in more distal segments, proliferation and phenotypic maturation of precursor smooth muscle cells such as pericytes and intermediate cells (21,41). Circulating PAF, and PAF synthesized by endothelial cells lying immediately adjacent to the precursor smooth muscle cells, may contribute to the vascular remodelling.

Previous studies of PAF's actions in the lung have focussed on its effects on vascular tone, permeability, and structure. However, the effects of PAF on proliferation of individual constituent cells of the pulmonary microvasculature have not been examined. We have therefore studied the effects of several PAF molecules and a PAF receptor

on rat lung pericyte growth in-vitro.

2.4 **MATERIALS and METHODS**

2.4.1 Pericyte culture

Pericytes were extracted from the lungs of male Sprague-Dawley rats (Charles River Laboratories, Saint Constant, Quebec, 200-400 grams), using a modification of previously described methods (11,17). All animals were handled in compliance with the guidelines of the Canadian Council on Animal Care. After inducing deep anesthesia with chloroform, the heart and lungs were removed en-bloc, and the lungs dissected free. Peripheral, subpleural pieces of lung tissue were minced with a tissue chopper (McIlwain, Brinkmann Inc, Rexdale, Ontario) and then suspended in Ca^{++} and Mg^{++}-free Hanks Balanced Salt Solution (HBSS, Life Technologies, Grand Island, N. Y.), containing 0.01% W/V type CLSPA collagenase (Worthington Biomedical, Freehold, N. J). After a 30 minute incubation at 37° C, the tissue suspension was passed through a 100 μm Nitex nylon mesh (Thompson Inc, Mont-Royal, Quebec), and the filtrate was centrifuged and resuspended twice using exchanges of MCDB-131 medium (23) containing 10% fetal bovine serum (FBS, Hy-Clone Laboratories, Logan, Utah) as a wash solution. The cell pellet was finally resuspended in MCDB-131 medium with 10% FBS, plated onto T25 plastic tissue culture flasks, and incubated at 37° C, in

5% CO_2 in air during initial culture and for all subsequent experiments. Culture media were subsequently changed twice weekly. Pericytes were passaged using 0.05% trypsin and 0.02% EDTA in HBSS, with the immediate addition of serum-containing medium following passage. Cells in first passage were used for all experiments.

The pericytes demonstrated previously described morphologic characteristics, including ragged margins, a lack of contact inhibition, and formation of macroscopic mounds at high density (11,17). As assessed by immunoperoxidase staining, they contained smooth muscle actin (antibody MA-935, Enzo Biochemical, New York, N. Y.), and did not produce factor VIII coagulant (Dako Corp., Santa Barbara, CA). Pericytes did not have angiotensin-converting enzyme activity (Hycor Biomedical, Portland, ME). Bovine pulmonary artery endothelial cells and human skin fibroblasts served as control cell populations for the characterizations.

2.4.2 Platelet-activating factors

It is now recognized that several molecules share the biologic activity of PAF (27,36), and they have the general structure of 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (AGEPC) (3,12). In the present studies, we have used a semi-synthetic 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (PAFs 7568 and 9525) (Sigma Chemical, St Louis MO,

catalogue nos. P7568 and P9525) prepared from bovine heart lecithin, as originally described by Demopoulos et. al. (12). PAF 7568 is a lyophilized preparation and PAF 9525 is a chloroform solution. In addition, we have studied the effects of synthetic PAF C16, 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphorylcholine, and PAF C18, 1-O-octadecyl-2 acetyl-sn-glycero-3-phosphorylcholine, (Bachem California, Torrance, CA, Catalogue numbers LPAF10 and LPAF20, respectively). Racemic PAF (rac-PAF) (Sigma Chemical, cat. no. P1402) and a competitive PAF receptor antagonist, CV-3988 (BIOMOL, Plymouth Meeting, PA) were also utilized.

PAF9525, supplied in a chloroform solution which was evaporated under dry nitrogen, was redissolved in 100% ethanol and stored at -20° C until use. PAF 7568, PAF-C16 and C18, rac-PAF, and CV-3988 were all dissolved in 100% ethanol and stored at -20° C. Immediately prior to use of all agents, the ethanol was evaporated in a Univapo-100H speed-vac (Uni Equipment, Martinsmed, Germany) for 10 minutes, and the agents were then resuspended in phosphate-buffered saline containing 0.125% bovine serum albumin (BSA, fraction V, Boehringer Mannheim, Mannheim, Germany).

2.4.3 Experimental Design

Pericytes, in a non-proliferative state in the normal pulmonary bed,

proliferate as part of the remodelling process in disease. PAF exposure as part of lung injury might occur before the release of other mitogenic mediators, with PAF acting alone on pericytes, or after other mediator release, with PAF acting in concert with the other mediators. Therefore, we studied the effects of PAF on both quiescent and proliferating pericytes, with the quiescent pericytes simulating an in-vivo microvascular bed that had not yet been exposed to other cytokines, and with serum-exposed, proliferating pericytes simulating a microvascular bed that had already been primed by other mitogens and then exposed to PAF.

Pericytes were passaged into 24 well tissue culture plates (8 wells per experimental condition, unless otherwise stated), and allowed to attach for 24 hours in MCDB-131 + FBS. The medium was then removed.

To study growth response of proliferating pericytes to varying PAF dosage, fresh MCDB-131 + 10% FBS was added, containing 0 to 10^{-6} M concentrations of the various PAF molecules. For other non-growth-arrest experiments, fresh MCDB-131 + 10% FBS was added, containing 0 or 10^{-9} M concentrations of PAF 7568, without (Control) or with 10^{-7} M CV-3988. In other wells, to study the effects of PAF 7568 on serum-exposed cells in the subsequent absence of serum, MCDB-131 +0.3% BSA was used.

For initial growth-arrest experiments, after a 24 hour attachment period in MCDB-131 + FBS, the growth phase of the pericytes was synchronized by changing the medium to MCDB-131 + 0.3% BSA for 72 hours. After this period, at the start of the experiments, the medium was changed to MCDB-131 + 10% FBS, without (Control) or with PAF 7568 or CV-3988. To examine the effects of PAF on growth-arrested pericytes in the ongoing absence of serum, the medium in other wells was replaced with MCDB-131 + 0.3% BSA after growth arrest.

The culture dishes were examined daily for cell detachment or lysis. On day 7 of each experiment, the pericytes were detached using 0.25% trypsin, and the cell counts per well were determined using a haemocytometer.

2.4.4 Thymidine incorporation studies

In other experiments, to measure the effects of PAF on ^{3}H-thymidine uptake, pericytes were growth arrested for 72 hours by serum starvation in MCDB-131 + 0.3% BSA. For each time point and treatment, six wells were utilized for measurement of ^{3}H-thymidine uptake, and two identically treated wells were used for measurement of total protein concentration. The pericytes were then exposed to thymidine-free MCDB-131 + 0.3% BSA, with or without 10^{-9} PAF 7568 for 12, 24 and 30 hours. Methyl ^{3}H-thymidine (100 µCi/well,

Amersham, Buckinghamshire, UK) was subsequently added for a two hour incubation. The medium was removed, the cells were washed with cold phosphate-buffered saline, 1 ml of cold 10% trichloroacetic acid was added per well, and the dishes were kept at 4° C for one hour . The wells were then washed twice more with 10% trichloroacetic acid, the acid was removed, 1ml 1N NaOH was added per well, and the well contents well mixed. After a one hour solubilization at room temperature, 200 µl of the solution was added to 5 ml scintillation fluid (EcoLite +, ICN Biomedicals, Mississauga, Ont.) and the radioactivity measured in a scintillation counter (Pharmacia Wallac, Turku, Finland). For measurement of total protein concentration, the two other wells were washed with cold PBS and then the protein precipitated with cold 10% trichloroacetic acid at 4°C for one hour. The protein was then solubilized with 1N NaOH at room temperature for one hour. Protein concentration was measured with a spectrophotometer at 750λ absorption wavelength, using the Lowry method.

2.4.5 Studies of cell apoptosis

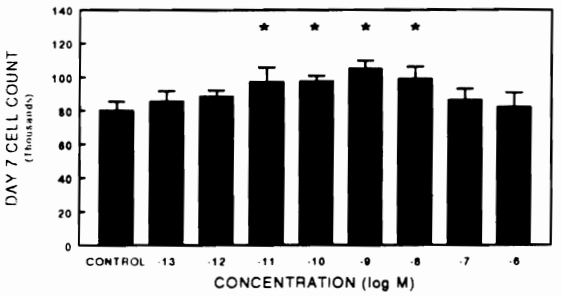
The nuclear DNA- laddering technique was used to detect whether cellular apoptosis was caused by exposure to PAF 7568 (14,39). Pericytes were passaged into 100mm tissue culture dishes (n=3 for each condition and time point), and allowed to grow to subconfluence in

MCDB-131 + 10% FBS. Next, the pericytes were exposed to PAF 7568 (0 or 10^{-9} M), with or without CV-3988, for 3 or 7 days. The medium was then removed, the plates rinsed in PBS, and 1 ml of lysis buffer (TRIS 10 mM, EDTA 10 mM, NaCl, 100mM) containing 200 ng/ml proteinase K (Sigma) was added for 2 minutes at room temperature. The cells were scraped off with a rubber policeman, and the lysate was frozen at -20°C. in sterile storage tubes. To extract DNA, the lysate was thawed, and sodium dodecyl sulfate was added to achieve a 1% V/V final concentration. The solution was incubated for three hours, and an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added. After thorough mixing and centrifugation for 10 minutes (2000g at 20° C.), the upper aqueous fraction was removed and subjected to another phenol-chloroform-isoamyl alcohol extraction. The DNA was then precipitated by adding 2.5 volumes of ice cold 100% ethanol, gentle agitation, and incubation at -20°C. for 20 minutes. After centrifugation (1400g) to include all fragmented DNA, the supernatant was aspirated and the pellet was washed twice in ice cold 70% ethanol, air dried, and resuspended in sterile water. For each experimental time point, the DNA samples (5µg each) were then loaded onto a 1.5% nonreducing agarose gel with Tris 0.1M, boric acid 0.1M, EDTA 20 mM, along with a 100bp DNA ladder marker (Pharmacia Biotech, Uppsala, Sweden) and DNA from a leukemia cell line rendered apoptotic by exposure to retinoic acid.

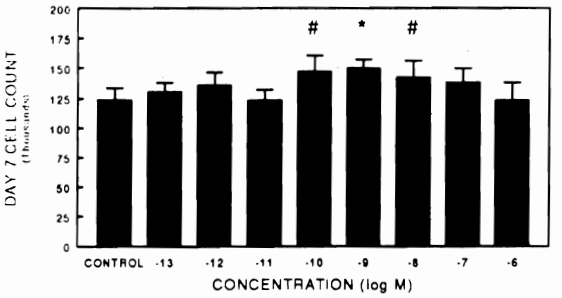
Figure 1.

Day 7 cell counts of pericytes grown in absence (control) or presence of various platelet-activating factor (PAF) molecules at varying concentrations. PAF 7568 (top) and PAF 9525 (middle) stimulate pericyte growth. *P<0.001 and #P=0.05 vs. control. Bottom: racemic PAF (rac-PAF) has no effect on growth. Bars are means +/- SD; n=8 per group.

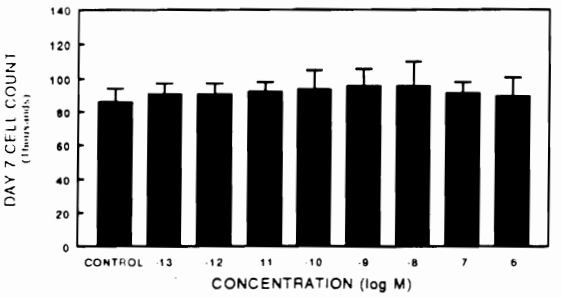
PAF 7568



PAF 9525



rac-PAF



After electrophoresis (75V, 50mA, 4 hours), the gels were stained with ethidium bromide and photographed under ultraviolet light.

2.4.6 Data analysis

Data are presented as the group mean ± 1 standard deviation (SD). To compare differences between groups, one-way analysis of variance was performed followed, where appropriate, by the Tukey-Kramer analysis for multiple comparisons. P values < 0.05 are taken to be significant.

2.5 **RESULTS**

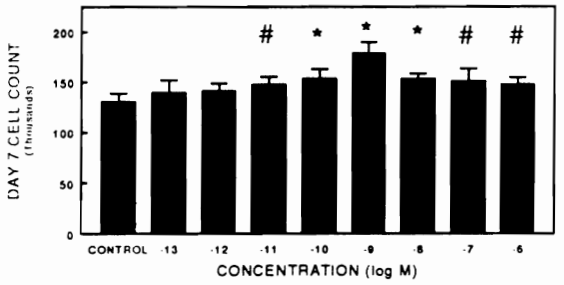
2.5.1 Growth response of proliferating pericytes to various PAF molecules

At concentrations of 10^{-11} to 10^{-8} M, PAF 7568 increased pericyte growth as compared to control growth (figure 1, top). The most potent concentration was 10^{-9} M, which increased mean growth by 31%. For subsequent experiments, PAF 7568 was studied at 10^{-9} M. PAF 9525 increased growth in a similar fashion (figure 1, middle), although less effectively. Racemic-PAF, as expected, did not alter proliferation (figure 1, bottom). Synthetic PAF C16 also stimulated pericyte growth, by a mean of 37% at 10^{-9} M (figure 2, top). Synthetic PAF C18 did not stimulate growth at any concentration. (Figure 2, middle). A mixture of

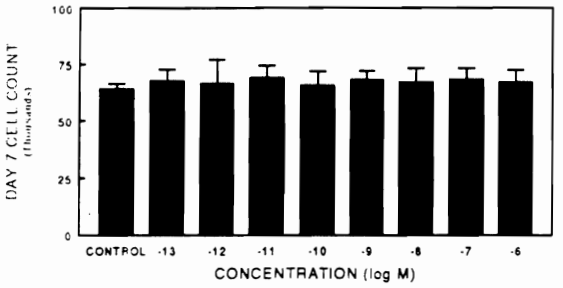
Figure 2.

Day 7 cell counts of pericytes grown in absence (control) or presence of synthetic PAF molecules at varying concentrations; n=8 per group. PAF C16 (top) and a mixture of PAF C16 and C18 (bottom) stimulate pericyte growth. *$P<0.001$ and #$P<0.02$ vs. control. Middle: PAF C18 alone has no effect on growth.

BACHEM PAF C16



BACHEM PAF C18



BACHEM PAF C16-C18 MIX

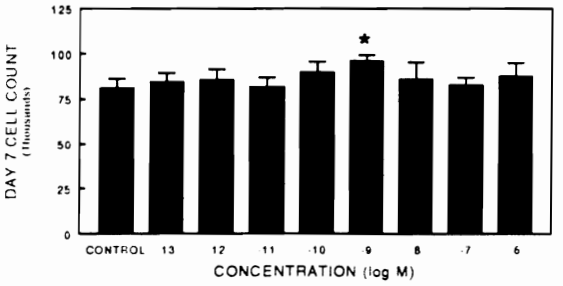
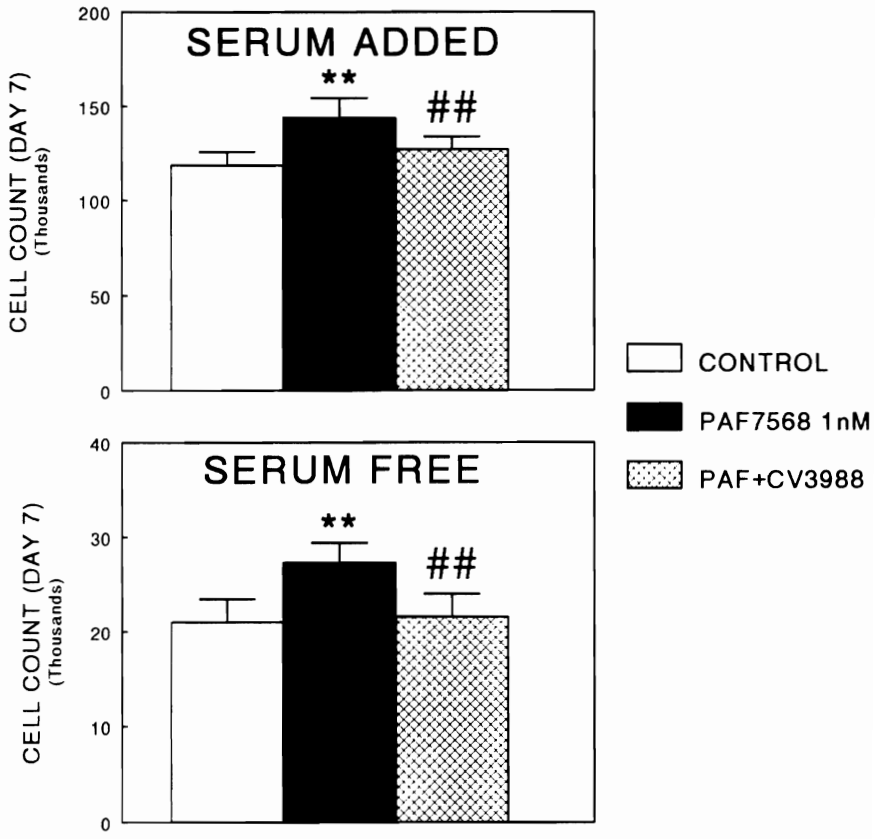


Figure 3.

Day 7 cell counts of pericytes that were not growth arrested, then exposed to PAF 7568 (10^{-9}M; solid bars) in presence (top) or absence (bottom) of serum in culture medium. PAF 7568 induces growth (**P<0.0001 vs. control). PAF-receptor blocker CV-3988 added to PAF (crosshatched bars) blocks this effect. ##P=0.002, PAF and NS vs. control; n=8 per group.


SERUM ADDED
CELL COUNT (DAY 7)
(Thousands)
200
150
100
50
0
**
##
SERUM FREE
CELL COUNT (DAY 7)
(Thousands)
40
30
20
10
0
**
##
CONTROL
PAF7568 1nM
PAF+CV3988

70% PAF C16 and 18% PAF C18 stimulated growth only at 10^{-9} M, although the growth seen at 10^{-10} M was nearly significantly different from that of control cells (p=0.058).

2.5.2 Non-growth-arrest experiments (pericytes initially proliferating)

Pericytes that had not been previously growth-arrested multiplied in MCDB-131 + FBS, to 118625 ± 7170 cells on day 7 (figure 3, top). PAF 7568 increased this growth by 21% (p<0.001). Addition of CV3988 to the PAF 7568 resulted in significant growth inhibition, and this latter group did not proliferate more than the control group. Addition of serum-free medium to the pericytes at the start of the experiment (figure 3, bottom) resulted in a complete inhibition of growth during the 7 day incubation. However, addition of PAF 7568 stimulated growth by 29% (p<0.001), an effect which was completely blocked by CV3988.

2.5.3 Growth-arrest experiments (pericytes initially quiescent)

Pericytes that had been previously growth-arrested and then exposed to MCDB-131 + FBS over the 7 day experiment (figure 4, top) grew to a mean count of 183250 ± 16585 cells on day 7. PAF 7568 increased this growth by 12% (p=0.02), and CV3988 prevented the PAF 7568-induced growth. Pericytes which were not exposed to serum over the 7 days did not grow (figure 4, bottom). Addition of PAF 7568 stimulated growth of these pericytes by 23% (p=0.001), and CV3988

Figure 4.

Day 7 cell counts of pericytes that were growth arrested by serum deprivation, then exposed to PAF 7568 (10^{-9}M; solid bars) in presence (top) or absence (bottom) of serum in culture medium. PAF 7568 induces growth (*P=0.02 and **P=0.001 vs. control). PAF-receptor blocker CV-3988 blocks this effect (#P<0.02 and ##P=0.001 vs. PAF, and NS vs. control); n=8 per group.

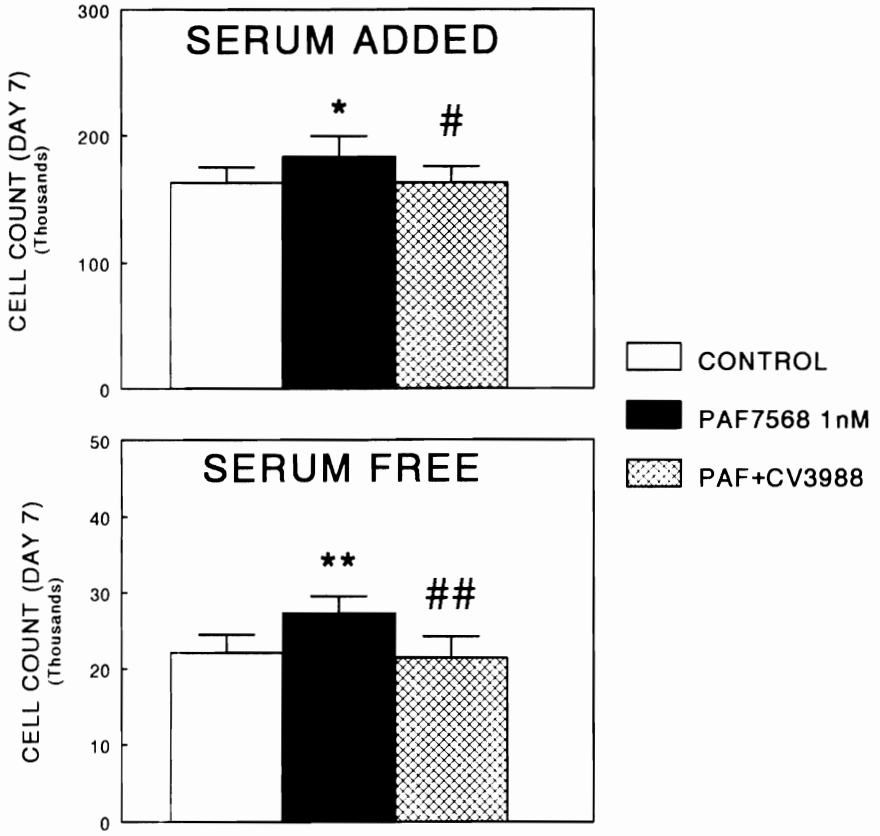

SERUM ADDED
CELL COUNT (DAY 7)
(Thousands)
300
200
100
0
*
#
SERUM FREE
CELL COUNT (DAY 7)
(Thousands)
50
40
30
20
10
0
**
##
CONTROL
PAF7568 1nM
PAF+CV3988

Figure 5.

(^{3}H) Thymidine incorporation (dpm/μg protein) in serum-free medium by growth-arrested pericytes at 12, 24, and 30 h after exposure to PAF 7568 ($10^{-9}M$) alone (solid bars) or with CV-3988 (hatched bars). *$P<0.001$ vs. control (open bars); n=6 per group.

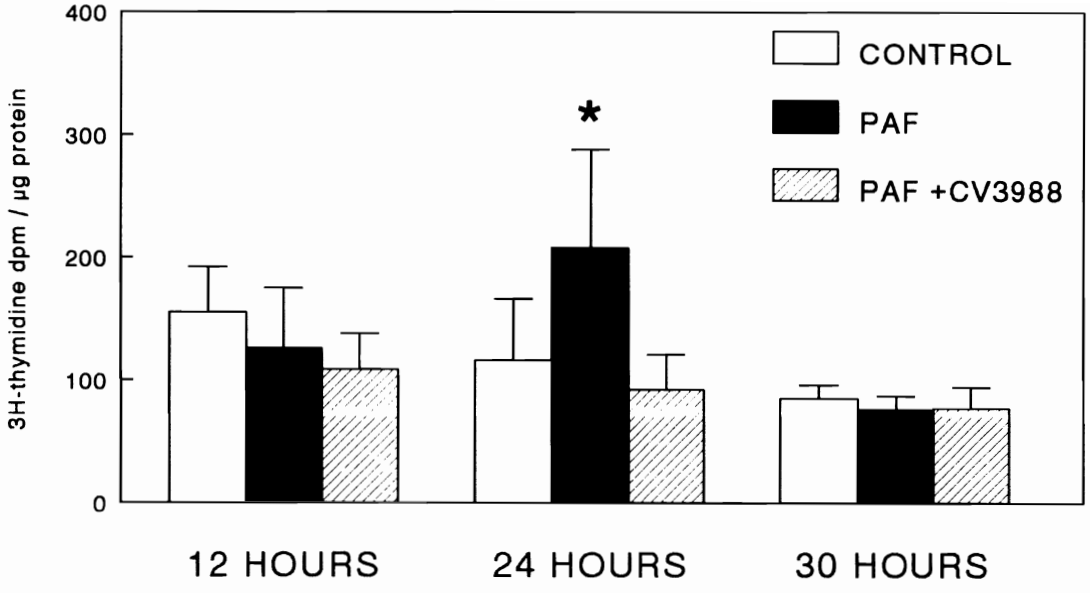
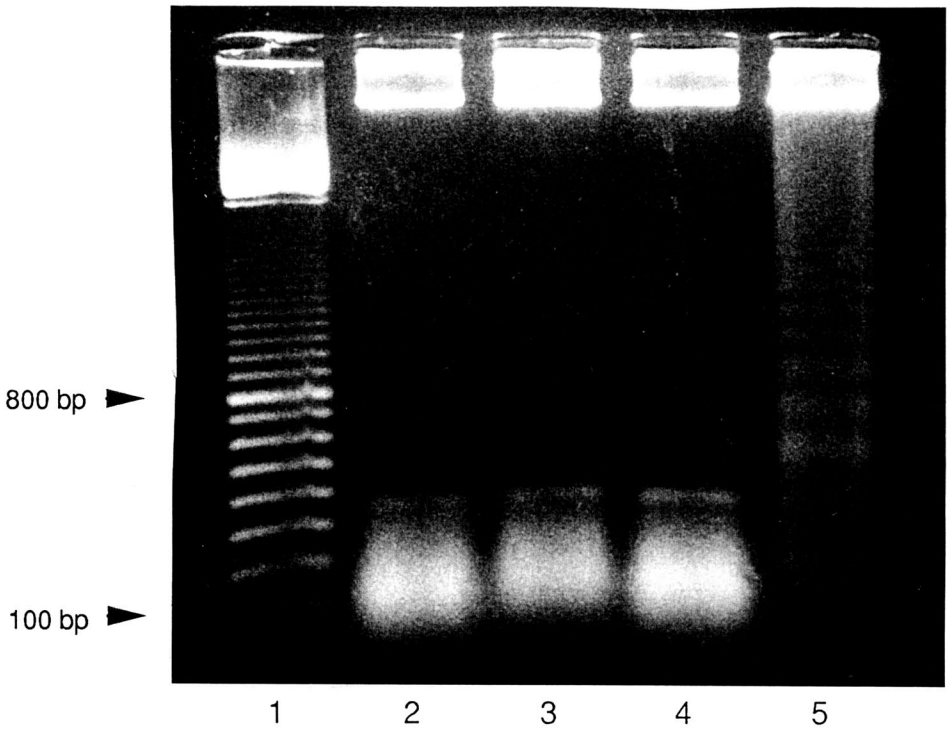

400
300
200
100
0
3H-thymidine dpm / µg protein
CONTROL
PAF
PAF +CV3988
*
12 HOURS
24 HOURS
30 HOURS

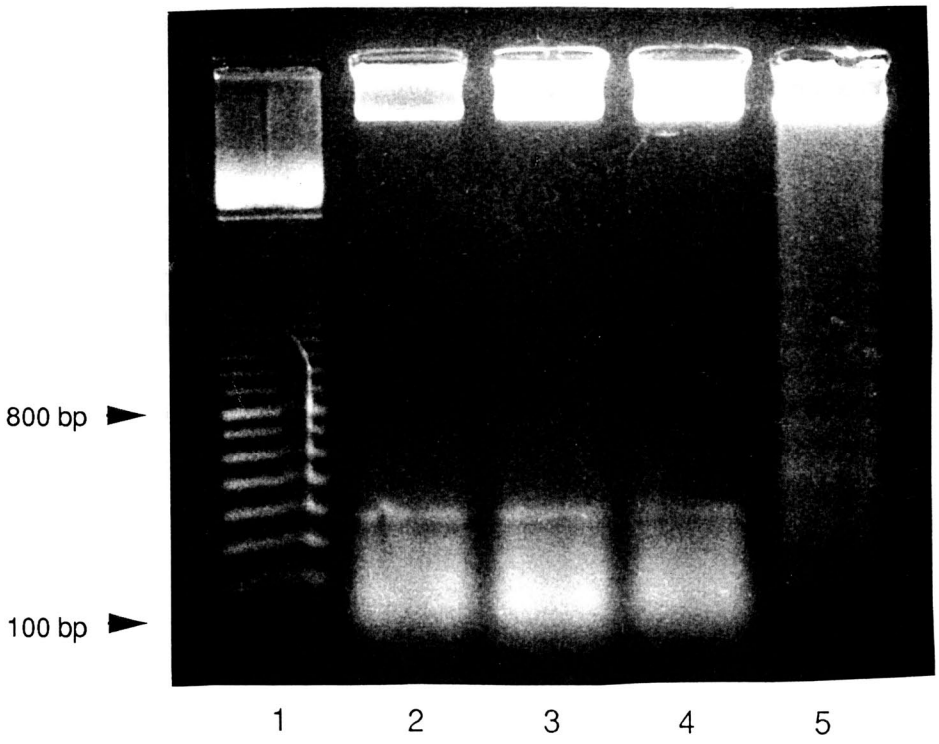
Figure 6.

DNA from pericytes exposed to PAF 7568 (10^{-9}M) for 3 days (A) and 7 days (B). In A and B: lane 1, 100-base pair DNA ladder marker; lane 2, control; lane 3, PAF; lane 4, PAF + CV-3988; and lane 5, apoptotic DNA control. PAF did not induce fragmentation or laddering of DNA.

Day 3



Day 7



stopped the proliferation.

2.5.4 ^{3}H-thymidine uptake

Protein levels were similar in all treatment groups, at all time points over the 30 hour study. After 12 hours of PAF 7568 exposure, pericytes did not take up more ^{3}H-thymidine than controls (figure 5). However, after 24 hours of exposure to PAF 7568, ^{3}H-thymidine incorporation by the pericytes increased by 79%, as compared to control levels. By 30 hours, however, ^{3}H-thymidine uptake in PAF 7568-treated cells had returned to control levels.

2.5.5 Studies of apoptosis

As assessed by DNA electrophoresis, PAF 7568 exposure did not induce DNA fragmentation and laddering at either day 3 or day 7 (figure 6).

2.6 **DISCUSSION**

PAF was originally described in 1972 as a leukocyte-derived platelet activator (2). However, PAF was quickly recognized to be an important mediator in a variety of inflammatory processes (1), acting on many cells types including pulmonary endothelial cells (13,19). Circulating PAF and PAF production by lung tissue have been detected in

experimental models of sepsis (4,5,8,9,38) and in human ARDS (15). The lung is often injured in sepsis and shock, being exposed to circulating mediators and activated cells, leading to ARDS and pulmonary hypertension.

This pulmonary hypertension is due not only to vasoconstriction, but also to gradual remodelling and narrowing of the pulmonary vascular bed by muscularization of small arteries and neomuscularization of the microvasculature (21,41). Precursor smooth muscle cells, including pericytes and intermediate cells, are thought to play an important role in the remodelling process, through proliferation and phenotypic conversion to smooth muscle (21). In a model of ARDS in the rat, from lung injury by monocrotaline, PAF antagonists given early after the injury are able to prevent subsequent remodelling and pulmonary hypertension (34,35).

PAF contributes to the inflammatory response in the lung, but its direct effects on the cells of the vessel wall involved in vascular remodelling have not been defined. In this study, we report that semisynthetic PAF and synthetic 16:0 PAF stimulate lung pericyte growth in-vitro, and that they act via the PAF receptor, since their effect is blocked by a specific PAF receptor blocker, CV3988. PAF's growth stimulatory effect on pericytes appears to be a feature common to cells of the smooth muscle phenotype (40) and also of pericyte-like mesangial cells (29).

Although PAF is 1-0-alkyl-2-acetyl-sn-glycero-3-phosphocholine, the length of the alkyl chain may vary from 15:0- to 22:2-acetyl-glycero-phosphocholine (3,12,19,27). While stimulated cells can produce multiple species of PAF simultaneously (27,36), 16:0-PAF is thought to be the predominant active species (19). We demonstrated that PAF C16 is an active mitogen for pericytes, while PAF C18 is not. PAFs 7568 and 9525, which are semisynthetic, and composed of predominantly 16:0 PAF with a lesser amount of 18:0 PAF and other unidentified fatty acid fractions (20), were also active. PAF 7568 was utilized for subsequent experiments, since it may more closely represent the heterogeneous PAF found in-vivo (3, 12).

PAF exerted its growth effects on proliferating pericytes, and this may be partly due to a recognized synergy of PAF with growth factors in serum, such as platelet-derived growth factor (40). Moreover, the actions of other cytokines, such as endothelin-1, may part be mediated through synthesis and release of PAF in an autocrine fashion (29). That PAF can stimulate growth of quiescent pericytes in the ongoing absence of serum, suggests that PAF might be able to initiate cell growth in-vivo in the absence of other cytokines.

Endothelial cells are targets for PAF (6,13,19), and injury may alter their barrier function to allow circulating PAF to enter the vessel wall (40). Once PAF has reached the interstitial space, it may be more readily

taken up and metabolized by muscle cells than by the abluminal surface of endothelial cells (40). Moreover, endothelial cells themselves produce PAF in a highly regulated fashion when they are activated by mediators circulating during sepsis and lung injury, such as thrombin (44), histamine, bradykinin, ATP (25,42), factor VIII, angiotensin II, vasopressin (7), and leukotrienes (24). This PAF is present on the endothelial surface within 10 minutes of cell activation (37). It may remain partially intracellular (23) and partially associated with the endothelial cell surface (24,37). Pericytes, lying immediately adjacent to endothelial cells, and making cell surface contact with them, could easily be exposed to diffusion or cell-to-cell transfer of circulating or locally-produced PAF.

In the monocrotaline model of lung injury, PAF levels in lung tissue were significantly elevated at 7 and 21 days, but not between those times (34). Moreover, administration of a PAF antagonist during brief, discrete periods after monocrotaline injury attenuated vascular remodelling (34). Thus, as with other mediators, the in-vivo pattern of PAF release in lung injury may be as an acute short burst or brief phasic periods, rather than chronic ongoing release. This might explain the different growth response to PAF in the present study as compared to the series of elegant studies by Ohar et al which used a constant infusion of synthetic PAF for up to 8 weeks in rabbits (30-33). In those studies, although pulmonary

hypertension developed, morphometric studies showed atrophy of the media of muscular arteries without muscularization of the distal microvasculature. Our data do not support a role for PAF in inducing apoptosis in pericytes. Ohar's findings suggested a lack of chronic PAF-induced muscular proliferation, but are not inconsistent with the present studies, in that the period of exposure to PAF differed greatly. In-vitro, PAF is rapidly taken up and metabolized by smooth muscle cells, up to 65% in 15 minutes (40). Thus, although we did not change the cell culture medium during the 7 day experiments, PAF may have produced its actions quickly, then been rapidly inactivated. Our thymidine uptake studies are consistent with a rapid action of PAF. It may be that constant replenishment of the PAF for 7 days would have inhibited pericyte growth. However, we felt that a brief exposure to PAF represented a better simulation of in-vivo events in lung injury.

The study of PAF's effects on lung cells is at an early stage (19). The results of the present experiments suggest a new role for PAF, as a direct cytokine on cells involved in vascular remodelling associated with lung injury.

2.7 ACKNOWLEDGMENTS

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Chapter 3

Effects of Endotoxin and Inflammatory Cytokines on Lung Pericyte Proliferation In-Vitro

3.1 **Preface**

LPS shed from gram-negative bacteria, starts an inflammatory response, causing the release of potent cytokine and phospholipid mediators. In the first paper, we studied the effects of various PAF molecules on the growth of lung vascular PC. In this paper, we show that LPS alone can directly affect PC growth. This is the first such demonstration showing that LPS can act as a direct mitogen of PC.

Cytokines released in the systemic inflammatory response were studied in this paper. Individually, the cytokines had no effects of growth on PC. Combined, to simulate *in vivo* conditions, they inhibited growth of PC in the absence of serum.

As shown previously, LPS also causes the release of PAF. In combination, PAF acts synergistically with LPS, possibly progressing pulmonary injury.

ABSTRACT

Lipopolysaccharide and inflammatory cytokines released during bacterial sepsis cause acute lung injury and ARDS. Pulmonary microvascular injury is a feature, and vascular remodelling develops, contributing to pulmonary hypertension. Pericytes in the lung circulation proliferate and contribute to the remodelling. It is unknown whether endotoxin or cytokines can directly stimulate pericyte growth. We show that lipopolysaccharide from *Escherichia coli*, *Psuedomonas aeruginosa*, and *Klebsiella pneumoniae* increases rat lung pericyte proliferation in vitro by up to 72% on day 7 of exposure ($p<0.001$). Endotoxin's mitogenic effect requires the presence of serum. Interleukin-1ß, interleukin-6, interleukin-8, interferon-γ, and tumor necrosis-factor-α do not affect pericyte proliferation. A mixture of these cytokines does not alter pericyte proliferation in the presence of serum, but reduces it in the absence of serum. Addition of platelet activating-factor to endotoxin increases proliferation. Thus, endotoxin, but not inflammatory cytokines, is a direct mitogen for lung pericytes in vitro, and synergizes with platelet activating-factor. Endotoxin, present in lung tissue early during sepsis, might directly contribute to the vascular remodelling in sepsis-induced lung injury.

INTRODUCTION

The inflammatory response to gram-negative sepsis is triggered by bacterial lipopolysaccharide (LPS, endotoxin), and is mediated by release of potent cytokines, including interleukins, interferon-γ, and tumor necrosis factor-α (TNFα) (3,11,37). Although these cytokines were originally implicated in cell-cell signalling between immune cells, it is now recognized that a variety of non-immune cells, including endothelial cells and airway cells, can release them during sepsis and acute lung injury (3,11,37,43). This cytokine production magnifies the inflammatory response, and it may have direct effects on other vascular cells in the injured lung (36).

The lung microvasculature is a vulnerable target in sepsis, and the Acute Respiratory Distress Syndrome (ARDS) often develops (12,28). Pulmonary vascular remodelling and pulmonary hypertension are prominent features of progressive ARDS (5,24,40,44), and they worsen its prognosis. This vascular remodelling results in part from proliferation and phenotypic conversion of lung pericytes, leading to muscularization and narrowing of the vascular bed (24). The role of circulating inflammatory mediators, or of those locally produced in the vessel wall, on pericyte proliferation is not well defined. Our hypothesis is that the endotoxin and other circulating cytokines directly stimulate lung pericyte growth. We have recently shown that platelet-activating factor (PAF),

which is also released in inflammation, acts as a mitogen for lung pericytes in-vitro (25). However, the mitogenic effect on pericytes of LPS itself, and of the other cytokines, has not been examined. We have therefore studied the effects of LPS from several bacterial species, and inflammatory cytokines, alone and in combination to simulate the complex mixture of mediators present in lung injury (34).

MATERIALS AND METHODS

Pericyte culture

Using a modification of previously described methods (10,19,25), pericytes (PC) were cultured from the lungs of male Sprague-Dawley rats (Charles River Laboratories, Saint Constant, Quebec) weighing 200-400 g. All animal care and handling complied with the guidelines of the Canadian Council on Animal Care. In brief, the rats were anaesthetized with chloroform, the heart and lungs were removed en-bloc, and peripheral pieces of lung were minced with a tissue chopper (McIlwain, Brinkmann Inc, Rexdale, Ontario). After incubating the tissue in Ca^{++} and Mg^{++}-free Hanks Balanced Salt Solution (HBSS; Life Technologies, Grand Island, N.Y.), containing 0.01% w/v type CLSPA collagenase (Worthington Biomedical, Freehold, N.J.) and 0.02% v/v penicillin-streptomycin supplemented with L-glutamine (Life Technologies) at 37°C for thirty minutes, the tissue suspension was passed through a 100 mm

Nitex nylon mesh (Thompson Inc, Mont-Royal, Quebec) and the filtrate centrifuged and resuspended twice using MCDB-131 medium (27) containing 10% fetal bovine serum (FBS; Hy-Clone Laboratories, Logan, Utah). The cell pellet was resuspended and plated into T25 plastic tissue culture flasks and incubated at 37°C, in 5% CO_2 in air. The culture medium was changed twice weekly and pericytes were allowed to grow to confluency before being passaged for experiments. Pericytes were passaged using 0.05% trypsin and 0.02% EDTA in HBSS, which was inactivated by immediate addition of MCDB-131 + 10% FBS after passage. Cells in first passage were used for all experiments.

The pericytes demonstrated previously described morphologic characteristics, including formation of macroscopic mounds at high density, ragged margins, a lack of contact inhibition (10,19). They did not have angiotensin-converting enzyme activity (Hycor Biomedical, Portland ME). By immunoperoxidase staining, the cells produced smooth muscle actin (antibody MA-935, Enzo Biochemical, New York, NY), but not factor VIII coagulant (Dako Corp., Santa Barbara, CA). Bovine pulmonary artery endothelial cells and human skin fibroblasts were used as control cell populations for the characterizations.

Endotoxin and Cytokines

LPS extracted from *Escherichia coli* strain 026:B6, *Pseudomonas*

aeruginosa, and *Klebsiella pneumoniae* (catalogue nos. L-3755, L-7018, and L-1519, respectively; Sigma Chemical, St Louis, MO) were resuspended with sterile water to a stock solution of 1mg/ml, alliquoted and stored at -20°C. Human recombinant Interleukin-1ß (IL-1ß; catalog no. I-3763; Sigma Chemical), recombinant human interleukin-6 (catalog no. 13240-015, Life Technologies) and interleukin-8 (Catalog no. 208-IL; R&D Systems, Minneapolis, MN), in phosphate buffered saline, were aliquoted and stored at -20°C. Rat interferon-γ and human TNFα (Life Technologies) were dissolved with phosphate-buffered saline containing 0.125% bovine serum albumin (BSA, fraction V; Boehringer Mannheim, Mannheim, Germany). Platelet-activating factor (PAF; catalog no. P7568, Sigma Chemical) and the PAF-receptor antagonist CV-3988 (BIOMOL, Plymouth Meeting, PA), were dissolved in 100% ethanol and stored at -20°C.

Experimental Design

Pericytes were passaged into 24 well plates (n=8 wells per experimental condition, except for result in figure 4, where n=6 wells) and allowed to attach for 24 hours prior to onset of the experiments.

For dose-response studies, the medium was then removed and fresh medium + FBS was added, containing LPS (0-250 µg/ml) from the various bacterial species, or interleukin-1ß (0-100 U/ml), interleukin-6

(0 - 100 U/ml), interleukin-8 (0 - 10 ng/ml), interferon-γ (0 -200 U/ml) or TNFα (0-1000 U/ml). The dose ranges were chosen to overlap those reported in patients with sepsis and acute lung injury, and in animal models of lung injury and pulmonary hypertension.

To examine the effects of a cytokine mixture (CYTOMIX; interleukin-1ß 5U/ml, TNFα 500U/ml, and interferon-γ 100U/ml) simulating that found in the milieu of an inflamed lung (34). Pericytes were used either without a prior growth arrest and, after attachment, immediately exposed to medium + FBS or BSA, with or without the addition of *E. coli* LPS and/or CYTOMIX, or they were subjected to a prior growth-arrest by incubating them in MCDB-131 + 0.3% BSA for 72 hours. Subsequently, fresh medium containing FBS or BSA, with or without LPS and CYTOMIX, was added.

In other experiments, to explore the possible contributions of PAF in LPS-induced pericyte proliferation, pericytes were allowed to attach as above, then the medium was replaced with medium + FBS with or without *E. coli* LPS (10 µg/ml), PAF and the PAF-receptor antagonist CV-3988 (10^{-7} M).

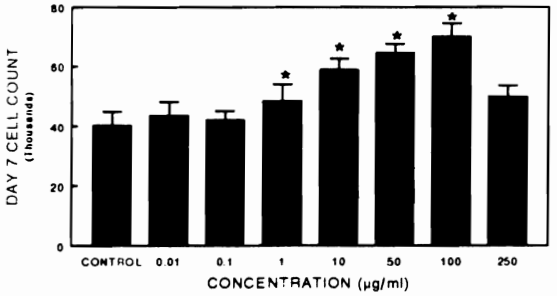
Culture dishes were examined daily for cell lysis or detachment. In each experiment, on day 7, the pericytes were detached with 0.25% trypsin + 0.02% EDTA and counted using a hemacytometer.

Studies of cell apoptosis

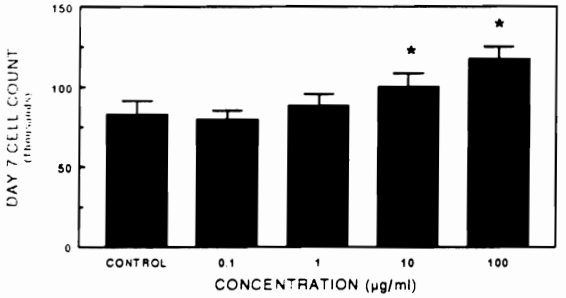
The nuclear DNA laddering technique was used to detect the development of cellular apoptosis (14). Pericytes were passaged into 100mm tissue culture dishes (n=2 per condition) and allowed to grow to subconfluence. LPS (10 μg/ml), or CYTOMIX with LPS, CYTOMIX without LPS, TNF-α (500 u/ml), interleukin-1ß (50u/ml) or interferon-γ (100u/ml) were then added for 72 hours. After exposure, the medium was removed and the cells were washed with cold phosphate-buffered saline (PBS) and lysed with 1ml of DNA lysis buffer [10 mM tris(hydroxymethyl) aminomethane (Tris), 10 mM EDTA, 100 mM NaCl, 200 ng/ml proteinase K (Sigma)] for two minutes at 20° C. The cells were scraped using a rubber policeman and the lysate placed in tubes. Sodium dodecyl sulfate (SDS) was added to make a 1% vol/vol concentration, and the tubes were incubated at 37° C for four hours. Two phenol-chloroform-isoamyl alcohol (25:24:1) extractions were performed, with centrifugations at 2000 g for 10 minutes at 20° C and then the DNA was precipitated by adding 2.5 volumes of ice cold 100% ethanol to the upper aqueous phase. After gentle agitation, the DNA formed strands, and the suspension was kept at -20° C for 20 minutes then centrifuged (1400 g) to collect all fragmented DNA. The supernatant was removed and the DNA pellet was washed in 70% ice cold ethanol, air dried and resuspended in sterile water. One μg of RNaseA (Sigma) was

Figure 1. Effect of various concentrations of lipopolysaccharide (LPS), from three bacterial species, on lung pericyte proliferation in-vitro. Bars are mean day 7 cell count + 1 SD. *, $p<0.01$ versus control.

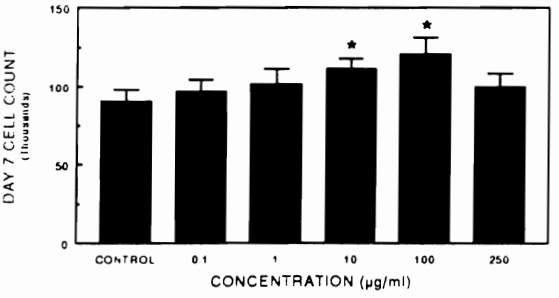
E. coli 026:B6 LPS



P. aeruginosa LPS



K. pneumoniae LPS



added per 50 µg DNA, at 37° C for 30 minutes, to remove any contaminating RNA. Each DNA sample (5 µg) was then loaded onto a 1.5% non-reducing agarose gel with 1xTBE (0.1M Tris, 0.1M boric acid, 20mM EDTA), along with a 100-base pair DNA ladder marker (Pharmacia Biotech, Uppsala, Sweden), a λ-Hind III marker (Promega, Madison, WI), and a positive control from a leukemia cell line rendered apoptotic by exposure to retinoic acid. After electrophoresis (75V, 50 mA, 4h), the gels were stained with ethidium bromide and photographed under ultraviolet light.

Statistics

Data are presented as group mean ± 1 SD. To compare groups, one-way analysis of variance was used, followed, where appropriate by Tukey-Kramer analysis for multiple comparisons. P values less than 0.05 are considered significant.

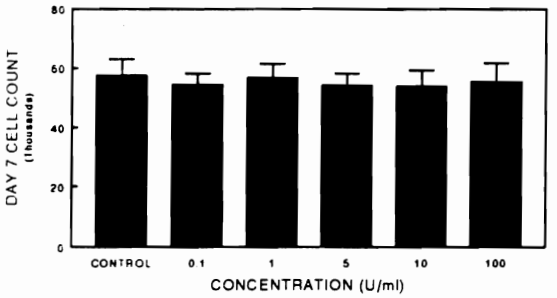
RESULTS

Growth response of proliferating pericytes to various LPS and cytokine molecules

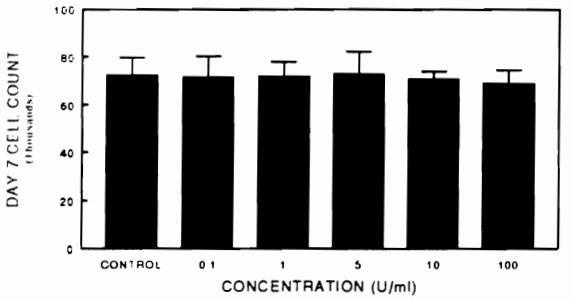
All LPS molecules increased pericyte proliferation as compared to control growth (Fig. 1). *E. coli* LPS stimulated proliferation over a wide concentration range, with a 20% increase at 1 µg/ml and a 72% increase

Figure 2. Effect of interleukins 1ß, 6 and 8, in various concentrations, on lung pericyte proliferation in-vitro. Bars are mean + 1 SD. The interleukins do not affect proliferation.

INTERLEUKIN-1 ß



INTERLEUKIN-6



INTERLEUKIN-8

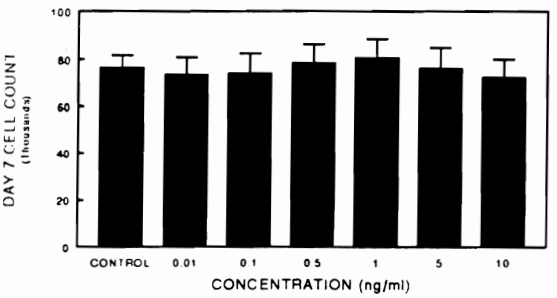
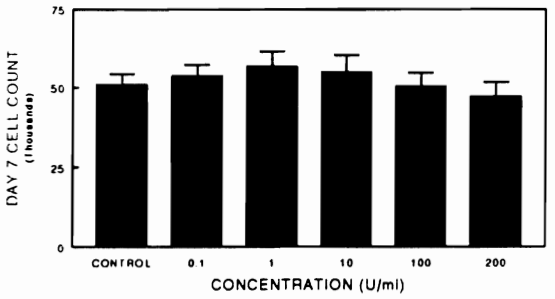


Figure 3. Effect of interferon-γ (top) and tumor necrosis-factor-α (bottom), in various concentrations, on lung pericyte proliferation in-vitro. Bars are mean + 1 SD. These cytokines do not alter pericyte proliferation.

INTERFERON-γ



TUMOR NECROSIS FACTOR-α

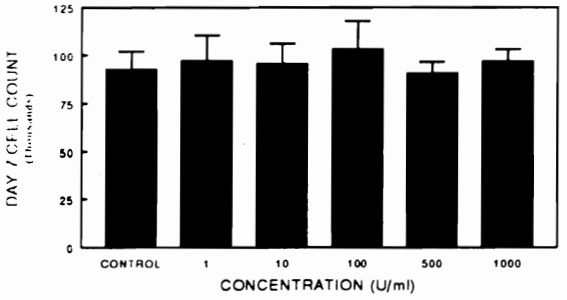
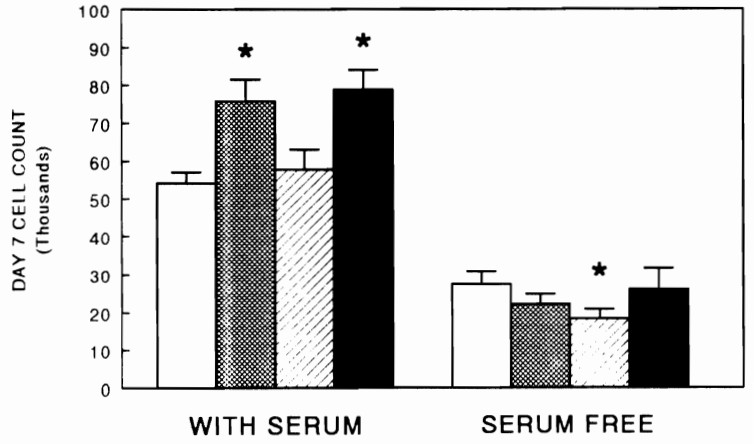
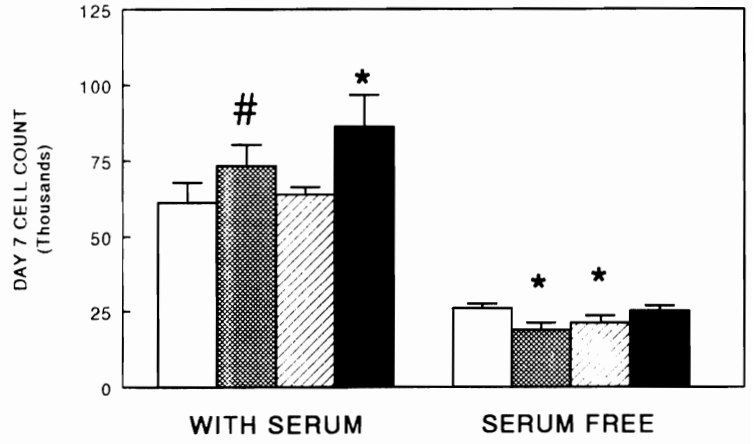


Figure 4. Effects of a cytokine mixture (CYTOMIX) on pericyte proliferation in-vitro, without (diagonal-hatched bars) or with LPS (hatched bars), and LPS alone (black bars). Control values are represented by the white bar. Pericytes had either been subjected to no prior growth (upper), or had been growth arrested by serum deprivation prior to the experiment (lower). *, $p<0.01$ versus control; #, $p=0.04$ versus control and $p=0.02$ versus LPS.

NO PRIOR GROWTH ARREST



PRIOR GROWTH ARREST



at 100 μg/ml (Fig. 1, top). *P. aeruginosa* LPS was less effective, increasing proliferation by 20% at 10 μg/ml and 41% at 100 μg/ml (Fig. 1, middle), while *K. pneumoniae* LPS increased proliferation by 23% at 10 μg/ml and by 34% at 100 μg/ml (Fig. 1, bottom).

Interleukin-1ß, interleukin-6 and interleukin-8 neither stimulated nor inhibited pericyte proliferation at day 7 (Fig. 2). At 1 U/ml, interferon-γ induced a nonsignificant (p=0.09) 11% increase in mean growth (Fig 3, top) TNFα had no effect on pericyte proliferation (Fig. 3, bottom).

Effects of cytokine mixture and LPS on pericyte proliferation

Exposure of pericytes to CYTOMIX in the absence of LPS and in the presence of serum did not affect proliferation as compared to CONTROL (Fig. 4, top and bottom), but, in serum-free medium, it reduced proliferation. Addition of *E. coli* LPS to CYTOMIX increased proliferation in the presence of FBS, by 39% as compared to CONTROL in cells not subjected to a prior growth arrest and by 19% in cells subjected to a prior growth arrest. This effect of LPS was not seen in the absence of serum, either with or without a prior growth arrest. However, the growth of pericytes in LPS alone was always greater than or equal to pericyte growth in the presence of CYTOMIX.

Figure 5. Effects of LPS, platelet activating-factor (PAF) and the PAF-receptor antagonist CV-3988 on pericyte proliferation. *, $p<0.001$ versus control; #, $p=0.02$ versus LPS; &, $p<0.01$ versus LPS+PAF.

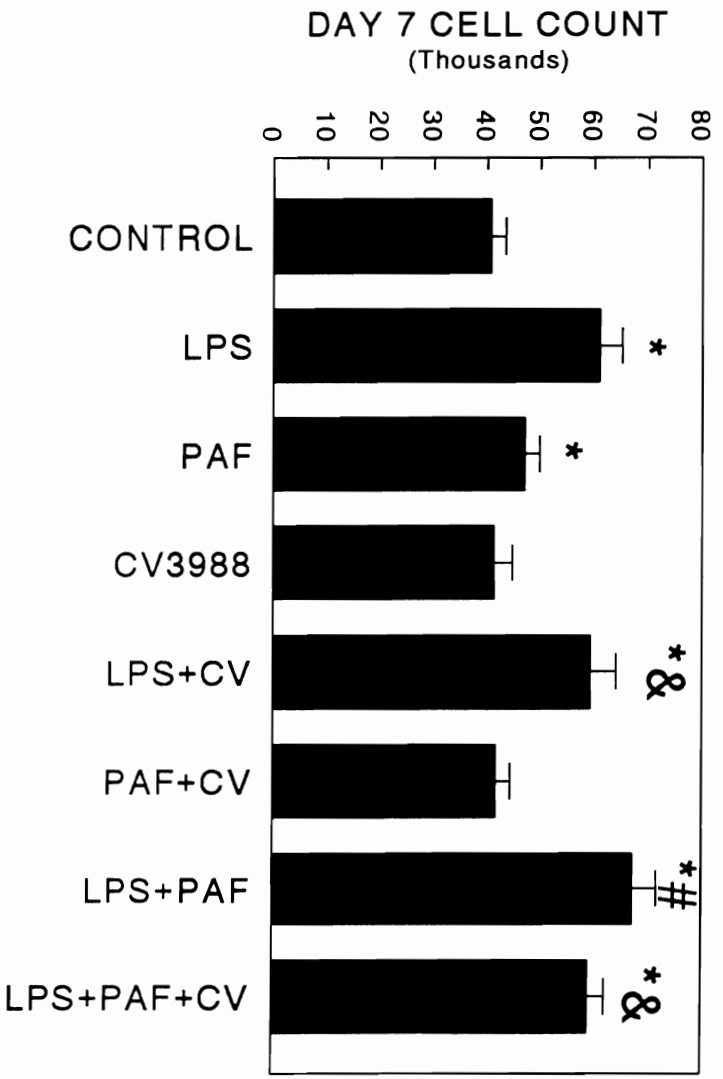

DAY 7 CELL COUNT
(Thousands)
0
10
20
30
40
50
60
70
80
CONTROL
LPS
*
PAF
*
CV3988
LPS+CV
&*
PAF+CV
LPS+PAF
#*
LPS+PAF+CV
&*

Figure 6. DNA from pericytes exposed to various cytokines and LPS for 72 h. Lane 1: λ-Hind III marker; lane 2: 100-base pair DNA ladder marker; lane 3: LPS (10 μg/ml); lane 4: tumor necrosis factor-α (500 U/ml); lane 5: interleukin-1ß (50 U/ml); lane 6: interferon-γ (100 U/ml); lane 7: apoptotic DNA control. The cytokines did not induce DNA fragmentation or laddering.

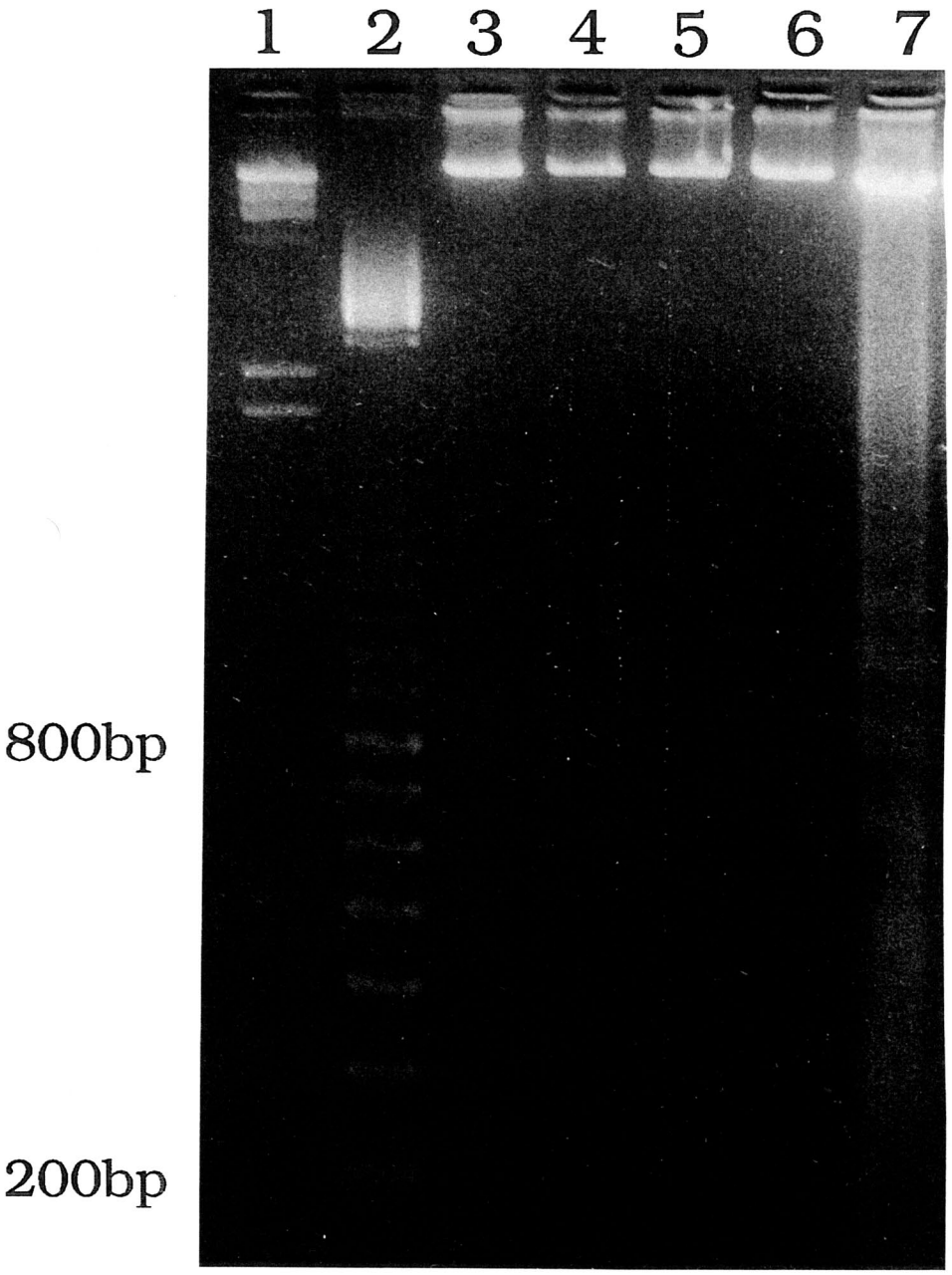
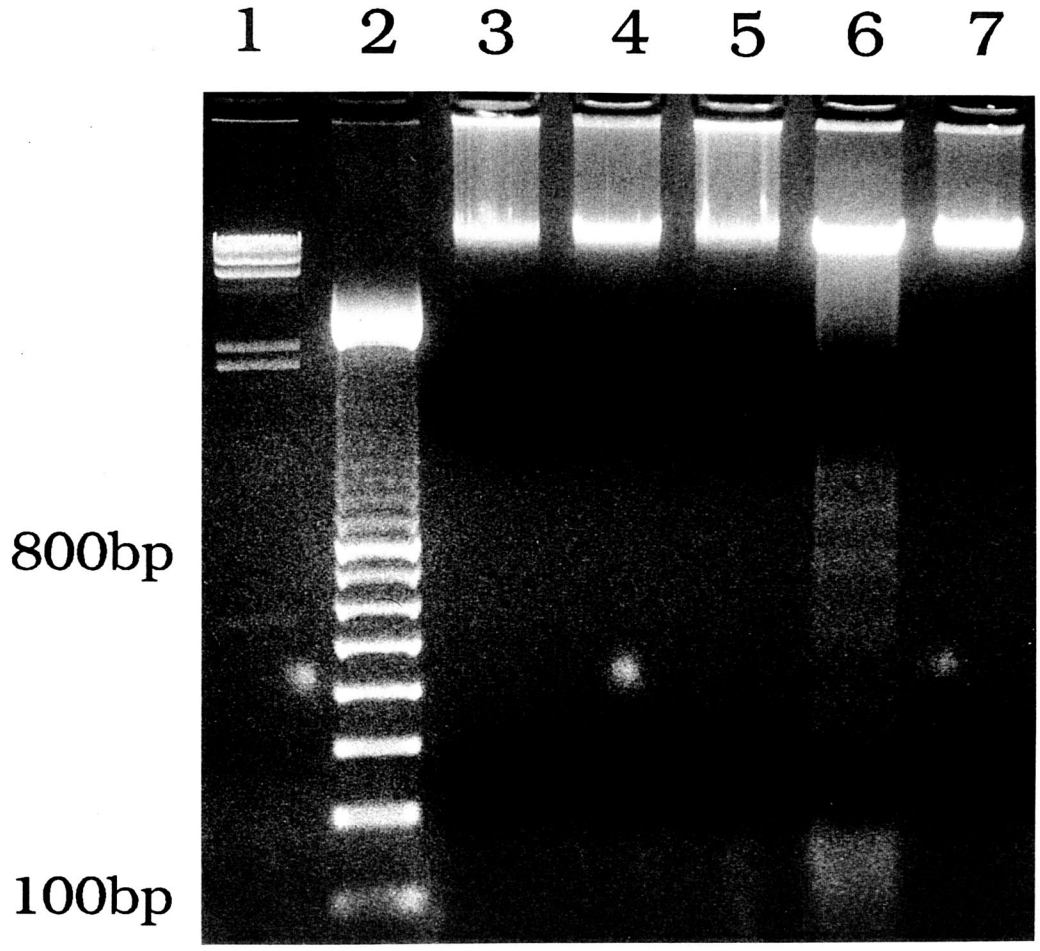

1 2 3 4 5 6 7
800bp
200bp

Figure 7. DNA from pericytes exposed to LPS or a cytokine mixture (CYTOMIX) for 72 h. Lane 1: λ-Hind III marker; lane 2: 100-base pair DNA ladder marker; lane 3: LPS (10 μg/ml); lane 4: CYTOMIX; lane 5: CYTOMIX with LPS; lane 6: apoptotic DNA control; lane 7: control (no LPS or CYTOMIX added). CYTOMIX does not induce DNA fragmentation or laddering.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Effects of LPS and PAF on pericyte proliferation

In these experiments, exposure to *E. coli* LPS increased pericyte proliferation by 50% at day 7, as compared to control growth (Fig. 5). PAF (10^{-9} M) induced a more modest 16% increase in proliferation. Addition of the PAF-receptor antagonist CV-3988 completely abolished the effects of PAF, but did not significantly reduce the effects of LPS. The combination of LPS + PAF was more effective than LPS alone, resulting in a 65% increase in proliferation as compared to control, and a 10% increase as compared to LPS alone ($p=0.02$). Moreover, the combination of PAF and LPS increased proliferation significantly more than LPS + CV3988 ($p=0.002$).

Studies of apoptosis

Individual cytokines (figure 6), or the CYTOMIX mixture (figure 7), did not induce apoptosis of pericytes, as assessed by the DNA laddering technique.

DISCUSSION

Without the toxic effects of endotoxin and of secondary cytokine and inflammatory mediators, an episode of systemic sepsis might have minimal impact on the lung. However, in most instances of severe sepsis, the lung is injured by endotoxin, and by the very mediators and

inflammatory cells activated to defend it (3,5,37). The pulmonary circulation is particularly vulnerable, and pulmonary hypertension develops through a combination of vasospasm, thrombosis and microvascular remodelling (40). Endothelial damage is a hallmark, and retraction of endothelial cells from the underlying basement membrane is a frequent finding (5,23). Narrowing of the microvasculature results from increased muscularization, including proliferation and phenotypic conversion of pericytes to smooth muscle, a process which takes days to fully develop. Attention has previously focussed on the effects of endotoxin and cytokines on the endothelium and inflammatory cells, but not on the direct effects of endotoxin and cytokines on cells which contribute to vascular remodelling.

Our studies demonstrate that lipopolysaccharide, particularly that from *E. coli*, is a direct potent stimulator of pericyte growth *in vitro*. The degree of increase in pericyte proliferation, over 70% in some experiments, could have great impact on narrowing of the microvascular lumen, where a small degree of reduction in diameter results in a greatly reduced cross sectional area and increased vascular resistance. Infusion of endotoxin in animal models of sepsis results in pulmonary vascular remodelling (26), although it is impossible to determine in these models the direct effects of endotoxin as compared to the effects of other mediators activated by endotoxin.

The levels of LPS required to increase pericyte proliferation *in vitro* were higher than the free endotoxin levels reported in the plasma of septic patients (22,41). However, circulating endotoxin is rapidly bound by the lipopolysaccharide-binding protein (LBP), which presents LPS to the CD14 receptor, and the *Limulus* amebocyte lysate assay, generally used to measure endotoxin levels in blood, measures only unbound lipopolysaccharide when high levels of LBP are present (38). Thus, the total (i.e. bound and unbound) levels of endotoxin in vivo may be much higher than previously reported. In addition, we heat-treat (56° C for 30 min) the serum used in our culture medium, to inactivate complement. This also reduces the activity of lipopolysaccharide-binding protein (32) and may thus reduce the efficiency of LPS presentation to the cultured pericytes, requiring higher LPS levels in the medium to drive the LPS-cell interaction.

After intravenous injection in rats, LPS is rapidly taken up and stored in pulmonary microvascular intimal cells, diffusely in the cell junction region, and membrane-bound in cytosomes (15). The concentration of LPS in the vascular wall may thus be much higher than that measured in circulating plasma. Pericytes lie immediately abluminal to endothelial cells, and make cell-surface contact with them. They could easily be exposed to cell-to-cell transfer of endotoxin, or release of endotoxin into the extracellular matrix. Furthermore,

endothelial disruption and retraction are prominent features of septic lung injury (23), exposing the underlying cells to circulating molecules such as endotoxin, clotting factors and platelet activating factor.

The LPS-receptor, CD14, is found on many inflammatory cells as a membrane bound receptor (20). CD14 also exists in a circulating soluble form, found in blood and bronchoalveolar lavage fluid (1,39). It appears that vascular smooth muscle does not express endogenous membrane CD14, relying instead on serum-derived soluble CD14 to mediate the effects of LPS (30). We found that pericytes were stimulated by endotoxin to proliferate only in the presence of serum, and not in serum-free conditions, consistent with their characterization as a smooth muscle-like cell.

The doses of inflammatory cytokines that we studied spanned concentrations detected in serum and bronchoalveolar lavage fluid from patients and animal models of sepsis and ARDS (3,11,16,18,22,31,35). Pericytes themselves can produce interleukins 1 and 6 (13). At least one of the inflammatory cytokines, interleukin-6, induces pulmonary hypertension in rats (33), and interleukin-1 receptor blockade inhibits monocrotaline-induced pulmonary hypertension (42). Interleukin-1 and TNF-α stimulate smooth muscle cell growth and cytokine production (4,29). However, we did not detect a mitogenic effect on pericytes with any of the cytokines tested. Moreover, the mixture of cytokines

(CYTOMIX), simulating *in vivo* conditions, inhibited proliferation as compared to CONTROL, and it also reduced lipopolysaccharide-stimulated growth under some conditions. A similar cytokine mixture induces apoptosis of vascular smooth muscle cells *in vitro* (17), and interferon-γ inhibits smooth muscle cell proliferation (21). Although this could explain the reduced growth seen in our experiments, we were not able to detect apoptosis in our DNA laddering experiments.

Other growth factors are also released in sepsis and inflammation, including those from platelets and immune cells (7). Endotoxin has been shown to stimulate PAF release in many cell types, including endothelial cells (8), and we have previously shown that PAF stimulates lung pericyte growth (25). In the present studies, the PAF-receptor antagonist CV-3988 did not significantly blunt the endotoxin-induced growth increase. However, it is interesting that PAF acted synergistically with endotoxin. Other growth factors might have similar synergistic effects.

International studies on sepsis and ARDS offer hope that the incidence and morbidity of sepsis-induced lung injury will be reduced (2,6,9). Some inflammatory cytokines we studied clearly moderate the LPS effect. But others, such as platelet-activating factor, amplify the LPS effect. Most importantly, our experiments identify a new potential role for LPS, as a direct mitogen in the process of vascular remodelling.

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Chapter 4

General Discussion

Discussion

Gram-negative sepsis can lead to ARDS and PH, two syndromes with high morbidity and mortality (5,14,17). LPS is released from the outer membrane of gram-negative bacteria (22,24). LPS is believed to act as a trigger, acting on various cells such as macrophages, neutrophils, and endothelial cells causing the production of numerous mediators including cytokines and the phospholipid mediator platelet-activating factor (PAF) (23,24,30).

Endothelial cells are targets for these mediators (4,7,11,17), and injury decreases endothelial barrier function which might permit the circulating mediators to enter the vessel wall (17,23,26). Pericytes, SMC precursors, are sub-endothelial cells attached through the basement membrane (20). Due to their proximity to endothelial cells, we hypothesize that PC play an important role in the vascular remodelling and neovascularization seen in acute lung injury.

We therefore set out to study several hypotheses, each one will now be dealt with.

- PAF has direct effects on PC growth and it acts via the PAF receptor.

Our work shows that PAF has a direct proliferative effect on PC. This effect is not mediated via secondary cells or mediators. By showing the addition of the PAF-receptor antagonist CV-3988 has an almost complete

inhibition of proliferation as compared to PAF alone, we show that PAF works via a receptor. Therefore, the list of cells which have PAF receptors can now include pericytes. Furthermore, semisynthetic PAF, similar to PAF found in vivo, has a greater effect than those of synthetic PAF. Synthetic 16:0 PAF and a combination of 70% 16:0 PAF and 18% 18:0 PAF have significant, although less increases as compared with semisynthetic PAF. 18:0 PAF or rac-PAF, the inactive PAF isomer, have no effect on PC growth in vitro alone.

- PAF elicits its effects in both serum and serum free media.

PAF elicits its effects on proliferating PC, this may by partly due to a recognized synergy of PAF with growth factors in serum, such as PDGF (26). Quiescent PC simulate an in vivo microvascular bed that has not yet been exposed to mitogens, and in the absence of serum, a microvascular bed lacking growth factors. That PAF can stimulate growth of quiescent PC suggests that PAF might be able to initiate cell growth in vivo in the absence of other growth factors and mitogens.

- The individual cytokines have a proliferative effect on PC.

The sepsis induced cytokines we studied were IL-1β, IL-6, IL-8, TNFα, and IFNγ. These cytokines cause various effects on cells and tissues. They are generally known for their role in inflammation (2,12,15). Certain cytokines induce pulmonary hypertension and septic shock syndrome (8,28,29). Surprisingly, none of the individual cytokines had

any proliferative nor any inhibitory effect on PC.

- A mixture of cytokines similar to those found in vivo has a proliferative effect on PC.

A mixture of cytokines (CYTOMIX) simulating that found in an inflamed lung (21) is added to lung PC in vitro. This CYTOMIX has no effect on PC with serum, but has an inhibitory effect on PC without serum as compared to control. A similar mixture induces apoptosis of vascular SMC in vitro (9). This may explain the reduced growth seen in our experiments, however, we were not able to detect apoptosis in our DNA laddering experiments.

- LPS diffusing into the vessel wall might itself have a proliferative effect on PC.

LPS reduces the barrier function of endothelial cells (3,13), possibly allowing the direct contact of LPS on PC. LPS from *Escherichia coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* were added to PC in vitro, and day 7 cell counts were performed. Although they varied in effectiveness, the three species of LPS are able to induce proliferation in PC in vitro. They vary in effectiveness from 1 to 100 μg/ml, and they all show inhibition at 250 μg/ml as compared to 100 μg/ml. Although it is believed that LPS may elicit its effects in ng/ml concentrations, the threshold level in our studies started at 1 μg/ml. This may be due to an absence of LPB (22), or to the decreased activity of LBP due to heat

inactivation of serum (18). LPS produces its effects on macrophages in very low doses in the presence of LBP, but requires greater concentrations in the absence of LBP (22).

- LPS elicits its effects in both serum and serum free media.

LPS is only capable of eliciting its effects on PC in serum. This shows that the LPS-receptor, CD14, must be present. CD14 exists in two forms, a membrane bound (mCD14) receptor found on many inflammatory cells, or as a circulating soluble form (sCD14) found in blood and bronchoalveolar lavage fluid (1,6,10,27). Vascular SMC do not have membrane bound CD14 (16). Pericytes do not appear to have mCD14, therefore requiring sCD14 found in serum to mediate the effects of LPS.

- A combination of LPS and PAF will result in increased proliferation compared to each individually.

LPS and PAF acted in a synergistic manner, causing a greater increase of proliferation as compared to each individually. The addition of the PAF-receptor antagonist CV-3988 did not significantly inhibit this proliferation, it did, however, seem to inhibit equally the proliferative synergism caused by PAF. Although there are reports showing that pre-treatment of animals by PAF-receptor antagonists greatly decreased septic shock syndromes caused by LPS infusion (25), we show that the simultaneous addition CV-3988 is not effective enough against the quick

actions of LPS (19).

- A combination of LPS and the mixture of cytokines will result in increased proliferation compared to each individually.

The addition of LPS to CYTOMIX did not increase proliferation, in fact, it decreased the proliferation significantly as compared to LPS alone in quiescent cells upon return to serum, and inhibited growth as compared to control in quiescent cells in the continued absence of serum. This inhibition is similar to that of CYTOMIX alone on pericytes.

- The addition of LPS or the mediators will not cause cell death or apoptosis of the PC.

Our studies suggest that apoptosis of PC was not induced by addition of any of the combination of LPS, PAF, and the cytokines. However, these studies were performed on proliferating pericytes in the presence of serum. Growth arrest and serum free studies have not been performed.

The significance of this work shows that, in the case of lung pericytes, LPS does not only act as a trigger, but also as a direct mediator on PC growth and proliferation. This is the first such finding implicating LPS as a direct mediator. PAF can also act directly on PC. Pericytes have now been shown to posses a PAF-receptor, as the specific PAF-receptor antagonist CV-3988 blocks the effects elicited by PAF. The inflammatory cytokines do not have a proliferative effect individually, but may actually have an inhibitory effect in concert. Finally, our hypothesis

that PC play an important role in the vascular remodelling and neovascularization seen in acute lung injury is supported by these studies.

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