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Vascular Derived Growth Factors: Cell Biology, Pathophysiology, and Pharmacology

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I. Introduction

Within the developing vasculature, both endothelial and smooth muscle cells influence the growth and the final structure of blood vessels by secreting a variety of growth stimulatory and inhibitory polypeptides. These substances exert their effects by either paracrine or autocrine mechanisms. During neovascularisation, atherogenesis and, on occasion in hypertension, nonvascular cells (in particular macrophages, platelets, and T-lymphocytes) also play a major role by secreting polypeptides that directly influence vascular growth as well as stimulate growth factor production by endothelial and smooth muscle cells. During these processes, there is also local degradation of the extracellular matrix or basement membrane, followed by migration, proliferation, and/or hypertrophy of the vascular cells. Thus, vascular cells, either alone or in concert with blood-borne cells, have the capability to reorganise the structure of existing blood vessels or create new vessels.

Because many vascular cells in diseased vessels recapitulate many of their characteristics exhibited during early embryonic and foetal development, in this review we initially focus on the characteristics possessed by endothelial and smooth muscle cells during normal vessel development and growth. This is followed by a discussion of the effects on vascular cells of such disease processes as atherosclerosis, hypertension, restenosis after luminal damage, and angiogenesis. The structure, biosynthesis, receptor-mediated interactions, and potential effects of the different vascular derived growth factors and regulators are described and then are considered with regard to the future therapeutic potential of agents that either limit or enhance the actions of specific growth factors involved in cardiovascular disorders.

II. Development of Vascular Endothelial and Smooth Muscle Cells

A. Embryonic Origins of Endothelial and Smooth Muscle Cells

Blood vessels first develop as a budding network of small endothelial lined channels (Sethi and Brooks, 1971; Noden, 1989). In this process, cords of angioblasts, which display some endothelial and haemopoietic immunological properties, develop vacuoles that coalesce to form the lumen of the developing vessel. Subsequent extension of the network occurs by a combination of in situ formation of new vessels, sprouting and fusion of established neighbouring vessels, and invasion of angioblasts from other regions. As the endothelial lined tube develops, a basement membrane is established that contains collagen types IV and V, laminin, entactin, heparan sulphate proteoglycan, and a variable amount of fibronectin. Early basement membrane initially contains large amounts of fibronectin which promotes proliferative and migratory activity of endothelium. Later, the endothelium synthesises more laminin which aids differentiation, cell attachment, and junction formation; proliferation is suppressed (Ausprunk et al., 1981; Navaratnam, 1991).

These early events are promoted by a variety of growth factors expressed during embryonic development. In some tissues, bFGF† appears to be an initial stimulus for vascularisation of developing tissues, and later it also plays a role in differentiation (Risau and Ekblom, 1986). Other FGF-related protooncogenes, such as *int*-2, also may be involved (Wilkinson et al., 1988).

After the vascular channels are ensheathed by extracellular matrix, they become surrounded by locally derived, irregularly shaped mesenchymal cells (Manasek, 1971). The mesenchymal cells, while actively proliferating and producing elastic lamellae and other extracellular matrix components, morphologically resemble fibroblasts in that they contain few filaments but have large amounts of rough endoplasmic reticulum and free ribosomes. Later, the elastic lamellae become more developed in large arteries, and the mesenchymal cells take on a more characteristic muscle appearance (Gonzales-Crussi, 1971).

Very little is known about the mechanisms in early embryogenesis by which surrounding mesenchymal cells are first recruited by the endothelium to form a media and then later proliferate and differentiate into smooth muscle cells. However, the heparan sulphate proteoglycan formed by the endothelial cells may serve to bind growth factors, such as PDGF and bFGF, that are derived from endothelium and which can function as chemoattractants and mitogens for smooth muscle. These growth factors could act either alone or in concert with TGF-β (see section IV). All three growth factors are expressed in tissues during angiogenesis.

Later in development, the increase in blood pressure may directly or indirectly influence the thickness of the vessel wall, as may innervation by autonomic nerves (Lee et al., 1991; Korner et al., 1992).

† Abbreviations: FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; aFGF, acidic fibroblast growth factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; $V_v$myo, volume density of myofilaments; IL, interleukin; TNF, tumour necrosis factor; CSF, colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; SHR, spontaneously hypertensive rats; EGF, epidermal growth factor; IGF, insulin-like growth factors; IGF-BP, insulin-like growth factor-binding protein; IFN, interferon; ET, endothelin; ACE, angiotensin-converting enzyme.

B. Structural and Growth Characteristics of Endothelial Cells

The ability of vascular endothelial cells to either produce or respond to vascular derived growth factors is to a large extent dependent on their origin, their phenotype, and the environmental conditions. For example, capillary endothelial cells produce and respond to PDGF, whereas those from larger vessels are refractory to this mitogen (Beitz et al., 1991). There is a wide variation in morphology and function of endothelial cells throughout the vascular tree, and these cells are usually divided into three major categories: *Continuous*. (*a*) Endothelium possessing both tight and gap junctions (e.g., blood vessels of muscle) and (*b*) endothelial cells with tight junctions alone (e.g., vessels of the brain). *Fenestrated*. Endothelial cells perforated by small pores or fenestrae, with or without closing diaphragms (e.g., capillaries of endocrine glands). *Discontinuous*. Endothelial cells with intercellular and transcellular discontinuities (e.g., sinusoidal capillaries of the liver).

Endothelial cells rest on a well-defined basement membrane containing an electron-lucent zone (lamina rara) immediately below the endothelium and an electron-dense zone or (lamina densa) close to the interstitial connective tissue (Palotie et al., 1983). Fibronectin, laminin, collagen types IV and V, entactin, and heparan sulphate proteoglycan are present in the basement membrane (Foidart et al., 1980; Bender et al., 1981; Kanwar and Farquhar, 1979), and the underlying reticular layer, when present, contains interstitial collagen types I and III produced by both endothelial cells and smooth muscle (Madri et al., 1980). The precise composition of the basement membrane and the underlying reticular layer is to a large extent dependent on the actions of local vascular derived growth factors, such as TGF-β and the PDGFs (see individual growth factors, section IV).

The endothelial surface of large vessels (e.g., aorta) is generally flat with low protrusions corresponding to underlying nuclei (Garbarsch et al., 1982). Finger-like projections of cytoplasm (microvilli) are located either randomly over the entire cell surface or condensed in the proximity of the underlying nucleus (Smolich et al., 1984). There also are minute projections of endothelial cytoplasm (marginal flaps) along the intercellular borders (Garbarsch et al., 1982). Primary cilia sometimes occur on the surface of endothelial cells in normal vessels and overlying early atherosclerotic plaques (Haust, 1987). Endothelial cells are normally quiescent in vivo, but following injury the cells undergo phenotypic changes that enable them to migrate, proliferate, and effect repair. Endothelial cells in culture can grow in a variety of states or phenotypes. When seeded into primary culture, endothelial cells from large vessels or microvessels proliferate until a confluent monolayer is produced (Gimbrone et al., 1974). The rate of proliferation then declines and the cells assume a cobblestone pattern of similar morphology to the contact-inhibited layer in vivo. When these cultures are maintained at postconfluent densities for long times, some of the cells exhibit a second growth pattern or phenotype. They become elongated and form branching cords as part of a process termed "sprouting" due to its similarity to the pattern of angiogenesis observed in wound healing and tumour formation (Cotta-Pereira et al., 1980). The growth of phenotypically attenuated endothelial cells in vitro is associated with altered biosynthetic functions. Sprouting endothelial cells synthesise different quantities of fibronectin and collagen types compared to cells in a confluent monolayer (McAuslan et al., 1982), consistent with the changes in the subendothelial extracellular matrix during angiogenesis (Nicosia and Madri, 1987). Sprouting endothelial cells also exhibit increased production of prostaglandins (Hahn and Polgar, 1984).

The morphological phenotype expressed by cultured endothelial cells may be influenced by a variety of factors including the type of medium used, availability of growth factors, age of cultures, and the composition of the surface on which the cells are grown (McGuire and Orkin, 1987). For example, endothelial cells cultured on dishes coated with basement membrane/basal lamina collagens (types IV and V) or laminin aggregate and form tube-like structures similar to capillaries at early culture times. Cells grown on interstitial collagens (types I and III) or fibronectin proliferate, form a confluent monolayer, and then, if cultured for extended periods, form occasional capillary tubes (Madri and Williams, 1983; Lawley and Kubota, 1989). Cultured endothelial cells retain some of the region-specific phenotypic modifications that are present in vivo. For example, cells from brain microvessels retain their ability to function as a more effective permeability barrier than those from other locations (Bottaro et al., 1986), and the considerable heterogeneity in cell size and shape observed in endothelial cells from adult human vessels is preserved in primary culture (Antonov et al., 1986). The ability of endothelial cells to retain their characteristics in cell culture is also reflected in their production of and response to a variety of growth factors and cytokines (Pober and Cotram, 1990). In essence, cell culture is an ideal model system in which to compare many of the properties of endothelial cells from different vascular beds.

C. Structural and Growth Characteristics of Smooth Muscle Cells

Smooth muscle cells throughout the vascular tree are functionally diverse depending on the size and nature of the vessel (e.g., elastic artery, muscular artery, arteriole, venule, vein). These differences in function are due to different organisation of the cells, innervation patterns, membrane properties, and receptors (Creed, 1979).

Smooth muscle cells can exist in a spectrum of phenotypes (Chamley-Campbell et al., 1979). As discussed

earlier, in developing blood vessels the smooth muscle cells resemble fibroblasts in that they contain few myofilaments but do have large amounts of rough endoplasmic reticulum, free ribosomes, and Golgi apparatus (Gonzales-Crussi, 1971). With time, thin filament bundles with associated dark bodies increase in size, thick myofilaments appear, the number of organelles decreases, and basal lamina plasmalemmal vesicles appear (Campbell and Chamley, 1976). In the newborn rat's aorta, the $V_v$myo in the smooth muscle cytoplasm is 7.7%; this increases to about 70% in the adult (Gerrity and Cliff, 1975). The major type of actin present in the foetal and newborn smooth muscle cells is the β-isoform, but this changes to predominantly the α-isoform with development (Kocher et al., 1984, 1985). Likewise, in the foetal aorta, 87% of medial smooth muscle cells contain only vimentin, 13% contain both vimentin and desmin, and none contain desmin alone; with development, there is a gradual decrease in the number of cells containing vimentin alone and an increase in those in which vimentin and desmin coexist.

When the adult vessel wall is damaged, e.g., by balloon catheterisation, the smooth muscle cells in the region of the wound undergo phenotypic changes to again resemble the cells of the developing vessel. These cells are highly responsive to the mitogenic effects of growth factors (Cuevas et al., 1991) and are capable of secreting a variety of structurally unrelated polypeptide growth factors (Sjölund et al., 1990) and inhibitors (Majewsky et al., 1991). Ultrastructurally, these cells contain large amounts of rough endoplasmic reticulum, free ribosomes, and mitochondria; there is a decrease in the $V_v$myo from approximately 70% to 37% 2 weeks after endothelial denudation. Six to 8 weeks after injury, when the overlying endothelial layer is reestablished, the $V_v$myo increases to 54% and by 26 weeks after injury has returned to levels equivalent to that of control media (Manderson et al., 1989a). Proliferation reaches a maximum in the neointima during the first week following damage and returns to baseline by 8 weeks when the intima is reendothelialised (Clowes et al., 1983a,b). Concomitant with a change in smooth muscle phenotype is a change in actin from the predominant α-isoform to the β-isoform and a change in the predominant 10-nm filament protein desmin to vimentin. When the endothelium has regenerated and the $V_v$myo of the smooth muscle cells increases to control levels, the cytoskeletal elements of the neointimal smooth muscle cells again become similar to those of the media (Gabbiani et al., 1984; Kocher et al., 1984). There is also a selective increase in responsiveness to some platelet-derived autacoids in the injured artery which may be related to the changes in smooth muscle phenotype (Manderson et al., 1989b).

In primary cell culture, medial smooth muscle cells that have been enzyme dispersed and seeded at densities below confluency undergo similar changes in phenotypic expression during the first 5 days after isolation (Campbell et al., 1989), accompanied by distinct changes in biology. Prior to change in phenotype, while the cells have a high $V_v$myo and a high α-actin mRNA expression as in the normal vessel wall, the cells do not proliferate in response to mitogens from serum, synthesise minimal collagen, and accumulate little lipid even after several days of exposure to high concentrations of β very low-density lipoprotein. In contrast, smooth muscle cells of low $V_v$myo have a decreased expression of α-actin mRNA as a percentage of total actin mRNA, proliferate logarithmically in response to mitogens from a variety of sources, synthesise 26-fold the amount of collagen (particularly collagen type I), 5-fold the amount of glycosaminoglycans (particularly chondroitin sulphate), and accumulate 3-fold the amount of lipid when exposed to β very low-density lipoprotein (Chamley-Campbell and Campbell, 1981; Campbell and Campbell, 1985; Ang et al., 1990; Campbell et al., 1989; Merrilees et al., 1990). If the cells undergo fewer than five cumulative population doublings to reach confluency, then the phenotypic change is reversible and the $V_v$myo of the cells returns to control levels. If, however, more than five cumulative population doublings occur prior to confluency, then the cells remain, apparently permanently, with a low $V_v$myo and low α-actin mRNA and respond immediately to mitogens when subcultured (Campbell et al., 1989). After approximately nine cumulative population doublings, the normal diploid cells have exhausted their replicative capacity and become senescent. These cells synthesise 45-fold the amount of collagen as high $V_v$myo cells and accumulate 7-fold the amount of lipid. They also secrete large amounts of TGF-β_3 (J. Saltis, A. Agrotis, and A. Bobik, unpublished data).

D. Pericytes

Pericytes are approximately 150 to 200 μm long and 10 to 25 μm wide. They form a discontinuous layer extending from the terminal arteriole to the postcapillary venule, often lying within the basement membrane of the endothelial cell (Forbes et al., 1977). Like smooth muscle cells, they contain α-actin in their microfilament bundles (Skalli et al., 1989). Cells with an appearance transitional between smooth muscle and pericytes exist at both arteriolar and venular ends of the microvasculature (Simionescu and Simionescu, 1983). The roles most frequently attributed to pericytes are contraction, phagocytosis, and as a source of undifferentiated mesenchymal cells. They participate in the activation of local endothelial derived TGF-β_1 (Sato and Rifkin, 1989) and respond to the mitogenic signals of bFGF.

E. Pseudoendothelium

Smooth muscle cells are capable of forming a "pseudoendothelium" which lines the luminal surface of vessels in which large areas of endothelium have been removed. The central body of these cells is embedded

within the neointima and contains large amounts of rough endoplasmic reticulum and free ribosomes; the filament bundles are small and localised toward the luminal surface of the vessel (Campbell and Campbell, 1985). These luminal smooth muscle cells, like true endothelium, are nonthrombogenic and with time produce increasing amounts of prostaglandin I_2 (Eldor et al., 1981). Like the endothelial cells, these luminal smooth muscle cells are capable of producing nitric oxide (Beasley et al., 1991; Schini et al., 1991), a vascular relaxant and potential growth inhibitor.

III. Pathophysiological Aspects of Blood Vessel Growth, Lesions, and Remodeling

A. Vascular Lesions in Atherosclerosis

Atherosclerosis is a response of the artery wall to a variety of initiating agents, with multiple pathogenetic mechanisms contributing to the formation of the plaques. Increased serum cholesterol is the most important factor in the development of plaques; hypertension accelerates their formation, and severe lesions are more likely to develop in patients with diabetes mellitus. Lesions consist of a fibrous cap of proliferated smooth muscle cells and the extracellular matrix they have produced. Beneath the fibrous cap is a highly cellular region that contains lipid-laden smooth muscle cells and macrophages and activated T-lymphocytes; all are capable of secreting a large variety growth stimulatory and inhibitory factors. Beneath this cell-rich region, there is usually a necrotic core containing cell debris, cholesterol crystals, and calcium deposits (Munro and Cotran, 1988).

Any concept of atherogenesis must account for the stimulation of smooth muscle proliferation, which is fundamental to the development of the plaque. The "response to injury" hypothesis of atherosclerosis (Ross and Glomset, 1976) is based on the proposals by Virchow in the last century. This hypothesis proposes that injury to the arterial endothelium by mechanical, chemical, toxic, viral, or immunological agents causes endothelial denudation, followed by platelet adhesion to the exposed subendothelial connective tissue, platelet aggregation, and release of PDGF. The PDGF stimulates migration of medial and smooth muscle cells into the intima, their proliferation, and the synthesis of extracellular matrix. With repeated injury, it is postulated, the atherosclerotic plaque forms.

It soon became apparent, however, that actual denudation of the endothelium is not a consistent early feature of atherosclerosis induced by hypercholesterolemia alone and that platelets and their stored growth factors are not necessary for proliferative lesions to develop. Since the time of its proposal, the hypothesis has been modified to account for the facts that the endothelium can respond to injurious stimuli not only by necrosis but also by subtle changes in function and that platelets are not the major source of growth factors for smooth muscle. Indeed, substances that influence smooth muscle and endothelial growth are derived from platelets, monocyte/macrophages, T-lymphocytes, and endothelium and smooth muscle cells themselves (see section IV).

Our understanding of the sequence of events that leads to atherosclerosis in humans has been derived, to a large extent, from animal models of the disease such as hypercholesterolemia in nonhuman primates, swine, and the Watanabe heritable hyperlipidemic rabbit. In monkeys, high plasma cholesterol concentrations similar to those observed in patients with the hereditary homozygous familial hypercholesterolemia initiate the attachment of leukocytes, principally monocytes, to the surface of the arterial endothelium. This involves enhanced IL-1 production by either the monocyte or endothelial cells and the expression of adhesion molecules on both cells (Pober et al., 1986; Warner et al., 1987). After the monocytes have adhered to the arterial endothelium, they are chemoattracted into the subendothelium where they differentiate into macrophages. The major chemoattractant is lipoprotein that has been oxidised either by the endothelium during its passage into the arterial wall, by smooth muscle cells, or by early-arriving monocyte/macrophages (Quinn et al., 1987). Other macrophage products are chemotactic for leukocytes including leukotriene B4, complement fragment C5a, TGF-β, IL-1, TNF-α, GM-CSF, and PDGF (Munro and Cotran, 1988). GM-CSF is also a growth stimulator of macrophages and is produced by the endothelium in response to oxidised low-density lipoprotein (Rajavashisth et al., 1990). Gerrity (1981) suggested that in the early stages of lesion development these monocyte/macrophages have a lipid-clearing function by becoming phagocytic, accumulating lipid and then migrating back into the blood stream by again penetrating the arterial endothelium. However, such monocyte/macrophages, particularly when lipid-laden and producing toxic oxidised lipids and superoxide anions, may further exacerbate an already dysfunctioning endothelium, as occurs in severe cases of hyperlipidemia; endothelial cell retraction occurs over these lipid-laden "foam cells." Also, many of the macrophages and smooth muscle cells, which have been overloaded with lipid, die, releasing their contents into the arterial wall to create the necrotic, lipid-rich core of the lesion. The secretion of matrix-degrading enzymes, cytokines, and growth factors by dysfunctioning endothelial cells and by invading monocyte/macrophages and T-lymphocytes initiates smooth muscle cell phenotypic changes (see section IV) and proliferation. Autocrine mechanisms may also contribute to smooth muscle cell proliferation which, together with the deposition of extracellular matrix by the smooth muscle, leads to fibrous cap formation.

B. Intimal Thickening

Intimal thickening is observed in injured arteries of humans and has generally been attributed to smooth

muscle proliferation and matrix synthesis (fig. 1A). Injury-induced intimal thickening can be the consequence of surgical trauma such as carotid endarterectomy (Cossman et al., 1978) or coronary angioplasty (Nobuyoshi et al., 1991). Restenosis following these procedures continues to be a serious clinical problem, and inhibition of the intimal thickening using some type of adjuvant pharmacology is a major challenge (see section VI). There is no doubt that an understanding of the pathophysiological changes that occur following balloon injury will provide the basis for appropriate drug design to keep vessels patent, but to date only limited information has been obtained from humans concerning the time course of changes in vessel wall architecture that leads to restenosis (Nobuyoshi et al., 1991). Knowledge of the sequence of events has come from studies of experimental animals, in particular rabbits and rats (Spaet et al., 1975; Fingerlie et al., 1990).

The time course of changes in cellular composition and extracellular matrix that occurs following balloon injury to vessels has been studied in the carotid artery (Fingerlie et al., 1990), the aorta (Spaet et al., 1975), and the iliac and femoral arteries (Consigny et al., 1986) (see also section II.C). Immediately after removal of the endothelial cell layer, the denuded vessel wall becomes covered with platelets, a major source of growth factors (see section IV.D.4); within 24 hours there is also a scattering of neutrophils and monocytes that have attached themselves to the luminal surface of the vessel (Reidy et al., 1985). Approximately 1 week later the platelet population has largely disappeared, and, in the absence of a regenerated endothelial layer, smooth muscle cells, which have migrated from the media through fenestrations in the internal elastic lamina and undergone proliferation, cover much of the luminal surface (Spaet et al., 1975; Reidy et al., 1985).

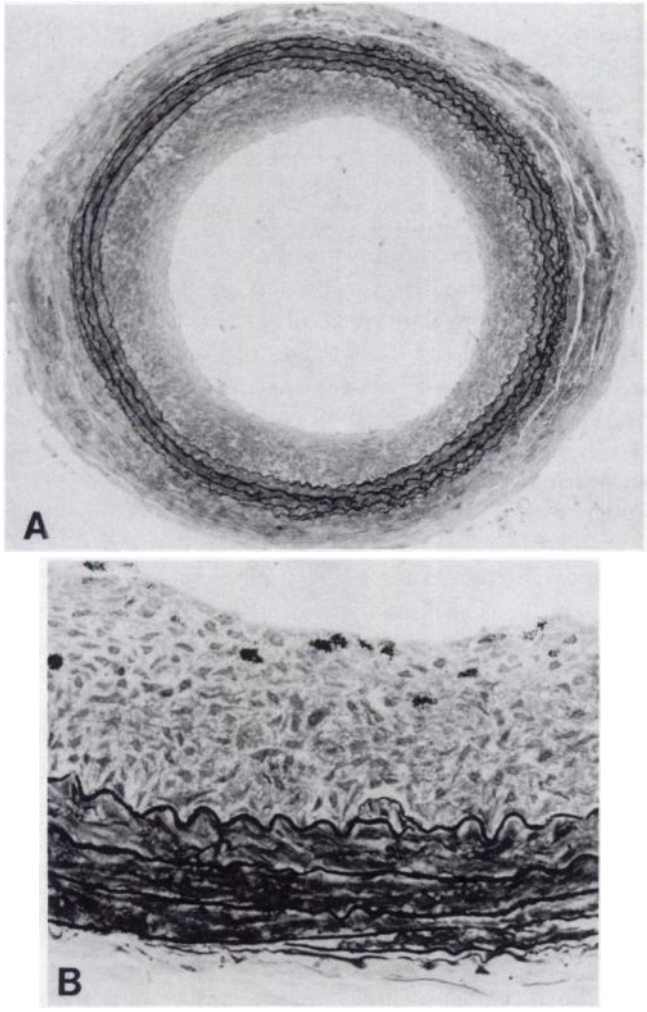

FIG. 1. Histological cross-sections of a left carotid artery from a rat 2 weeks after balloon catheter injury. Top, Note the wrinkled lamelli, a consequence of marked vasoconstriction. Bottom, Active proliferation of smooth muscle cells at the surface of the newly formed neointima (demonstrated by the dark grains of [^{3}H]thymidine), the pseudoendothelium.

The initial stimulus for the smooth muscle cells to migrate is closely associated with an increase in vessel urokinase activity and tissue-type plasminogen activator (Clowes et al., 1990), whereas the proliferative response occurs in the presence of increased PDGF-A chain (Majewsky et al., 1990) and bFGF expression (Lindner et al., 1991). Direct evidence implicating bFGF in the proliferation of migrating smooth muscle cells has recently been obtained using antibodies against bFGF (Lindner and Reidy, 1991). The production of TGF-β_1 is also increased at this time (Majewsky et al., 1991). The smooth muscle cells forming the neointima are in the synthetic phenotype; they possess a great abundance of rough endoplasmic reticulum and free ribosomes and a low $V_v$myo (Manderson et al., 1989a). After a few weeks the areas that endothelial cells have been unable to repopulate become entirely covered by round, unorientated smooth muscle, termed a pseudoendothelium (see section II.E). During this time virtually all of the thickening that has occurred in the injured vessel wall is the consequence of smooth muscle migration from the media and proliferation in the intima. Subsequently, only smooth muscle at the surface continues to secrete PDGF-A and (Majewsky et al., 1990) maintain a high level of proliferative activity, whereas those deep within the intima and media return to quiescence (fig. 1B) and no longer possess large amounts of rough endoplasmic reticulum but regain their original $V_v$myo.

During the first few weeks following endothelial denudation, active contraction of this region of the vessel accounts for the bulk of the luminal encroachment (Clowes et al., 1983a). The pathophysiological significance of this prolonged contraction, which may be maintained for more than a month, is unknown but is analogous to that produced by myofibroblasts in open skin wounds (Gabbiani et al., 1971). Vessels are known to undergo marked constriction when endothelial cell-derived nitric oxide production is inhibited (Moncada and Higgs, 1991). Thus, removal of this natural vasodilator

from the vessels could also account for the vasoconstriction observed in regions denuded of endothelial cells. Recently, it was shown that nitric oxide synthetase can be induced in vascular smooth muscle of the synthetic phenotype (Beasley et al., 1991; Schini et al., 1991), and therefore, in the absence of endothelium, the modified luminal smooth muscle of the pseudoendothelium may reverse this constriction by producing nitric oxide.

Under normal circumstances, rapid reendothelialisation of vessels prevents myointimal thickening due to reductions in smooth muscle cell proliferation and the accumulation of extracellular matrix (Teida and Reidy, 1987). However, the endothelium only appears capable of covering a small area of a large denuded region before its replication rate decreases dramatically. Thus, small lesions may be completely reendothelialised, whereas in the large lesions luminal smooth muscle becomes the predominant surface cell (see fig. 1 and section II.E). The reason for this inability of the endothelial cell to regenerate over the surface of the lesion is unknown but may be related to the appearance of the modified smooth muscle on the luminal surface together with the synthesis of a matrix containing factors (e.g., TGF-β homologues) that are unfavourable for endothelial cell growth.

C. Vascular Hypertrophy in Hypertension

In hypertension, vascular hypertrophy can be primary in nature, occurring before the onset of hypertension and contributing to its subsequent development. In addition, and possibly more important, hypertension can initiate, and then exacerbate, abnormalities in arterial wall structure. Increases in vessel wall thickness in response to elevated blood pressure initially tend to be protective in nature, enabling vessels to better withstand the increase in wall tension imposed by the elevated intravascular pressure. These changes in vessel architecture differ markedly from those usually associated with the normal blood pressure elevation that occurs during development, when there is a rapid increase in the number and size of the lamellar units containing medial smooth muscle cells, allowing the tension per unit to remain constant.

In contrast, in hypertension, vessel wall thickness is not achieved by increasing the number of lamellar units but, rather, by increases in muscle mass and connective tissue. Vascular smooth muscle hypertrophy, polyploidy, and proliferation account for the increase in muscle mass, and increases in the synthesis of collagen, elastin, proteoglycans, and fibronectin by the smooth muscle account for most of the increase in extracellular matrix (Owens and Reidy, 1985; Owens et al., 1988a,b; Black et al., 1989, Chobanian, 1989; Mulvany et al., 1978). By encroaching on the lumen of blood vessels, these changes in wall architecture become the major determinant of the increase in vascular resistance and, hence, blood pressure elevation in established hypertension (Folkow et al., 1970). The prevention and reversal of these changes greatly reduce the severity of hypertension in both experimental animals and in humans (Adams et al., 1990; Black et al., 1989). The full potential of normalising the vascular structure in established hypertension will only be realised when there is a better understanding of how growth factors interact with endothelial and smooth muscle cells, with the matrix, and with each other.

Extensive changes in endothelial cell morphology and functioning are associated with hypertension. In most instances, they appear to be secondary to the elevated blood pressure; however, they have the potential to profoundly influence vascular structure through the secretion of a variety of growth-promoting and inhibitory factors (see section IV). Because of difficulties in procuring viable human material, most of the knowledge of endothelial cell function in hypertension has been derived from laboratory animals with either primary hypertension or various types of experimentally induced hypertension. In steroid-dependent salt-induced hypertension in rats, the changes in endothelial cell morphology can be seen with only moderate increases in blood pressure. Initially, the subendothelial zone becomes wider and is filled with loose extracellular material, a thickened reticular basement membrane, and, possibly, precipitated plasma proteins, and endothelial cells bulge toward the lumen but still exhibit intact functional complexes. Subsequently, dense material, including reticulated basement membrane and collagenous extracellular material, completely fill the widened subintimal zone (Still et al., 1968; Haudenschild et al., 1981). The endothelial cells continue to increase in height and their nuclei become very nonuniform and frequently bizarre in configuration.

Majno et al. (1969) first interpreted this degree of nuclear folding as being a morphological equivalent of endothelial cell contraction, an observation that is consistent with an increased amount of actin-containing filaments in these cells. With the widening of the subendothelial zone, the adhesion sites of the endothelial cells may be reduced to a few semidesmosomes found on endothelial extensions near the internal elastic lamina. The endothelial cell layer also becomes more permeable to a variety of agents including albumin, lipoproteins, and colloidal carbon. An increase in adherence of blood-borne monocytes, lymphocytes, and granulocytes to the endothelial cells has also been reported (Kowala et al., 1988; Haudenschild et al., 1981). This increase in adhesion to the endothelial cells is most probably related to a reduction in their ability to produce nitric oxide, because endothelium-dependent relaxation is frequently reduced in hypertension (Moncada and Higgs, 1991). Subsequent additional increases in intimal area are associated with the appearance of subendothelial cells of different origins, e.g., monocytes, and probably modified smooth muscle that often exhibits swollen cytoplasmic

extensions and increased amounts of organelles (Greditzer and Fischer, 1978).

The mechanisms responsible for these initial changes in endothelial structure and function are unknown but probably involve mechanical factors because a similar course of events occurs in the large vessels of SHR with established hypertension. These changes initially appear to be focal but with time extend to affect large areas of the vessels. Although not extensively studied, endothelial cell dysfunction in small resistance arteries could be expected to be similar to that seen in the larger vessels. In many instances, the ability of endothelial cells in such vessels to produce nitric oxide is also impaired (Moncada and Higgs, 1991), and this in turn could increase monocyte adhesion and initiate a course of effects similar to that seen in the larger arteries. Damaged endothelial cells also produce copious amounts of growth factors (see section IV). In very severe hypertension, this may even initiate microvessel rarefaction (Hansen-Smith et al., 1990).

The changes in smooth muscle ultrastructure and size that occur in hypertension are, to a large extent, dependent on the size of the vessels and the nature of the hypertension. In the large muscular and elastic arteries, increases in cell size account for much of the increase in medial wall diameter. The aortic smooth muscle cell hypertrophy that occurs in primary hypertension in SHR, as well as in renal and steroid- and salt-dependent hypertension, is frequently accompanied by an increase in DNA ploidy, implying that a subpopulation of cells has entered into the mitotic cell cycle (Black et al., 1989; Owens and Schwartz, 1983; Lichtenstein et al., 1986). A large proportion of these polyploidy smooth muscle contain tetraploid DNA and, on occasions, may even contain octaploid DNA. Polyploid vascular smooth muscle cells also contain considerably more protein than do diploid cells; the increases in actin and myosin that occur in these cells are in proportion with increases in other cellular proteins. The precise factors responsible for the induction of polyploid cells in blood vessels have not been identified, although TGF-β_1 and PDGF-AA are capable of inducing polyploidy in vascular smooth muscle cells (see section IV.A.C). The increased frequency of polyploid cells in vessels need not be solely the consequence of hypertension. In large human elastic arteries, the increases in the frequency of polyploid smooth muscle appear more related to age. Again, this may be the consequence of age-dependent vascular remodeling induced by cytokines.

Increases in cell size may also occur through partial dedifferentiation of the smooth muscle cells. Dedifferentiated smooth muscle cells ("synthetic" phenotype) occupy two to three times the volume of those in the differentiated "contractile" phenotype (Campbell et al., 1989). Although low in frequency, synthetic phenotype smooth muscle, characterised by a large increase in endoplasmic reticulum and a low $V_v$myo, has been consistently found in the large vessels of hypertensive animals. On occasion, these cells may protrude through fenestrations in the internal elastic lamina and migrate into the subendothelial space (Haudenschild et al., 1980).

It has been suggested that in some vessels an increase in the wall thickness may occur by a reorganisation of the smooth muscle cells around a narrower lumen rather than as a result of cellular growth (Baumbach and Heistad, 1985; Short, 1966). The secretion of a wide variety of proteolytic enzymes by the smooth muscle would be a critical event in this type of vascular remodeling. It is highly likely that growth factors would be involved in this remodeling because they are potent stimuli for the production of proteolytic enzymes by vascular smooth muscle cells and play a major role in controlling the composition of the resynthesised matrix (see section IV).

D. Angiogenesis

Angiogenesis (the formation of new blood vessels) may occur in response to a variety of physiological or pathophysiological stimuli. Insights into the complex processes that occur during new vessel formation have been obtained from a variety of pharmacological animal model systems, including the avascular cornea of the rabbit and the chick embryo chorioallantoic membrane (Muthukkaruppan and Auerbach, 1979; Klagsbrun et al., 1976).

Initiating angiogenic factors (e.g., angiogenin, bFGF, TGF-β_1) that stimulate new blood vessel formation may be secreted either by avascular tissues or, in the case of hypoxic tissue, by macrophages or T-lymphocytes (Folkman and Klagsbrun, 1987). Initially, the endothelial cells respond to the angiogenic stimulus by secreting plasminogen activator and a variety of proteolytic enzymes, causing fragmentation of the basal lamina (Folkman, 1982). Endothelial cells closest to the angiogenic stimulus then commence to migrate through the fragmented lamina and into the perivascular space. After a few days the capillary buds have elongated to an extent that cross-sections of new vessels can be seen a short distance from the parent vessel. The capillary sprouts then begin to branch at their tips and join to form loops. Blood then commences to flow through the newly formed capillary. Pericytes subsequently cover the abluminal endothelial cell surface.

A role for vascular derived growth factors in angiogenesis is suggested by the observation that DNA synthesis in endothelial cells commences while they are still within the parent vessel. Mitosis also occurs in cells following the "leader" endothelial cells, but these cells, at the tip of the new capillary, do not undergo mitosis themselves (Folkman, 1982).

bFGF is one potentially important growth factor that can initiate mitogenesis in the endothelial cells. It is stored in high concentrations within the extracellular matrix as an inactive complex and is released when the

matrix is dissolved by the activated endothelial cells (see section IV.B). Heparin, which enhances bFGF binding to its membrane receptor, enhances angiogenesis (Yayon et al., 1991; Folkman and Klagsbrun, 1987). Migrating endothelial cells participating in angiogenesis also produce copious amounts of inactive TGF-β, which subsequently prevent further migration and proliferation (Sato and Rifkin, 1989). Microvascular endothelial cells also synthesise and respond to PDGF (Beitz et al., 1991; see section IV.A).

IV. Endothelium- and Smooth Muscle-Derived Growth Factors

A. Platelet-Derived Growth Factor

PDGF was discovered in 1974 by two groups (Kohler and Lipton, 1974) as the principal mitogen of whole blood serum acting on cultured mesenchymal cells. It is not the only mitogen released by activated platelets, but it accounts for 50% of the mitogenic activity (Heldin et al., 1981) (see section IV.D.4). The term PDGF is now known to be inaccurate because it is produced by many diploid cells such as endothelial cells (Dicorleto and Bowen-Pope, 1983), vascular smooth muscle (Seifert et al., 1984; Nilsson et al., 1985), and activated monocyte/macrophages (Shimokado et al., 1985; Martinet et al., 1986).

PDGF exhibits multiple molecular weight forms, ranging in size from 28,000 to 35,000 Da, and is composed of two distinct, but homologous, polypeptide chains, denoted A and B, linked by disulphide bonds (Antoniades and Hunkapiller, 1983; Waterfield et al., 1983). All possible dimeric combinations of PDGF chains (PDGF-AA, PDGF-BB, and PDGF-AB) have been found. For example, human platelets contain 70% PDGF-AB heterodimer with the remainder being PDGF-BB homodimer (Hammacher et al., 1988); porcine platelet PDGF is of the BB homodimer form only (Stroobant and Waterfield, 1984); conditioned media from several tumour cell lines contain only the AA homodimer (Heldin et al., 1986).

Each PDGF molecule contains 16 cysteine residues (eight per chain), and the A chain is 51% homologous to the B chain over 109 amino acids. The mature A and B chains are hydrophilic and contain a relatively large number of basic residues (Heldin et al., 1977; Ross et al., 1979). Both the A and B chains are synthesised as precursors in which a number of regions of the polypeptides including the N-terminal region share up to 50% homology. Nonhomologous regions are largeley confined to exons 3 and 6 (see fig. 2 for details) (Raines et al., 1989). Carbohydrate analysis indicates that 4% to 7% of PDGF consists of neutral or amino sugars (Deuel et al., 1981), which probably accounts for the discrepancy between the predicted molecular weight of the A chain (12,000) and the molecular weight estimated from sodium dodecyl sulphate-polyacrylamide gel electrophoresis (16,000 to 18,000). Sugar residues include mannose, galactose, glucosamine, galactosamine, fucose, and sialic acid. A possible glycosylation site, Asn-Thr-Ser, exists at residue 48 of the mature A chain. No sites for N-linked glycosylation exist on the mature B chain, and the difference in predicted molecular weight (12 kDa) from that estimated from sodium dodecyl sulphate-polyacrylamide gel electrophoresis (14 to 16 kDa) is due to O-linked glycosylation or some other posttranslational modification.

In 1983, it was shown that the B chain of PDGF is 93% homologous with $p28^{sis}$, the transforming gene product of v-*sis* from simian sarcoma virus (Doolittle et al., 1983; Waterfield et al., 1983; Johnsson et al., 1984). Indeed, the PDGF-B chain is encoded by the c-*sis* protooncogene, the cellular homologue of the oncogene transduced by both the simian sarcoma virus and Parodi-Irgenes feline sarcoma virus (Gelmann et al., 1981; Doolittle et al., 1983; Josephs et al., 1984). The B-chain gene is located on human chromosome 22 and contains seven exons spacing 24 kilobases of genomic DNA (fig. 2). It has a close structural relationship with the A-chain gene, located in the proximal long arm of chromosome 7, which suggests that they stem from a common ancestral gene.

PDGF mRNA is expressed by a wide variety of cells (Raines et al., 1989). In the normal vessel wall, PDGF-A chain transcripts are primarily associated with the smooth muscle-rich media, whereas PDGF-B chain transcripts are expressed in the adventitia localised to macrophages and to the endothelium of vasa vasorum (Collins et al., 1985; Barrett and Benditt, 1988). However PDGF-A chain is expressed in microvascular endothelial cells (Starksen et al., 1987). Expression in vivo by endothelial and vascular smooth muscle cells, however, is only a small percentage (usually <10%) of that seen when the same cells are grown in culture (Barrett et al., 1984; Barrett and Benditt, 1987; Majesky et al., 1988). This indicates that expression of PDGF is tightly regulated and that caution must be taken when extrapolating culture studies to mechanisms in vivo.

All the same, the level of constitutive expression and secretion of PDGF appears to be affected by the state of the cells when isolated in vivo. For example, medial smooth muscle cells isolated from 13- to 18-day-old (pup) rats and intimal smooth muscle cells isolated from adult rat carotid arteries 2 weeks after balloon catheter injury secrete higher levels of PDGF into the culture medium than do smooth muscle cells isolated from adult arterial media (Seifert et al., 1984; Majesky et al., 1988; Walker et al., 1986). The pup cells continue to secrete increased levels of PDGF for up to 20 passages in vitro and to express high levels of both A- and B-chain mRNA (Seifert et al., 1984). Adult rat aortic smooth muscle cells kept in serum-free medium in primary culture show high levels of PDGF-A chain mRNA and high PDGF receptor activity but do not secrete detectable amounts of PDGF-like mitogen (Sjölund et al., 1988). Upon exposure to

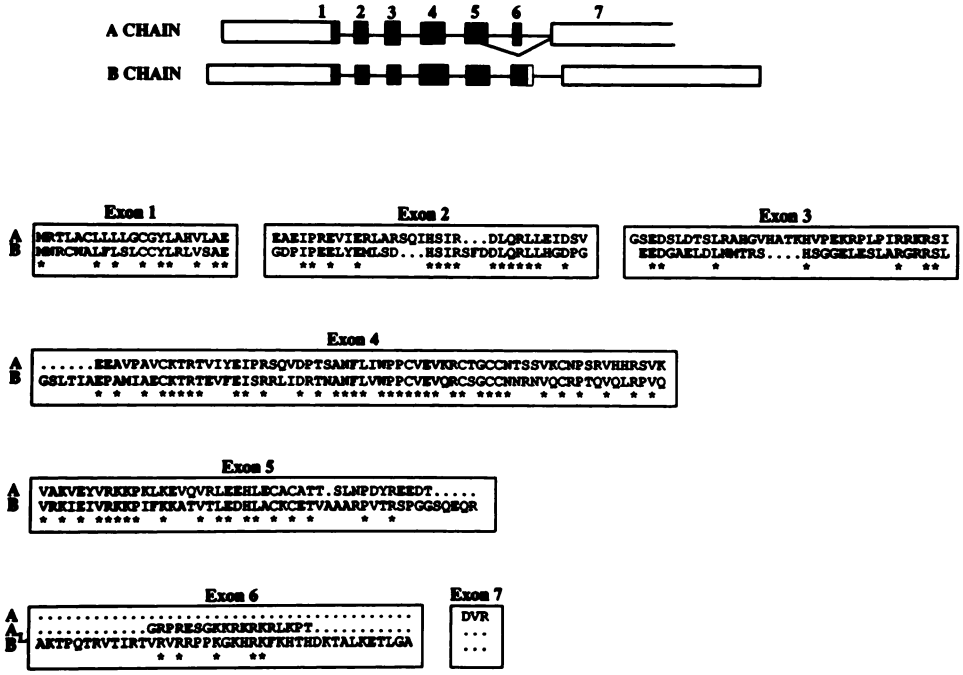

FIG. 2. A, Diagram shows the intron-exon structures of PDGF-A and PDGF-B chains. B, Amino acid sequences of the PDGF-A and PDGF-B chains depicting that nonhomologous regions are largely confined to exons 3 and 6. The PDGF-A chain depicted is the short form of the A chain isolated from human endothelial cells (Collins et al., 1985). A_L represents the long form of PDGF-A chain isolated from a human glioma cell line (Betsholtz et al., 1986). The A_L form appears to be only a minor component of the PDGF produced by endothelial cells.

PDGF, a PDGF-like mitogen is released into the culture medium. Concomitantly, there is a decrease in the amount of A-chain transcripts per cell and the ability of cells to bind radiolabeled PDGF.

The expression and secretion of PDGF by cultured cells can be modulated by several factors. For example, the constitutive, stable secretion of PDGF by endothelial cells is decreased by some modified low-density lipoproteins and fish oils (Fox and DiCorleto, 1986, 1988; Fox et al., 1987) and by forskolin, an activator of adenyl cyclase (Daniel et al., 1987). Transient induction of PDGF occurs in the presence of TGF-β, TNF-α and by two products of the coagulation cascade, thrombin and activated factor X, all physiological mediators present at sites of injury (Harlan et al., 1986; Gadjusek et al., 1986). Thrombin initiates release of PDGF from an intracellular storage pool in endothelial cells and then causes a transient increase in transcript levels, followed by secretion of PDGF. The effect of thrombin on PDGF secretion is mimicked by exposure of endothelial cells to phorbol myristrate acetate (Daniel et al., 1986); however, phorbol myristrate acetate is more effective in inducing B-chain transcripts, and thrombin is more effective in inducing A-chain transcripts (Starksen et al., 1987). TGF-β increases the transcriptional rate of both A and B chains (Kavanaugh et al., 1988). This indicates that the forms of PDGF produced by a given cell may vary with the inducing stimulus. At present little is known about how these different agents initiate PDGF gene transcription.

There is also only limited information concerning the processing and cellular localisation of PDGF in normal diploid cells. However, results of transfection experiments have indicated that PDGF-AA and PDGF-AB are secreted as 30-kDa products, whereas PDGF-BB is processed into a 24-kDa product that remains associated with the cells, primarily within the endoplasmic reticulum and Golgi complex. Eventually, it may also be transferred to lysosomes for degradation. In contrast, PDGF-AA follows a pathway for bulk flow, with rapid passage through the endoplasmic reticulum and Golgi complex, package in secretory vacuoles, and extracellular release by exocytosis (Thyberg et al., 1990).

PDGF binds with high affinity to its receptors (K_D approximately 10^{-9} to 10^{-11} M) (Williams et al., 1982; Bowen-Pope and Ross, 1982). These early studies were generally interpreted in terms of PDGF binding to a single form of PDGF receptor, a membrane-bound glycoprotein of molecular weight 170 to 180 kDa, possessing an extracellular ligand-binding domain and an intracellular tyrosine kinase domain (Raines et al., 1989). However, with the availability of recombinant PDGF-AA and PDGF-BB, monoclonal antibodies to the PDGF receptor, and cDNA clones for a PDGF receptor came additional information, and it is now proposed that the PDGF receptor is a dimer of α-subunits, which can bind A or B chains, and/or β-subunits, which can bind only B chains (Seifert et al., 1989). The α- and β-subunits of the PDGF receptor appear to be approximately 170 to 190 kDa in size (Seifert et al., 1989); both are acidic in nature with isoelectric points of approximately 4.5 (Claesson-Welsh

et al., 1987). The gene for the α-subunit has been mapped to the long arm of human chromosome 5, and the β-subunit has been mapped to the long arm of chromosome 4 (Yarden et al., 1986; Matsui et al., 1989). Three possible dimeric receptors can form: $\alpha\alpha$, $\alpha\beta$, and $\beta\beta$. $\alpha\alpha$ binds PDGF-AA, PDGF-AB, and PDGF-BB; $\alpha\beta$ binds PDGF-AB and PDGF-BB; and $\beta\beta$ binds only PDGF-BB. In vascular smooth muscle, PDGF-BB does not apparently bind to all three dimeric receptors. Although PDGF-AB and PDGF-BB are equipotent in terms of their ability to stimulate DNA synthesis in vascular smooth muscle, PDGF-AB is very ineffective in displacing PDGF-BB from its membrane-binding sites (fig. 3) (Bobik et al., 1990a). In the absence of PDGF, the receptor subunits exist as separate monomers or as unstable dimers. With the addition of PDGF there is ligand-induced subunit dimerisation or stabilisation (Heldin et al., 1989; Li and Schlessinger, 1991). Under these conditions the half-life of the cell surface PDGF receptor is reduced from 3 to 4 hours to approximately 30 minutes (Hart et al., 1987, 1989; Huang and Huang, 1988). All three isoforms of PDGF are internalised via PDGF receptors and subsequently degraded in lysosomes with similar kinetics.

Structure-activity studies have been carried out with the different PDGF isoforms. Replacement of Asn-115, Arg-154, and Ile-158 of the PDGF chain with the corresponding A-chain amino acids leads to a dramatic decrease in the affinity for the β-receptor. Conversely, introduction of B-chain amino acids into the A-chain in the region spanning from Asn-115 to Ile-158 yields a product with high affinity for the β-receptor, indicating that Asn-115, Arg-154, and Ile-158 are likely to be part of the active binding site of the PDGF-B chain (Ostman et al., 1991). No other growth factor or substance has been shown to bind to the PDGF receptor with high affinity. Basic proteins such as protamine, histone, and polylysine bind with low affinity at relatively high concentrations (Huang et al., 1982). Suramin, a small acidic molecule that inhibits many protein-protein interactions, efficiently strips off prebound PDGF (Williams et al., 1984). It is not specific for PDGF and is toxic when used for several hours at 37°C (Hawkins, 1978).

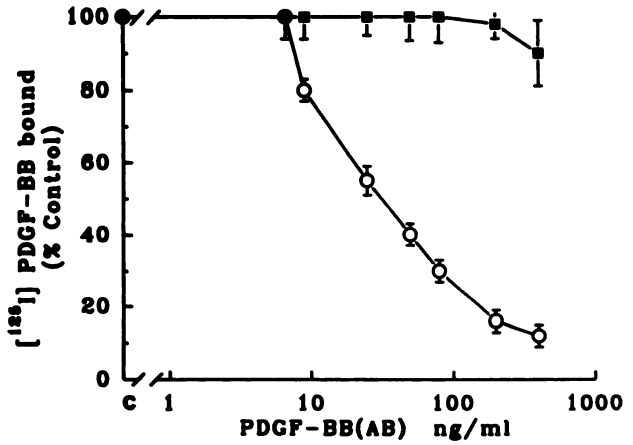

FIG. 3. Displacement of [^{125}I] PDGF-BB from high-affinity binding sites on primary cultures of rat aortic smooth muscle by increasing concentrations of PDGF-BB (○) and PDGF-AB (■). Despite being equipotent with respect to their mitogenic effects on aortic smooth muscle, PDGF-BB and PDGF-AB do not appear to bind to the same high-affinity receptors (Bobik et al., 1990a). Reproduced with permission.

The number and proportion of each of the two receptor subunits differ widely from cell to cell, with pup rat vascular smooth muscle cells in culture expressing only β-subunits, and most diploid fibroblasts have at least 5-fold as many β-subunits as α-subunits (Raines et al., 1989). Receptor expression in vivo is low in normal tissues and increases in response to various forms of disturbance including adaptation to culture (Terracio et al., 1988). Smooth muscle cells in normal large arteries express low levels of receptors, but smooth muscle in atherosclerotic lesions express higher levels (Rubin et al., 1988; Wilcox et al., 1988). The cells with high receptor levels in the atherosclerotic lesion are described as "mesenchymal appearing intimal cells." Deoxycorticosterone acetate-salt hypertension in rats induces a 3-fold increase in aortic steady-state PDGF-β receptor mRNA levels and is also higher in SHR compared with normotensive controls. PDGF receptor mRNA levels are not altered, indicating that the genes of the PDGF ligand/receptor system are differentially regulated in hypertension (Sarzani et al., 1989, 1991).

The interaction of PDGF with its receptor-type protein-tyrosine kinase induces dimerisation of the receptor subunits and cross-phosphorylation on tyrosine residues. These multiple tyrosine phosphorylation sites on the dimerised PDGF receptor provide specific binding sites for a variety of cytosolic enzymes containing specific SH-2-binding domains (for review, see Cantley et al., 1991), such as phospholipase C-γ, phosphatidylinositol-3 kinase, and ras-GAP, which are then activated by tyrosine phosphorylation. Serine/threonine protein kinases are also activated, which in turn activate ribosomal protein S_6 kinase II and nuclear lamin C, kinases involved in protein synthesis, as is nuclear lamin C. The activation of these intracellular pathways together with other auxiliary responses, such as the stimulation of protein kinase C, Na^+/H^+ exchange, and an elevation in intracellular Ca^{2+}, is responsible for the activation of the c-*myc*, c-*fos*, and c-*jun* genes. Activation of the latter two genes increases the nuclear concentration of the jun-fos protein complex which in turn activates the transcription of a large variety of genes (for review, see Hunter, 1991). Another "early" gene whose expression is regulated by PDGF is JE which codes for a protein with cytokine-like properties (Campbell et al., 1991). Excluding the auxiliary pathways, all mitogens activating receptor-type protein kinases stimulate this series of events during the cell's progress through the mitotic cell cycle.

The A chain of PDGF is 20-fold less efficient in inducing transformation of 3T3 cells than is the B chain

under the influence of the same promoter (Beckmann et al., 1988). There also are marked differences in the secretory and mitogenic properties of the two chains, which may account for the differences in transformation potential. PDGF-AA, however, is not intrinsically less mitogenic than PDGF-BB because the mitogenic potency of the different isoforms depends on the cell type and the ability of the cell to bind the isoform (Seifert et al., 1989). Thus, 3T3 cells, which express high levels of both receptor subunits, are equally stimulated by all three isoforms of PDGF, whereas human dermal fibroblasts, which express few α-subunits, are stimulated by PDGF-AB and PDGF-BB but only minimally by PDGF-AA.

As well as directly stimulating mitogenesis, PDGF can enhance the proliferative response by inducing the expression of other genes whose products then amplify the mitogenic response. An example of this is the stimulation by PDGF of somatomedin C-like substances by aortic smooth muscle cells (Clemmons and Van Wyk, 1985). There is a clear synergism of PDGF with a number of growth factors (Lynch et al., 1987; Lawrence et al., 1986). At high concentrations, TGF-β differentially down-regulates the expression of PDGF-binding sites and thus the mitogenic responsiveness toward the three PDGF isoforms (Gronwald et al., 1989). At low concentrations (1 to 2 fg/cell), TGF-β elicits maximal proliferation of cultured smooth muscle cells, presumably by stimulating autocrine PDGF-AA secretion (Battegay et al., 1990; Majack et al., 1990). Similarly, the mitogenic activity of IL-1 for smooth muscle cells is indirect and mediated by induction of the PDGF-A chain gene (Raines et al., 1989). Furthermore, both PDGF and EGF induce expression of PDGF-A chain. Angiotensin II increases PDGF-A chain expression in quiescent adult rat aortic smooth muscle cells in culture accompanied by a 15- to 20-fold increase in PDGF concentration in the culture medium (Naftilan et al., 1989). Also, angiotensin II and noradrenaline potentiate the submaximal, but not maximal, mitogenic effects of PDGF-BB, but not PDGF-AB, by an increase in BB-receptor number (Bobik et al., 1990a).

PDGF is a potent chemotactic agent for smooth muscle, and the concentration of PDGF required for maximal chemotactic activity coincides with that for maximal mitogenic stimulation (Seppa et al., 1982; Grotendorst et al., 1982). Cells expressing only the β-receptor subunit are responsive only to PDGF-BB, whereas cells expressing the α-receptor subunit are equally responsive to all three dimeric forms, PDGF-AA, PDGF-AB, and PDGF-BB (Ferns et al., 1990). PDGF is also chemotactic for mononuclear cells and neutrophils, which do not respond mitogenically to PDGF (Deuel et al., 1982).

In response to PDGF, (fig. 4) mesenchymal cells show an increased synthesis of both noncollagen protein and collagen, particularly type V collagen, although it also regulates the amount of type III versus type IV collagen synthesised (Canalis, 1981; Narayanan and Page, 1983). PDGF stimulates the activity of stromelysin and interstitial collagenase in mesenchymal cells and the synthesis of tissue inhibitor of metalloproteinases (Chua et al., 1985; Circolo et al., 1991) and modulates the expression and secretion of thrombospondin, a cell-associated matrix protein involved in cell-matrix interactions (Majack and Bornstein, 1984, 1985). PDGF can also induce concentration-dependent contraction of strips of rat aorta (Berk et al., 1986), although many other areas of the vascular tree are unresponsive (Bassett et al., 1988).

PDGF causes a decrease in smooth muscle α-actin synthesis and concentration in quiescent, postconfluent smooth muscle cells in culture concomitantly with their increasing in size and undergoing one synchronous round of DNA synthesis (Blank and Owens, 1990). PDGF acts

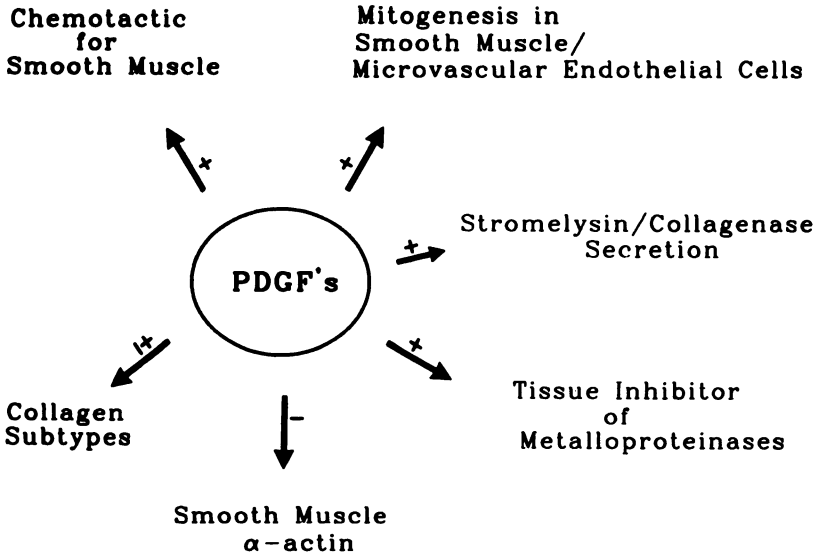

FIG. 4. Summary of the diverse actions of PDGFs on vascular cells.

by inducing a transcriptionally dependent destabilisation of the cytosolic smooth muscle α-actin mRNA pool, indicating that it may play a role in regulating smooth muscle differentiation via a posttranscriptional control mechanism (Corjay et al., 1990). As smooth muscle cells modulate phenotypically from a contractile (high $V_v$myo) state to a synthetic (low $V_v$myo) state in serum-free primary culture, α-receptors for PDGF appear (Sjölund et al., 1990). The β-receptor for PDGF is already expressed in vivo and increases further as the cells are isolated and cultured in vitro. The content of PDGF-A chain mRNA starts to increase, and the cells retain a high level of DNA synthesis in serum-free medium. As a result of this autocrine stimulation, the PDGF receptors are down-regulated. This supports the idea that phenotypic modulation of arterial smooth muscle cells prepares the cells to enter the cell cycle (Chamley-Campbell et al., 1979, 1981); when stimulated by an exogenous growth factor, they enter the cell cycle and, in the absence of other growth regulators such as TGF-β, begin to stimulate their own growth in an autocrine manner by production of PDGF-AA (Sjölund et al., 1990).

Although there is little information about the physiological significance or functions of the different PDGF isoforms in normal arteries, evidence is accumulating to indicate that these growth factors have important roles in angiogenesis, wound repair, arterial intimal thickening, vascular hypertrophy in hypertension, atherosclerosis, and neoplasia, all of which are related to its stimulation of chemotaxis, matrix reorganisation, and cell proliferation. PDGF may also be involved in scleroderma, a vascular disease in which there is proliferation of connective tissue surrounding the blood vessels (Takehara et al., 1987; Pandolfi et al., 1989). There is also some information indicating that developmentally regulated production of PDGF and its receptors contributes to autocrine stimulation of proliferation in embryogenesis.

B. Fibroblast Growth Factors

The existence of substances in the brain and pituitary that promote the growth of cultured fibroblasts was already well known by 1939 (Trowell et al., 1939). On the basis of these early findings, bFGF was isolated from bovine pituitary in 1975 (Gospodarowicz, 1975). This molecule has a high (basic) isoelectric point and is sensitive to acid and high temperature. aFGF was subsequently also isolated from the same tissue (Lemmon and Bradshaw, 1983). Both have a strong affinity for the anionic glycosaminoglycan heparin (Baird and Ling, 1987). This property greatly facilitated their final purification. The complete amino acid sequence of bovine pituitary bFGF was established by classical protein sequencing (Esch et al., 1985); it is a single-chain protein of 146 amino acids with an apparent molecular weight of 16.5 kDa. More recently, amino-terminally truncated or extended forms have also been reported. Results of Western blotting experiments have repeatedly suggested the existence of larger forms of bFGF (Presta et al., 1988) that appear to be equally bioactive (Florkiewicz and Sommer, 1989); however, their precise physiological function with respect to cell growth and differentiation remains to be determined. The amino-terminally truncated forms of aFGF and bFGF are generally regarded as extraction artifacts.

Recently, high sequence homology was found between the FGFs and three cellular oncogene products, suggesting the presence of a family of FGFs. The first related protein is encoded by the *hst/ks* oncogene present in fibroblasts transfected with Kaposi's sarcoma DNA (Yoshida et al., 1987); it was also identified in a stomach cancer DNA (Barr et al., 1988b). In both instances, the mRNA codes for an identical 206 amino acid protein whose carboxy-terminal portion has about 40% homology with bFGF. Studies with antagonists of bFGF indicate that *hst/ks* exerts its effect by interacting with FGF receptors (Baird et al., 1988; Halaban et al., 1988) It stimulates the proliferation of fibroblasts and endothelial cells as well as induces the release of plasminogen activator (Delli-Bovi et al., 1988). Like aFGF and bFGF, it also binds to immobilised heparin, but unlike the FGFs it is glycosylated and secreted (Delli-Bovi et al., 1987, 1988). The protein product of the *int*-2 oncogene is also closely related to FGF (Dickson and Peters, 1987). *int*-2 codes for a 243-residue protein whose amino terminus is homologous to the FGFs, and, like bFGF, it has no obvious signal sequence required for secretion. It appears to be very transiently expressed in early embryogenesis. FGF-5 is the fifth member of the FGF family (Zhan et al., 1988) and possesses about 50% structural homology with aFGF and bFGF, but unlike these it possesses a signal sequence. FGF-5 is mitogenic and also possesses heparin-binding properties (Zhan et al., 1988). More recently, FGF-6 (Marics et al., 1989) and FGF-7 (KFG) have also been described (Rubin et al., 1989).

In the cardiovascular system, arterial and capillary endothelial cells, vascular smooth muscle cells, and fibroblasts are all capable of synthesising bFGF and aFGF. The genes coding bFGF and aFGF are single-copy genes located on chromosomes 5 (Jaye et al., 1986) and 4 (Mergia et al., 1986), respectively. The bFGF gene contains three exons and spans at least 38 kilobases of genomic DNA. The close similarities between genes of the FGF family indicate that they originally evolved from a single ancestral gene with virtually no change in exon structure. The cDNAs that encode the mRNAs for mature bFGF and aFGF possess open reading frames that predict the existence of 155-residue proteins.

In most tissues, polyadenylated mRNA levels for bFGF and aFGF are low, suggesting either that they are unstable (Jaye et al., 1986) or that the FGFs are produced in low amounts. However, despite this the concentrations

of aFGF and bFGF present in tissues are relatively high. Because these proteins possess no obvious signal sequence, they appear to be secreted as a complex via carrier proteins or possibly in conjunction with extracellular matrix proteins (Vlodavsky et al., 1987). The latter appears to be the case, at least in cell culture. Little is known about the regulation of FGF production, and recent studies indicate that mechanisms other than its production, such as changes in receptor number, may regulate cell growth and differentiation.

Receptors that bind the FGFs with high affinity and high specificity have been identified on endothelial cells (Friesel et al., 1986), vascular smooth muscle cells (Winkles et al., 1987), myoblasts, and fibroblasts (Olwin and Hausehka, 1986). They appear to be single-chain polypeptides with molecular weights ranging between 110 and 150 kDa. In general, most bind bFGF more avidly than aFGF, with affinities ranging between 10 and 80 pM (Huang and Huang, 1986). These receptors contain an extracellular domain with either two or three immunoglobulin-like domains, a transmembrane domain, and a cytoplasmic tyrosine kinase domain (fig. 4). Four FGF receptor subtypes have been identified: type 1 (Dionne et al., 1990), type 2 (Dionne et al., 1990), type 3 (Keegan et al., 1991), and type 4 (Partanen et al., 1991). Type 4 binds aFGF with high affinity but does not bind bFGF. Recently, we have shown in cell culture that multiply passaged rat aortic smooth muscle expresses a 4.3-kilobase mRNA species encoding the FGF receptor homologous to *flg* (J. Saltis, A. Agrotis, and A. Bobik, unpublished data). The receptor nomenclature used in this review is that recommended by Keegan et al. (1991): the *flg* protein represents FGF receptor type 1, *bek* represents type 2, and *Cek2* has greatest homology with type 3.

The binding of bFGF to its receptors appears dependent on sequence locations near the amino terminus and the carboxy terminus. High concentrations of synthetic peptides possessing these sequences are capable of antagonising the effects of bFGF; however, in addition to their antagonist activity, they also possess partial agonist activities (Baird et al., 1988; Schubert et al., 1987). The sequence of amino acids from 105 to 115 has been identified using this structure-activity approach as crucial for bFGF binding to its receptor. Harper and Lobb (1988) identified Lys-118 as being involved in aFGF binding to receptors. The question as to whether these binding sites are important for FGFs to bind to different receptor subtypes still requires elucidation. It is possible that some of the receptor subtypes may more avidly bind other members of the FGF family such as FGF-5, *hst/Ks*, or *int*-2. However, the observation that synthetic peptide antagonists of bFGF also inhibit the effects of *hst/Ks* suggests that at least this FGF binds to the FGF receptors in a similar manner (Baird et al., 1988; Halaban et al., 1988).

FGFs, in addition to binding to their surface cell membrane receptors, also have a relatively high affinity for heparin and related sulphated glycosaminoglycans (Baird et al., 1988; Gospodarowicz and Cheng, 1986) as well as cell-derived heparan sulphate (Saksela et al., 1988). Heparan sulphate constitutes a major scaffolding glycosaminoglycan of extracellular matrix (see section V). It not only serves to protect the FGFs from degradation (Saksela et al., 1988) but also possesses the capacity to act as a large storage repository (Folkman et al., 1988). Bound FGF can be released from the heparan sulphate by the action of cell-derived heparinases (Vlodavsky et al., 1991). Because FGFs bind to heparan sulphate with a relatively low affinity (K_D approximately 10 to 50 nM) compared with the high affinity (K_D approximately 10 to 50 pM) for membrane FGF receptors, the stores may also serve as a continuous release receptacle. Heparan sulphates in the extracellular matrix also stabilise *hst/ks* (Delli-Bovi et al., 1988) and FGF-5 (Zhan et al., 1988). In addition, FGFs bind avidly to heparan sulphate on the cell surface, thereby localising the FGFs close to their site of action. These binding sites play a pivotal role in regulating the binding of FGFs to their membrane receptors (Yayon et al., 1991; Rapraeger et al., 1991). Thus, bFGF could become bioavailable to vascular target cells following the release by these cells of proteolytic enzymes capable of degrading the extracellular matrix, such as collagenase, plasminogen activator, or, more important, heparinase (Montesano et al., 1986; Nakajima et al., 1983; Vlodavsky et al., 1983). Following the action of heparinase, bFGF would only be transiently available to the vascular cells because it would now also be susceptible to proteolytic degradation. However, plasmin-released bFGF complexed noncovalently to either heparan sulphate proteoglycans or glycosaminoglycans is relatively stable and capable of interacting with its receptor in a manner identical with free bFGF (Moscatelli, 1987; Shing et al., 1985). bFGF can also be specifically released from these low-affinity-binding sites by an excess of heparin but not with closely related glycosaminoglycans, such as chondroitin sulphate (Moscatelli, 1988; Bashkin et al., 1989). This action of heparin could contribute to its ability to prevent myointimal thickening of vessels following balloon-mediated damage of the vascular endothelium (Clowes and Karnovsky, 1977).

Prevention of binding between cell surface heparan sulphate and bFGF substantially reduces both the binding of FGF to its cell surface receptors and the responses in fibroblasts (Rapraeger et al., 1991). Blockade of cell surface heparan sulphonation has an identical effect. It is also probable that protamine, a protein that binds strongly to heparin, inhibits FGF-induced endothelial cell migration (Azizkhan et al., 1980) and angiogenesis associated with embryogenesis and inflammation by preventing the association of FGF with its membrane receptors (Taylor and Folkman, 1982). On the basis of its

interactions with heparin, Yayon et al. (1991) suggested that bFGF can only bind to its high-affinity membrane receptor when it is bound to cell surface heparan sulphate proteoglycans or to free, soluble heparin or heparin-like molecules. Apparently, both free and cell surface-immobilised heparin-like molecules can interchangeably confer a stable, receptor-compatible conformational change on bFGF. Results of these studies suggest that specific analogues of FGF, which either prevent it from directly interacting with its high-affinity membrane receptor or prevent its binding to cell surface heparan sulphate proteoglycans, would be capable of inhibiting the biological effects of FGF. It is also conceivable that cell responsiveness to FGFs could be regulated at the membrane level either through changes in the number of FGF membrane receptors or through the expression of cell surface membrane heparan sulphate proteoglycans (fig. 5).

At present the normal function(s) of the different members of the FGF family remain to be defined. However, studies of cell culture indicate that the FGFs are multifunctional peptides (Sporn and Roberts, 1988). FGFs not only have the ability to stimulate mitogenesis in a variety of mesoderm-derived cells, including vascular endothelial and smooth muscle (Sato and Rifkin, 1988; Schweigerer et al., 1987; Winkles et al., 1987), but also possess chemotatic activity (Presta et al., 1986; Sato and Rifkin, 1988). They can induce or suppress cell-specific protein synthesis or secretion, regulate cell differentiation, and modulate both endocrine and neural functions (Baird et al., 1985; Walicke et al., 1986). On occasion, aFGF appears to be a weaker agonist than bFGF, possibly reflecting qualitatively different interactions of the two growth factors with their cell surface receptor species (Neufeld and Gospodorowicz, 1986). However, some receptor species exhibit the reverse order of potency (Partanen et al., 1991). Endothelial and vascular smooth muscle cells become bipolar when exposed to bFGF, and random migration is greatly increased (Terranova et al., 1985; Gospodarowicz, 1985). Similar effects have also been reported with aFGF (Neufeld and Gospodorowicz, 1986).

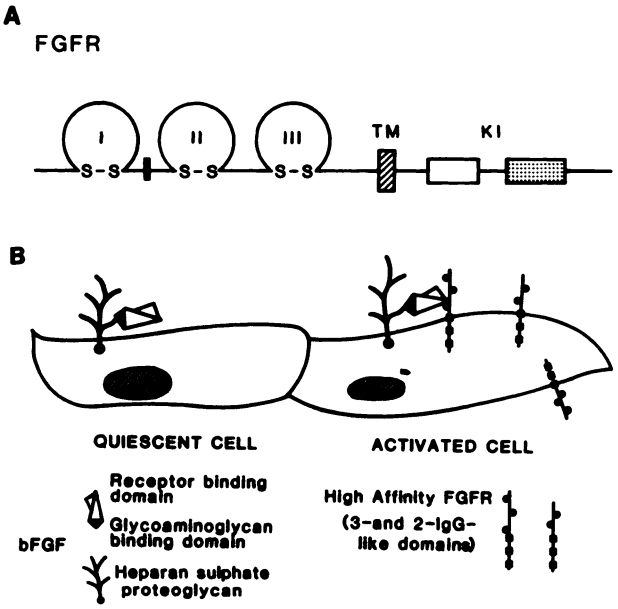

FIG. 5. Top, General structure of FGF receptor subtypes. S-S, immunoglobulin domains with cysteine residues; black box, acidic region; hatched box, transmembrane domain (TM); vertically striped boxes, kinase domains; KI, a kinase insert region. Homology between the extracellular domains, the immunoglobulins, varies between 14% and 87% between the four receptor subtypes. The tyrosine kinase domains exhibit the greatest degree of homology. Some truncated FGF receptors (FGFRs) possess only two rather than three immunoglobulin domains, which does not appear to affect the binding of bFGF. Bottom, A complex of FGF, heparan sulphate proteoglycan, and high-affinity receptor is required for mitogenic activation of endothelial and vascular smooth muscle cells.

bFGF can greatly influence phenotypic expression of many mesoderm-derived cells in culture. For example, endothelial cells from large vessels, cultured in the presence of bFGF, exhibit at confluency all of the morphological and functional characteristics of endothelium in the vessel from which they were derived. In the absence of bFGF, these cells no longer form a contact-inhibited cell monolayer with an antithrombogenic apical cell surface (Greenberg et al., 1980; Vlodavsky and Gospodarowicz, 1979; Vlodavsky et al., 1979). This ability of bFGF to influence endothelial cell differentiation correlates with its ability to stimulate the synthesis and deposition of extracellular matrix proteins, which in turn influences endothelial cell surface polarity and gene expression. bFGF has profound effects on endothelial cell collagen, fibronectin, and proteoglycan production (Tseung et al., 1982; Gospodarowicz, 1983; Gospodarowicz et al., 1987). Monolayers of quiescent capillary endothelial cells synthesise a basement membrane rich in type IV collagen, whereas migrating or sprouting cells, such as those exposed to bFGF, switch their synthetic pattern to types I and III collagen (Madri and Williams, 1983).

It is possible that the ability of bFGF to stabilise endothelial cell phenotype is also achieved through its effects on extracellular matrix production. When endothelial cells are grown on a collagen matrix, bFGF stimulates the cells to reorganise into tubules that resemble capillaries (see also section II, B). The cells also commence to produce a urokinase-type plasminogen activator and procollagenase (Banda et al., 1987). Capillary endothelial cells induced to migrate in response to angiogenic substances, such as the FGFs, produce a membrane-bound enzyme that degrades type IV collagen. Prostromelysin is also secreted by microvascular endothelial cells. When activated, this enzyme has a broad substrate specificity for a variety of basement membrane glycoproteins, including proteoglycans, fibronectin, laminin, and type IV collagen. Although the two metalloproteinases are frequently secreted together with a glycoprotein inhibitor, endothelial cells secreting these enzymes

have the potential to markedly remodel blood vessel structure or initiate new vessel formation (Banda et al., 1987).

Administered bFGF is a potent stimulator of angiogenesis. For example, implantation of a slow release preparation of bFGF into a pocket of the normally avascular cornea of the rabbit eye induces a massive ingrowth of capillaries from the periphery of the eye toward the bFGF implant (Baird and Bohlen, 1990). Similar neovascularisation is seen in the hamster cheek pouch model and the chick chorioallantoic membrane. Injected FGFs can also stimulate vascularisation of the brain (Cuevas et al., 1988). Despite these angiogenic properties of bFGF, its precise role in the physiological and pathophysiological regulation of new blood vessel growth is still largely circumstantial. Both aFGF and bFGF are expressed at a time when vascularisation begins in the developing chick embryo (Risau et al., 1988), and the embryonic mouse kidney synthesises an FGF-like substance that induces vascularisation of the kidney mesenchyme (Risau et al., 1988). FGFs also may play a role in the development in the neovascularisation of the ischaemic heart (Tomanek et al., 1989; Sasaki et al., 1989; McNeil et al., 1989).

The role of FGFs in tumour angiogenesis is controversial. Dennis and Rifkin (1990) reported that bFGF-neutralising antibodies are unable to block tumour growth. In contrast, Baird et al. (1986) reported that growth of chondrosarcoma, which express FGF, could be inhibited by antibodies to FGF.

Several lines of evidence indicate that bFGF contributes to restenosis of arteries following balloon angioplasty and endarterectomies. Lindner and Reidy (1991) demonstrated that systemic injection of a neutralising antibody against this growth factor prior to balloon catheterisation markedly reduced medial smooth muscle cell proliferation. It is conceivable that following balloon injury bFGF is released from mechanically damaged cells (McNeil et al., 1989). This release of bFGF, together with the matrix-bound FGF and an increase in the number of high-affinity receptors for FGF in the damaged region of the vessel, could play a major role in any subsequent restenosis following angioplasty (Naftilan, 1991). In principle, bFGF could also contribute to the progression of atherosclerotic lesions by similar mechanisms; cell necrosis, increased cell turnover, and growth of vasa vasorum into the intima and media are frequently observed in vessels affected by atherosclerosis (Cuevas et al., 1991).

The role of the FGFs in the development of vascular hypertrophy in hypertension remains to be elucidated. mRNA levels for bFGF do not appear to be increased in the aorta of rats with deoxycorticosterone acetate-salt-induced hypertension (Sarzani et al., 1989). To date there has been no attempt to determine whether high-affinity FGF receptor numbers are altered in vessels susceptible to hypertension-induced vascular hypertrophy, although an enhanced proliferative responsiveness to bFGF has been observed in the vascular smooth muscle of SHR compared to normotensive Wistar-Kyoto rats (Saltis et al., 1992a,b). Current therapeutic approaches to limit vascular smooth muscle growth focus on either preventing the ability of FGFs to bind to low-affinity extracellular matrix-binding sites, thereby accelerating their degradation, or exploiting any selective increases in high-affinity receptor number in damaged vessel areas (see section VI).

C. Transforming Growth Factors-β

TGFs were initially defined by their ability to induce reversible phenotypic transformation of cultured fibroblasts and were first characterised as sarcoma growth factor activity in conditioned media of murine sarcoma virus-transformed mouse fibroblasts (De Larco and Todaro, 1978). This activity was subsequently found to be made up of a mixture of TGF-α and TGF-β. The former, a homologue of EGF, exerts its effects through the EGF receptor and is structurally unrelated to TGF-β. TGF-β belongs to a large superfamily of factors involved in growth, differentiation, and morphogenesis (Sporn et al., 1987; Massagúe, 1990).

TGF-β (now called TGF-β_1) was first isolated from human platelets (Assoian et al., 1983) and subsequently cloned from a human cDNA library (Derynck et al., 1985). TGF-β_1 is a disulphide-linked dimer of two identical 112 amino acid chains. Each chain is synthesised as the carboxy-terminus domain of a 390-amino acid precursor with a signal sequence at the NH_2 terminus that is characteristic of secretory polypeptides. It also contains a hydrophobic signal sequence for translocation across the endoplasmic reticulum, is glycosylated (Derynck et al., 1985; Purchio et al., 1988), and shares a cellular recognition site for fibronectin/vitronectin, Arg-Gly-Asp. This sequence is found in all of the TGF-βs except TGF-β_2 (Ruoslahti and Pierschbacher, 1987). The precursor cleavage site consists of four basic amino acids immediately preceding the active domain. TGF-β_2 is in many aspects similar to TGF-β_1 (Cheifetz et al., 1987; Seyedin et al., 1987). The position of all nine cysteine residues are preserved, and there is 72% homology in the 112 amino acid chains of the two peptides. It is now clear that the TGF-βs are expressed in many normal tissues; both the type and the relative proportions of these secreted TGFs are greatly influenced by the cell type from which they are derived (Jakowlew et al., 1988). The mRNA for TGF-β_3 is expressed in a variety of mesenchymal cells of both human and rodent origin (Derynck et al., 1988); however, to date TGF-β_4 and TGF-β_5 of mammalian origin have not been detected.

Homology between the different TGF-βs ranges from 64% to 82% with all cysteine residue positions well conserved. As might be expected, the various TGF-β genes are located on separate chromosomes (Massagúe,

1990). In addition, the individual TGF-βs are extremely well conserved between species (Massagúe, 1990; Derynck et al., 1987; Jakowlew et al., 1988; Madisen et al., 1988). Despite this, the precise complexity of effects of this group of peptides on vascular cells still remains to be realised; the fact that different TGF-β gene products are coexpressed in the same cell (e.g., TGF-β_1, TGF-β_2, and TGF-β_3 in rat vascular smooth muscle cell) (A. Agrotis, J. Saltis, and A. Bobik, unpublished data) raises the possibility that heterodimers may also exist within the blood vessel wall. The existence of the TGF-β_1/TGF-β_2 heterodimer (TGF-$\beta_{1.2}$) has recently been found in porcine platelets (Cheifetz et al., 1987).

The human TGF-β_1 precursor is encoded by seven exons (Derynck et al., 1987). The positions of the introns/exon junctions of TGF-β_1 are conserved in TGF-β_2 and TGF-β_3 with the exception of the first which differs by three nucleotides (Derynck et al., 1988). There are two major transcriptional start sites for human TGF-β_1 mRNAs (Kim et al., 1989), and two promoter regions have also been characterised, one upstream of the first transcriptional start site and a second between the two start sites (Kim et al., 1989). Within the upstream promoter, a positive regulatory region, an enhancer-like region, and two distinct negative regulatory regions have been identified. The positive regions contain several binding sites for a number of transcriptional factors including nuclear factor 1, SP1, and AP-1 (Kim et al., 1989); the negative regulatory regions correspond to the presence of FSE 2-negative elements (Distel et al., 1987). Angiotensin II, ET-1, and TGF-β_1 activation of the TGF-β_1 gene in vascular smooth muscle appear to involve the AP-1-binding site (Hahn et al., 1991a,b).

A number of different mechanisms exist to tightly control the expression and activity of the TGF-βs. These include the regulation of TGF-β gene transcription, the production of TGF-βs as latent factors, and the sequestration of activated TGF-βs by extracellular matrix. Transcription of the TGF-β_1 gene can be stimulated by phorbol esters (Akhurst et al., 1988) and by TGF-β_1 itself (Van Oblerghen-Schilling et al., 1988). Promoter regions containing the transcriptional enhancer elements respond to both the phorbol esters and TGF-β_1 or transactivation by AP-1, the latter being mediated by the binding of the AP-1 (*jun-fos*) complex (Kim et al., 1990). There also appear to be phorbol ester-responsive elements in the 3′-flanking region of the TGF-β_1 gene (Scotto et al., 1990). At present it is not known whether the phorbol ester-mediated activation of the gene is mediated via protein kinase C activation. However, in vascular smooth muscle, angiotensin II, ET-1, and TGF-β_1 activate the gene, presumably by this mechanism. In the future it will be of interest to investigate whether TGF-β_1 is regulated through various hormones stimulating these various intracellular signals and whether the same mechanisms also regulate transcription of the other TGF-β genes.

After TGF-βs are synthesised, they appear to be released from endothelial cells, vascular smooth muscle cells, and fibroblasts via a constitutive secretory pathway as an inactive complex (latent TGF) that is unable to interact with its surface receptors (fig. 6) (Lawrence et al., 1985; Pircher et al., 1986). In fibroblasts this complex consists of the mature TGF-β_1 dimer noncovalently bound to two pro-region polypeptides disulphide-linked to a glycoprotein of 170 to 190 kDa. This glycoprotein contains multiple EGF-like repeats in tandem; their significance with respect to TGF-β_1 function is unknown (Kanzaki et al., 1990). The pro-region polypeptides are also disulphide linked to each other. These polypeptides appear to be essential for the correct folding of TGF-β_1 during synthesis. The TGF-β_1 pro-region contains mannose-6-phosphate (Purchio et al., 1988) through which it can bind to a cell surface cation-independent mannose-6-phosphate/IGF-II receptor (Purchio et al., 1988; Kovacina et al., 1989). The binding of latent TGF-β_1 to this receptor, on both endothelial and vascular smooth muscle, appears critical to its subsequent activation (see later) (Dennis and Rifkin, 1991). The pro-region also contains the Arg-Gly-Asp (RGD) sequence which in fibronectin, vitronectin, laminin, and other cell adhesion molecules recognise certain adhesion receptors of the integrin class (Ruoslahti and Pierschbacher, 1987).

The mechanisms that are responsible for activating the secreted form of TGF-β_1 in vivo are unknown. In culture, endothelial cells and vascular smooth muscle cells can only activate latent TGF-β_1 when they are in contact with vascular pericytes (Antonelli-Olridge et al.,

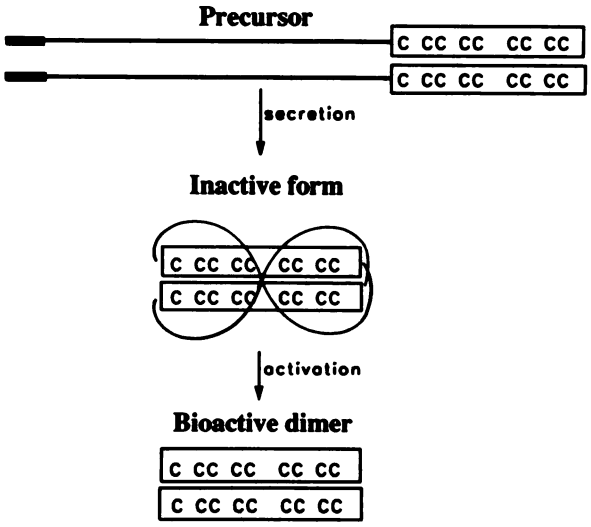

FIG. 6. Precursor, secreted, and active forms of TGF-β_1. The precursor of TGF-β_1 consists of an NH_2-terminal signal sequence (shaded box), a pro-region (heavy line), and the carboxy-terminal biologically active domain (box) containing nine cysteines (C). After secretion, the cleaved pro-region remains associated with the TGF-β_1 dimer in a biologically inactive form. Biological activity is attained when this complex is disassembled.

1989; Sato et al., 1990). The requirements for activation in these coculture systems are cell to cell contact (or the close apposition of the two different cell types) and plasmin and urokinase. It has been suggested that activation in vivo may involve the proteolytic action of plasmin or cathepsin D on the TGF-β_1 pro-region (Lyons et al., 1988; Sato et al., 1990) or the removal of carbohydrate residues in this region (Miyanzono and Heldin, 1989). Treatment of TGF-β_1 with endoglycosidase F or sialidase, and the addition of sialic acid or mannose-6-phosphate, results in its activation. Local acidic microenvironments, as might be found in wound healing or necrotic areas of atherosclerotic plaques, could also liberate active TGF-β_1. Activated macrophages secrete sialidase (Pilatte et al., 1987) and proteases and can lower the pH to 4 (Silver et al., 1988). These additional mechanisms would also contribute to its activation in diseased blood vessels. After TGF-β_1 is released from its inactive complex, it can bind to its specific cell surface receptors or it can bind to various extracellular matrix proteins (Andres et al., 1989) that protect it from degradation and act as a long-term repository; this provides a sustained release mechanism under normal physiological conditions and a rapid release when extracellular matrix proteins are hydrolysed.

The action of TGF-βs is mediated through specific cell membrane receptors whose affinities for the different TGF-βs are in the picomolar concentration range (Frolik et al., 1984; Massagúe and Like, 1985; Wakefield et al., 1987). Three distinct classes of proteins on the cell membrane have been shown to bind the TGF-βs with high affinity: class I receptor proteins have molecular masses of 65 kDa, whereas class II, depending on species, range from 85 to 110 kDa. TGF-β_1 binds to both the class I and class II receptors with an affinity 12- to 16-fold greater than TGF-β_2 (Cheifetz et al., 1988a,b; Myoken et al., 1990). All three forms of TGF-β bind with equal affinity to the class III receptor, which is the most abundant of the three. The class III receptor is a proteoglycan (Segarini and Seyedin, 1988; Cheifetz et al., 1988a,b) predominantly composed of heparan sulphate glycosaminoglycan chains with a smaller amount of chondroitin or dermatan sulphate attached to a core protein of approximately 100 to 140 kDa, the binding site for TGF-βs resides in this core protein. Because the cytoplasmic tail of this receptor has no obvious motif, it has been suggested that its main function may be to regulate the ability of the TGF-βs to bind to other TGF-β receptor subtypes (Wang et al., 1991). Alternatively, it may regulate the surface expression of the different TGF-β receptor subtypes. The class I and II receptors, like most growth factor receptors, are glycoproteins. The precise roles played by these various receptors are still poorly defined. It has been suggested that effects specific to TGF-β_1, such as inhibition of endothelial cell proliferation (Jennings et al., 1988), are mediated through the class I receptor. Confirmatory evidence for a role for the class I receptors in inhibiting cell proliferation comes from the finding that loss of these receptors is associated with the development of resistance to the growth inhibitory effects of TGF-β_1 (Boyd and Massagúe, 1989). Recently, the TGF-β type II receptor has been cloned and characterised as a serine/threonin kinase (Lin et al., 1992). Its function is yet to be defined.

Most of the current information concerning the action of the TGF-βs is derived from cell culture studies using either TGF-β_1, TGF-β_2, or TGF-β_3. The type of effect exerted by these agents on vascular cells, as on other cell types, is critically dependent on many parameters including their differentiation state, the growth conditions, and the presence or absence of other growth factors (Sporn et al., 1987). The TGF-βs are multifunctional growth factors, and many of their diverse effects on cell proliferation and phenotype are not susceptible to simple explanations (fig. 7).

Because the TGF-βs have profound effects on the composition of the extracellular matrix, it has been suggested that matrix interactions between the cells could be a major mechanism by which these peptides control growth, differentiation, and function of many mesenchymal cells. TGF-β_1 and TGF-β_2 stimulate the synthesis of many extracellular matrix proteins by vascular smooth muscle cells, fibroblasts, and endothelial cells. Both increase the synthesis of collagen types I (Ignotz et al., 1987; Roberts et al., 1986), III, IV, and V (Varga et al., 1987; Madri et al., 1988), thrombospondin (Penttinen et al., 1988), tenasin (Pearson et al., 1988), elastin (Lui et al., 1988), and chondroitin/dermatan sulphate proteoglycans (Chen et al., 1987; Falanga et al., 1987; Bassols and Massagúe, 1988; Hirake et al., 1988). TGF-β secreted by endothelial cells also markedly stimulates glycosaminoglycan synthesis by vascular smooth muscle cells (Merrilees and Scott, 1990). This effect is mimicked by the

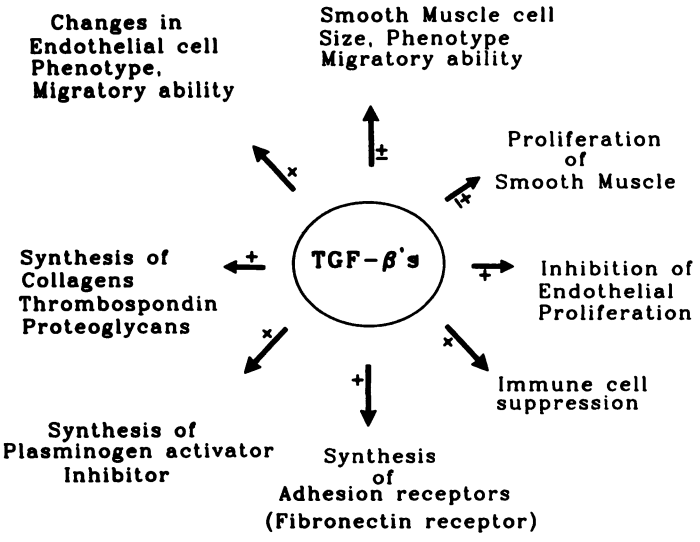

FIG. 7. Summary of the pharmacological properties of TGF-β on vascular cells in cell culture.

addition of exogenous TGF-β_1 which also increases versican-like chondroitin sulphate proteoglycan synthesis (Schonherr et al., 1991). This increase in chondroitin sulphate proteoglycan synthesis is the consequence of an increase in both core protein synthesis and chondroitin-4 and chondroitin-6 sulphate synthesis (Chen et al., 1991). Recently, TGF-β_1 has been implicated in the increases in fibronectin and collagens α_2 (type I) and α_1 (type III) which occur in the neointima of vessels damaged by balloon angioplasty (Majewsky et al., 1991). TGF-β can directly stimulate the activity of the collagen α_2 (1) promoter (Rossi et al., 1988) as well as the fibronectin promoter via an NF-1-binding site (Dean et al., 1988). Both the increase in transcription and stabilisation of the respective mRNAs contribute to the elevated mRNAs and the subsequent increase in matrix protein biosynthesis.

TGF-βs also influence vascular matrix proteins by controlling both the activities and the secretion of matrix degradative proteases. The expression of plasminogen activator inhibitor by endothelial cells is markedly enhanced by TGF-β_1 (Sawdey et al., 1989). This increase in plasminogen expression is closely associated with the ability of TGF-β_1 to prevent invasion of capillary endothelial cells into the human amnion basement membrane (Mignatti et al., 1989). Urokinase secretion is also reduced by TGF-β (Slivka and Loskutoff, 1991). TGF-β affects matrix degradative enzymes secreted by vascular smooth muscle cells in a similar manner. For example, plasminogen activator inhibitor-1 secretion is also increased by TGF-β_1 (Reilly and McFall, 1991).

TGF-β can also alter vascular wall properties by influencing the expression of cell adhesion receptors on vascular cells (Hynes, 1987). It can regulate both the α- and the β-subunits of the fibronectin receptor (Roberts et al., 1988), and this, together with its ability to increase fibronectin expression, increases cell adhesion to the pericellular matrix (Ignotz and Massagúe, 1986). Such a mechanism may account for the increase in blood vessel wall stiffness frequently seen in established hypertension (Folkow, 1982).

Attempts to fully characterise the effects of TGF-β on vascular endothelial and smooth muscle cell phenotype, migratory, and proliferative abilities have, to a large extent, been complicated by the nature of the tissue culture systems used and, in particular, the growing of cells on plastic. In vivo, TGF-β has been shown to be angiogenic (Roberts et al., 1985; Fiegel and Knighton, 1988). This property of TGF-β_1 appears, at least in part, to be related to its effect on endothelial cell phenotype and function. When microvascular endothelial cells are grown in three-dimensional collagen gels, TGF-β_1 promotes contraction of the gel and induces the formation of tube-like structures (mimicking angiogenesis); it does not inhibit cell proliferation (Madri et al., 1988). TGF-β_5 exerts a similar effect on endothelial cells in three-dimensional culture and is more potent than TGF-β_2 (Merwin et al., 1991a,b). In contrast, the proliferation of endothelial cells on either laminin, type IV collagen, or fibronectin-coated dishes is inhibited by TGF-β_1. Whether such differences in the responsiveness of endothelial cells to TGF-β under different tissue culture conditions are the consequence of alterations in the synthesis of extracellular matrix proteins or alterations in the expression of TGF-β receptor subtypes needs to be determined. Evidence for the latter possibility is suggested by the different responses of foetal bovine heart endothelial cells and human umbilical vein endothelial cells to TGF-β (Myoken et al., 1990). Low concentrations of TGF-β_1 stimulate the growth of heart endothelial cells, whereas high concentrations inhibit growth; these cells possess two distinct classes of TGF-β-binding sites. In contrast, the human umbilical vein endothelial cells possess only a single binding site for TGF-β_1, and their growth is only slightly promoted.

The differential effects of bFGF on TGF-β receptor subtypes are also consistent with multiple TGF-β receptors mediating different responses in endothelial cells (Fafeur et al., 1990). Multiple but specific receptor subtypes for TGF-β would explain why the different TGF-β homologues differentially affect endothelial cell migration (Merwin et al., 1991a). Endothelial cell morphology is also markedly altered by TGF-β with the cells losing their cobblestone appearance and assuming a pleomorphic shape (Coomber, 1991). Vascular smooth muscle can also be stimulated indirectly through the effects of TGF-β on endothelial cell function. Genes in endothelial cells encoding PDGF-A and PDGF-B chains, as well as those for FGF, are activated by TGF-β (Starksen et al., 1987; Daniel et al., 1987). In addition, TGF-β can directly affect vascular smooth muscle growth. TGF-β_1 can either inhibit or potentiate the mitogenic effects of other growth factors (Owens et al., 1988a; Majack et al., 1990; Saltis et al., 1992a,b,c). This is most probably related to the expression of distinct TGF-β receptor phenotypes (Goodman and Majack, 1989), although PDGF-A chain expression could also account for part of the response (Majack et al., 1990). It is also interesting to note that TGF-β_1 always potentiates the mitogenic effects of the tyrosine kinase-activating growth factors on vascular smooth muscle from SHR but inhibits these effects in smooth muscle of normotensive rats (Saltis et al., 1992b,c). Vascular smooth muscle structure (Majack, 1987), phenotype (Bjorkerud, 1991), cell size (Owens et al., 1988a), and migratory ability (Merwin et al., 1991b) are also affected by TGF-β_1.

Despite this multiplicity of effects of TGF-β_1 on vascular cells, its role and that of its homologues in the pathogenesis of vascular hypertrophy and the formation of atherosclerotic lesions, as well as in restenosis following balloon angioplasty, remain to be determined. TGF-β mRNA expression can be readily detected in normal

blood vessels, and its levels are increased in hypertension (Sarzani et al., 1989) and atherosclerosis (Ross et al., 1990). TGF-β_1 mRNA levels are also increased during the formation of the neointima following balloon angioplasty (Majewsky et al., 1991), whereas TGF-β_3 mRNA levels are decreased (R. J. Dilley, personal communication). However, levels of active TGF-β_1 or TGF-β_3 have not been measured; nor have their potential effects on vessel wall composition, particularly the extracellular matrix, been determined.

D. Miscellaneous

1. Cytokines. This class of compounds is generally composed of soluble (glyco)proteins, nonimmunoglobulin in nature, released by a variety of cells, which act via membrane receptors to influence cell function and growth. Invariably, they are involved in the remodeling of injured tissue including blood vessels. Vascular derived cytokines which have been implicated in altering blood vessel wall structure in atherosclerosis, hypertension, and other cardiovascular diseases include the ILs and the colony-stimulating factors.

a. INTERLEUKINS. IL-1 is a pleiotropic inflammatory mediator that affects the functioning of numerous cell types (Pober and Cotram, 1990). It consists of two major polypeptides: IL-1α encodes the polypeptide whose isoelectric point is 5.0, and IL-1β encodes for the isoelectric point 7.0 species. There is only limited homology between these two species that are encoded by two separate genes. Their primary translation products vary between 33 and 34 kDa in size. The primary translation product of IL-1α is active, whereas the β-form requires processing to an active form. The two mature species, IL-1α and IL-1β, are derived from the carboxy-terminal halves of the primary translation products. Neither IL-1 gene product contains any signaling sequence. Despite this, vascular endothelial cells produce and secrete IL-1α, whereas other vascular cells produce both IL-1α and IL-1β, the latter being the major secretory product (Shingu et al., 1991). TNF-α and endotoxin increase IL-1 production in both endothelial and smooth muscle cells (Libby et al., 1986; Shingu et al., 1991). Subsequent autoinduction can also contribute to this response. For example, in vascular smooth muscle, IL-1β induces itself as well as IL-1α, whereas in vascular endothelial cells, IL-1α secretion alone is enhanced (Shingu et al., 1991). Because the actions of IL-1α and IL-1β are similar, one would also expect IL-1α to induce its own production in endothelial cells.

IL-1 can influence vascular growth through a variety of mechanisms. Both IL-1α and IL-1β induce the expression of bFGF in vascular smooth muscle derived from the saphenous vein (Gay and Winkles, 1991). This effect is relatively slow and dependent on new protein synthesis. IFN-γ, as well as glucocorticoids, such as dexamethasone and hydrocortisone, inhibits this transcriptional response (Gay and Winkles, 1991). IL-1-activated smooth muscle also secretes copious amounts of IL-6 (Loppnow and Libby, 1990) which, in turn, stimulates the growth of these cells by mechanisms partially dependent on PDGF production (Raines et al., 1989; Morimoto et al., 1991).

IL-1 can also indirectly influence the development of vascular lesions in atherosclerosis by inducing endothelial cells to produce GM-CSF (Broudy et al., 1988; Sieff et al., 1987), G-CSF (Broudy et al., 1988), and IL-8 (Strieter et al., 1989) (see section IV.D). Endothelial cell morphology and function are also affected by IL-1. Morphologically, the organisation of the endothelial cells changes from a cobblestone "epitheloid" arrangement to take on a fibroblast-like spindle-shaped appearance (Montesano et al., 1985). These effects are associated with a reduction in the net amount of cellular RNA, protein synthesis and, as a consequence, attenuate endothelial cell growth (Cavender and Edelbaum, 1988; Norioka et al., 1987). Recently, IL-1 has been shown to inhibit bFGF-stimulated angiogenesis in the avascular rabbit cornea. Many of these effects are mediated through specific membrane-associated receptors. The binding affinities of the IL-1s to the IL-1 receptor type 1 is in the nanomolar range. It is interesting to note that the EC_{50} for many of the described effects of the IL-1s is in the picomolar range.

Vascular restructuring can also be influenced by other endothelial cell-derived ILs. Endothelial cell-derived IL-6 can directly stimulate vascular smooth muscle cell proliferation (Morimoto et al. 1991). Not only IL-1 but also IL-4, a T-helper lymphocyte-produced cytokine, can increase IL-6 production by endothelial cells. This increase in IL-6 production is similar to that induced by either IFN-γ or TNF-γ; in combination, IL-4 and IFN-γ synergise in their effect on IL-6 production. Thus, lymphocytes acting via the endothelium also have the potential to influence vascular wall structure during chronic inflammation, as might occur in atherosclerosis or severe hypertension. The secretion by endothelial cells of IL-8, a chemotactic factor for T-lymphocytes that can be stored attached to heparan sulphate moieties in the subendothelial matrix, could also participate in the development of vascular lesions in atherosclerosis.

b. COLONY-STIMULATING FACTORS. This family of growth factors is well known for its ability to affect the differentiation, survival, proliferation, migration, and metabolism of macrophages and granulocytes. More recently, CSFs have also been shown to affect other cell types including vascular endothelial cells (Bussolino et al., 1991). G-CSF was first purified from culture medium conditioned by the human bladder carcinoma cell line 5637. Its full-length cDNA codes for a protein of 207 amino acids of which 177 amino acids make up the mature G-CSF. It is extensively glycosylated and has a molecular weight of 19.6 kDa and an isoelectric point of

5.5 (Sieff, 1987). The DNA coding GM-CSF codes for a 144-amino acid precursor protein which is subsequently cleaved to yield the mature GM-CSF of 122 amino acids. Both G-CSF and GM-CSF are highly species specific. Vascular endothelial cells produce both G-CSF and GM-CSF (Rajavashisth et al., 1990), whereas vascular smooth muscle cells have to date only been shown to produce GM-CSF. Little is known about the regulation of GM-CSF production by vascular smooth muscle cells. In vascular endothelial cells, both G-CSF and GM-CSF production may be stimulated by TNF-α (Broudy et al., 1988), as well as oxidised low-density lipoproteins (Rajavashisth et al., 1990).

G-CSF, as well as GM-CSF, can indirectly influence the development of atherosclerotic plaques. Macrophages frequently present in damaged regions of vessels (see section III.A) and the production of G-CSF and GM-CSF by the vascular cells could stimulate macrophages and their progenitors to replicate. Macrophages within the atherosclerotic plaque are known to be an important source of PDGF-B and IL-1 (Ross et al., 1990). More recently, it has also been suggested that they may play a role in angiogenesis. Both G-CSF and GM-CSF induce human umbilical vein-derived endothelial cells to alter their phenotype, migration, and proliferation (Bussolino et al., 1991). G-CSF also induces angiogenesis in the avascular region of the rabbit cornea without any inflammatory reactions. Recently, macrophage CSF has been shown to increase DNA biosynthesis in intimal aortic smooth muscle cells of the atherosclerotic rabbit (Inaba et al. 1992).

2. Endothelins. ETs are a group of small peptides secreted by both vascular endothelial and smooth muscle cells (Sokolovsky, 1991). Three different isoforms of ET called ET-1, ET-2, and ET-3 have been identified. All three are made up of 21 amino acids interconnected by two Cys-Cys disulphide bridges. The major differences between the different ETs are found within the sequences of the inner Cys^3-Cys^{11} loop. Metalloproteinases, thought to be specific for the 39-amino acid precursor peptide and termed big ET-1, appear responsible for the generation of ET-1 (Matsumura et al., 1991). Subsequent secretion of ET-1 appears to involve the microtubular sytem (Kitazumi et al., 1991). ET-1 secretion from endothelial cells can be stimulated by hypoxia (Kourembanas et al., 1991) and by a variety of agents, including thrombin, TGF-β_1, arginine-vasopressin, and angiotensin II (Kurihara et al., 1989; Emori et al., 1991). ET-3 can also stimulate ET-1 production in cultured umbilical vein endothelial cells (Yokokowa et al., 1991). Because the increases in ET-1 secretion involve gene activation through the AP-1-binding site (Lee et al., 1991), it is possible that ET-1 can also induce its own production in endothelial cells.

Several lines of evidence implicate ET-1 in the pathophysiology of hypertrophied vessels and atherosclerotic lesions. ET-1 stimulates vascular smooth muscle cell proliferation (Bobik et al., 1990b) (fig. 8) and DNA synthesis (Hirata et al., 1989). Mitogenesis is dependent on both pertussis toxin-sensitive intracellular signals, presumably involving heterotrimeric G proteins as well as pertussis toxin-insensitive mechanisms (fig. 8) (Bobik et al., 1990a,b). ET-1, ET-2, and ET-3 are equally effective in potentiating PDGF-BB-stimulated DNA synthesis in vascular smooth muscle (Weissberg et al., 1990). Plasma levels of ET-1 are increased in hypertensive patients (Saito et al., 1990) as well as those with atherosclerotic lesions (Lerman et al., 1991). In the latter group, plasma ET-1 levels correlated directly with the number of vascular lesions, and ET-1 was also detected within the lesions, presumably in the smooth muscle cells. Nor-

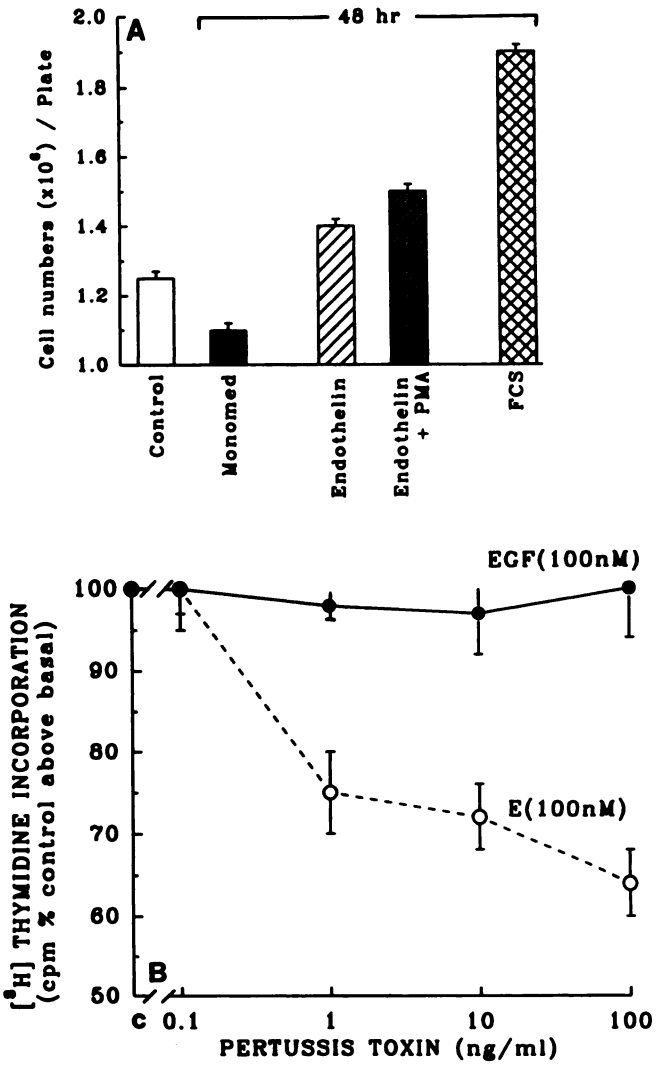

FIG. 8. Top, Growth factor properties of ET-1 on vascular smooth muscle cells. ET-1 stimulates vascular smooth muscle to replicate. Phorbol myristate acetate (PMA) further enhances its proliferative effects. Experiments were performed in Monomed (insulin/transferrin) or fetal calf serum (FCS) during a 48-hour period. Bottom, Mitogenic effects of ET-1 (E) are the consequence of pertussis toxin-sensitive (G protein dependent) and -insensitive mechanisms. A consequence of ET-1 interacting with multiple receptors on vascular smooth muscle? (Bobik et al., 1990b; used with permission).

mally, ET-1 is localised to the endothelial cell layer (Hiroe et al., 1989; Miyauchi et al., 1990). It appears that the mitogenic effects of ET-1 on vascular smooth muscle in the synthetic phenotype are mediated via the ET-1-selective G protein-coupled ETA receptor (Arai et al., 1990) and the nonselective ETB receptor (Sakurai et al., 1990).

3. Insulin-like growth factors. IGFs denote a class of peptides whose basic structure has many of the characteristics of proinsulin (e.g., approximately 50% homology) but whose functions differ markedly from that of insulin. Rather than stimulating glucose transport and a variety of other metabolic processes, the IGFs stimulate cell growth. IGF-I is a basic peptide with an isoelectric point of 8.6; it is composed of 70 amino acids and has three disulphide bridges. IGF-II is a shorter peptide of 67 amino acids whose disulphide linkages are identical with those of TGF-I. Both the A and B domains are located between the disulphide linkages; the A domain is the shorter of the two. The A domain appears to be associated with the mitogenic properties of IGF-I (Cascieri et al., 1988), whereas the binding regions for carrier proteins, the IGF-BPs, are located on the B domain of IGF-I and IGF-II (Sheikh et al., 1987). The IGF-BPs, which are also biosynthesised in several forms by endothelial and smooth muscle cells, are important regulators of the physiological actions of the IGFs, and any consideration of the physiological or pathophysiological role of IGFs should take into account their IGF-BPs. IGF-BPs can either inhibit (Gopinath et al., 1989) or enhance the effects of the IGFs on vascular smooth muscle and endothelial cells (Elgin et al., 1987; Bar et al., 1989).

The two genes that code for IGF-I and IGF-II possess three introns and four exons with most of the protein-coding sequence located within two of the exons. The IGF-I gene transcribes at least five distinct forms of mRNA from exons 1 to 3 with only the largest mRNA transcript not being translated (Pagter-Holthuizen et al., 1986). Alternate splicing accounts for two other minor forms of IGF-I, namely, IGF-IA and IGF-IB (Rotwein, 1986). The IGF-II transcripts also show evidence of alternate splicing. Posttranslational modification also appears to contribute to IGF-I heterogeneity (Francis et al., 1988).

Both the IGF-I and IGF-II genes have now been demonstrated to be expressed in a variety of cell types. In the human foetus expression is evident in connective tissues or cells of mesenchymal origin (Han et al., 1987). Vascular endothelial and smooth muscle cells also synthesise and secrete IGF-I (Delafontaine et al., 1991). Many trophic hormones appear to regulate IGF biosynthesis in these cells. For example, PDGF stimulates IGF-I production in porcine vascular smooth muscle cells (Clemmons, 1985). bFGF has also been shown to stimulate IGF-I production (Clemmons and Shaw, 1983), whereas glucocortocoids inhibit IGF-I expression (Adamo et al., 1988). Glucocortocoids also inhibit IGF-II expression, presumably by interacting with the glucocorticoid-responsive elements on the IGF-II gene, thereby inhibiting transcription (Beck et al., 1988).

A number of factors influence the expression of IGFs in blood vessels. For example, a chronic increase in vascular load is associated with marked increases in IGF-I production by endothelial and smooth muscle cells of the rat femoral artery (Hansson et al., 1987a). Increases in IGF-I expression have also been observed during vessel restenosis and in angiogenesis (see later). The stimuli responsible for these increases have not been identified. IGF-BPs secreted by vascular smooth muscle and endothelial cells are also subject to both positive and negative regulation. Insulin and IGF-I increase IGF-BP secretion in a manner dependent on protein synthesis; the increase in secretion correlates with the stimulation of cell proliferation by these hormones. In contrast, when cell division is inhibited, for example by dexamethasone, IGF-BP secretion by porcine smooth muscle is also attenuated (McCusker and Clemmons, 1988). This latter effect may be due to increased IGF-BP degradation rather than a consequence of reduced protein synthesis (Roeder and Gunn, 1987). At present there is no information concerning the expression of IGF-BPs in vascular disease.

The cellular effects of the IGFs are mediated via one of two distinct transmembrane glycoproteins. The IGF-I (type 1) receptor is structurally and functionally similar to the insulin receptor. It is composed of two peptides, one α-subunit (130 kDa) and one β-subunit (95 kDa), which are disulphide linked. The α-subunit which forms a major part of the extracellular domain is involved in IGF binding, and the β-subunit spans the cell membrane and contains the intracellular tyrosine kinase domain. The binding of IGF-I, IGF-II, or insulin to this receptor initiates autophosphorylation of tyrosine residues of the β-subunit (Morgan et al., 1986). The affinity of binding to this receptor is in the order IGF-I > IGF-II > insulin. In contrast, the IGF-II (type II) receptor is a single-chain peptide of 270 kDa which shares homologies with the type II region of fibronectin and the cation-independent mannose-6-phosphate receptor (Morgan et al., 1987; Lobel et al., 1987). Approximately 92% of the receptor protein forms the large extracellular domain. The short hydrophobic cytoplasmic domain possesses several potential phosphorylation sites. Only IGF-II, and to some extent IGF-I, binds with high affinity to this receptor, by interacting with the fibronectin-like insert.

The gene for the human IGF-I receptor is located on the long arm of chromosome 15 (Ullrich et al., 1986) and is expressed by vascular smooth muscle (Pfeifle and Ditschuneit, 1983), endothelial cells (Barr et al., 1988a,b), and pericytes (King et al., 1985). IGF-II receptors have also been identified on pericytes and microvascular endothelial cells (Jialal et al., 1985). Both IGF receptors appear to be regulated by a variety of mecha-

nisms. For example, expression of the IGF-I receptor on vascular smooth muscle is dependent on growth status (Pfeifle and Ditschuneit, 1983). Expression is high in rapidly dividing cells and low in quiescent cells. The findings of Pfeifle et al. (1987) that FGF and PDGF increase [^{125}I]IGF binding to smooth muscle suggest that this increase in IGF-I receptor number may be a consequence of direct induction through other growth factor receptors. The IGF-I receptor is also down-regulated according to the degree of receptor occupancy by the IGFs or insulin (Rosenfeld and Hintz, 1980). In contrast, the IGF-II receptor does not appear to undergo down-regulation (Ota et al., 1984) when interacting with its respective ligands. Both receptors undergo rapid internalisation and recycling.

The IGFs exert their effects continuously throughout life. Their properties may be considered to be primarily anabolic in nature, the type of response elicited by the IGF being dependent on the responsiveness of the target cell type. If cells have entered the mitotic cell cycle, then the IGFs stimulate proliferation; however, if the cells are quiescent or undergoing hypertrophic growth, then other anabolic processes such as protein synthesis only are stimulated. It has been suggested that the IGFs act as progression factors to stimulate cells through the S-phase of the cell cycle. This mitotic action occurs in conjunction with competence factors such as PDGF and EGF which initiate entry of cells into the mitotic cell cycle (Stiles et al., 1979).

In vascular smooth muscle, PDGF exhibits an additive proliferative response with either IGF-I or insulin (Banskota et al., 1989). mRNA for the protoncogene c-*myc* is also increased in an additive manner. Similar effects on proliferation have been observed with PDGF and IGF-I on rat A10 vascular smooth muscle (Cascieri et al., 1986). However, other growth factors, namely, aFGF and EGF, are not capable of initiating DNA synthesis in the absence of added IGF-I. Presumably, in these cells, neither aFGF nor EGF can induce IGF production. In contrast, we have always observed that in rat aortic smooth muscle most tyrosine kinase-activating growth factors, in particular, PDGF-AA, PDGF-AB, PDGF-BB, bFGF, and EGF, all stimulate cell entry into the S-phase (Saltis et al., 1992a,b,c). Microvascular, but not large vessel-derived, endothelial cells are also responsive to the effects of the IGFs. For example, DNA synthesis is stimulated only in microvascular endothelial cells exposed to IGF-I, IGF-II, or insulin (Bar et al., 1986). Other anabolic effects of the IGFs are also only observed in the microvascular endothelial cells (Bar et al., 1986; Barr et al., 1988a). However, IGF-I and IGF-II stimulate proteoglycan biosynthesis in both the microvascular and large vessel-derived endothelial cells (Bar et al., 1986).

Several lines of evidence suggest a role for vascular derived IGFs in both restenosis following vessel injury and angiogenesis. Cercek et al. (1990) reported a rapid increase in vascular IGF-I mRNA expression following balloon catheter deendothelialisation of blood vessels. IGF-I may particpate in forming the neointima by increasing the number of PDGF receptors on the vascular smooth muscle cells (Pfeifle et al., 1987). Peak elevation in mRNA was observed at 7 days and remained elevated throughout the period of rapid cell replication. Regenerating endothelial cells in injured vessels produce copious amounts of IGF-I (Hansson et al., 1987b, 1989b). Recently, insulin and IGF-I have been implicated in angiogenesis, stimulating endothelial cell migration, and tube formation (Nakao-Hayashi et al., 1992). However, whereas IGF-I immunoreactivity can be detected in low amounts in all normal vessels, it appears to be absent in vascular cells within malignant tumours (Hansson et al., 1988). The reason for this is not known.

Evidence for a role for the IGFs in the development of atherosclerotic lesions and vascular hypertrophy in hypertension is rather circumstantial. In atherosclerotic lesions, PDGF would contribute to any increase in IGF levels within the lesion as would the monocyte/macrophage-derived IGF-I; both the PDGF-stimulated smooth muscle cell-derived IGF and the monocyte/macrophage-derived IGF-I could affect smooth muscle cell differentiation and proliferation. Recently, IGF-I and IGF-II have been shown to be expressed in the vessels of rats with deoxycorticosterone acetate-salt-induced hypertension (Sarzani et al., 1989). The functional significance of this expression will only become apparent when other factors regulating the responsiveness of smooth muscle to the IGFs have also been examined, e.g., the expression of receptor subtypes and the characteristics and properties of the IGF-BPs.

4. Platelet-, monocyte/macrophage-, and T-lymphocyte-derived growth factors. Substances that influence the growth of smooth muscle and endothelium are derived from a variety of cellular sources. In this section, we briefly focus on those factors derived from cells that enter the wall in large numbers under pathological conditions, such as endothelial denudation/injury and hypercholesterolemia. These cells are platelets, monocyte/macrophages, and T-lymphocytes.

a. PLATELETS. As discussed in sections II and III, injury to the endothelium results in adhesion of platelets to the subendothelial connective tissue within seconds, followed by aggregation and release of their contents. Platelets contain a variety of biologically important molecules, including platelet factor 4, β-thromboglobulin, vasoconstrictor amines, arachidonic acid metabolites, heparitinase, and several growth factors. About half of the mitogenic activity of platelets is due to PDGF, with the remainder due mainly to EGF and TGF-β (Heldin et al., 1977; also see section III).

b. MONOCYTE/MACROPHAGES. One of the earliest events in experimental atherosclerosis induced by hypercholesterolemia is increased adhesion of monocytes to

an intact endothelium (Gerrity, 1981; Joris et al., 1983; Fagiotto et al., 1984), followed by their migration subendothelially where they differentiate into macrophages and accumulate lipid (see section III). A variety of nonlipid factors can also promote adhesion of leukocytes to endothelium by inducing adhesion molecules on the endothelium and/or leukocytes (Bevilacqua et al., 1987).

Activated macrophages have a large number of secretory products and biological activities (Nathan, 1987). They produce IL-I and TNF and, thus, contribute to the adhesiveness of the endothelium for monocytes and other leukocytes (Pober et al., 1986). They produce several products that are chemotactic for leucocytes, such as leukotriene B_4, complement fragment C5a, TGF-β, IL-I, TNF, GM-CSF, and PDGF (Assoian et al., 1987; Wang et al., 1987a,b; Deuel et al., 1982). Macrophages can produce toxic substances, such as reduced oxygen species and proteases, which injure neighbouring smooth muscle cells and endothelium and thus contribute to the central necrotic core often found in atherosclerotic plaques. The reactive oxygen species also oxidise lipoproteins, particularly low-density lipoprotein and β very low-density lipoprotein, which are avidly taken up by macrophages and smooth muscle cells converting them into foam cells (Parthasarathy et al., 1986). In addition to platelets, macrophages can produce 12-hydroxyeicosatetraenoic acid, an eicosanoid chemotactic for smooth muscle, which may be involved in attracting smooth muscle cells from the media into the intima (Nakao et al., 1982).

The principal mitogen for smooth muscle produced by macrophages is PDGF, which is also chemotactic for smooth muscle (Shimokado et al., 1985). Macrophages also secrete IL-I, bFGF, and EGF to stimulate vascular cell growth (Libby et al., 1986; see also section IV). IL-6 is produced by macrophages and stimulates T- and B-cell proliferation and the production of immunoglobulin by B-cells. In the vascular system, production of IL-6 can be induced by IL-I, endotoxin, and TNF. TNF and FGF produced by macrophages may also induce the neovascularisation characteristic of plaques and thus act as angiogenesis factors (Leibovich et al., 1987). Macrophages are also capable of producing potential growth inhibitory or modulatory substances such as prostaglandin E_2, IFN-α, and TGF-β (Nathan et al., 1987; Assoian et al., 1987; see also section IV).

Macrophages have been reported to stimulate matrix synthesis by smooth muscle cells by producing TGF-β (Chen et al., 1987). They can also degrade the matrix by producing a variety of proteases and heparan sulphate-degrading enzymes (Nathan et al., 1987).

c. T-LYMPHOCYTES. Perivascular lymphocyte infiltrates are often present in the adventitia of atherosclerotic arteries as well as in both early and advanced intimal lesions (Jonasson et al., 1986). The T-cells in lesions, together with activated macrophages, may represent a cell-mediated, delayed type hypersensitivity form of immune response (Hansson et al., 1989a).

The major cytokine produced by T-lymphocytes is γ-IFN which exerts a multitude of effects on cells of both the immune system and vessel wall. γ-IFN inhibits the proliferation of both endothelial cells and smooth muscle (Friesel et al., 1987; Hansson et al., 1988) and can inhibit the expression and production of PDGF and IL-I (Hansson et al., 1989a). Associated with this inhibition of proliferation is an up-regulation of high-density lipoprotein receptors (Oppenheimer et al., 1988). One of the major effects of γ-IFN in the vessel wall, however, is the induction of cell surface antigens on smooth muscle and endothelial cells; these belong to the class II major histocompatibility complexes and are, therefore, normally only expressed by the cells of the immune system (Warner et al., 1989). This induction occurs at the level of transcription; its significance is unknown, but it may indicate that endothelial and smooth muscle cells exposed to γ-IFN can act as antigen-presenting cells.

V. Heparan Sulphate: A Vascular Cell Growth Regulator

Heparan sulphate proteoglycans in blood vessels occur as part of the extracellular matrix and as a component of the basal lamina of both smooth muscle and endothelial cells (Fransson, 1987). The proteoglycan is attached to the cell surface in two distinct ways: the heparan sulphate side chains can be bound ionically to surface receptors and thus displaced by exogenous polyanions, e.g., heparin; alternatively, the proteoglycan can be attached to the cell membrane by intercalation of a hydrophobic region of the core protein directly into the lipid bilayer in a manner similar to integral membrane proteins or may be covalently linked to an inositol-containing phopsholipid (Kjellen et al., 1980, 1981). The membrane-intercalated proteoglycan binds to specific sites on collagen, laminin, and fibronectin through its heparan sulphate chains (Gallager et al., 1986; Saunders and Berndield, 1988). Heparan sulphate has many biological effects in the vessel wall. For example, it plays a major role in vascular hypertrophy by controlling the phenotype that smooth muscle cells express, inhibiting transit of the cells from G_1 to S in the cell cycle, and binding a variety of growth factors.

The effect of heparan sulphate on smooth muscle phenotype was discovered using cell culture techniques. Enzyme dispersion of vascular smooth muscle, followed by seeding at subconfluent density in primary culture, results in a gradual decrease in their $V_v$myo (Campbell et al., 1989; see also section II.B). However, if the basal lamina is rapidly reconstituted by seeding the cells onto basement membrane containing heparan sulphate proteoglycan, collagen type IV, laminin, and entactin, or if a microenvironment is provided by completely embedding the cells within a gel of collagen type I, then the

smooth muscle cells do not undergo a change in phenotype (Stadler et al., 1989). The contractile phenotype is also maintained by plating freshly dispersed cells at confluent density (Campbell et al., 1989) or placing sparsely seeded cells with a spatially separated feeder layer of confluent, high $V_v$myo smooth muscle cells or confluent endothelial cells (Campbell and Campbell, 1984). Both feeder layers are known to produce large amounts of an antiproliferative heparan sulphate species (Fritze et al., 1985; Castellot et al., 1981). Furthermore, a crude extract of glycosaminoglycans from the aortic intima and media maintains sparsely seeded smooth muscle cells with a high $V_v$myo, whereas treatment of the extract with heparinase destroys the active factor (Chamley-Campbell and Campbell, 1981). Also, addition of sodium heparin to culture medium will maintain smooth muscle in the contractile state (Campbell et al., 1981).

The mechanism by which heparan sulphate and the closely related glycosaminoglycan heparin exert this effect is unknown. One of the functions of the cell-associated heparan sulphate proteoglycan is to promote the organisation of actin filaments within the cell, which also has the effect of stabilising cell morphology (Le Baron et al., 1988). Thus, degradation of cell surface heparan sulphate by prolonged enzymatic treatment during cell dispersion may initiate a change in smooth muscle phenotype through disorganisation of actin filaments with subsequent influences on gene expression (Bissell and Barcelos-Hoff, 1987). However, the observation that low concentrations of trypsin alone (which releases the heparan sulphate proteoglycans from the cell surface) do not induce a change in smooth muscle phenotype suggests that the heparan sulphate chains must be completely destroyed or otherwise removed from the vicinity of the cell for this to occur (Campbell et al., 1991). The ability of free heparin to prevent a change in phenotype of those smooth muscle cells whose extracellular matrix and basal lamina have been degraded and removed during enzymatic isolation supports this view.

Heparan sulphate proteoglycans and free glycosaminoglycan chains, including heparin, bind to specific proteins at the cell surface, are internalised within endocytotic vesicles, and are degraded (Bienkowski and Conrad, 1984; Schmidt and Buddecke, 1988). A small fraction of the heparan sulphate/heparin is transported to the cell nucleus where it has been implicated in cell growth control (Fedarko and Conrad, 1986; Ishihara et al., 1986; Castellot and Karnovsky, 1977). Whether internalised heparan sulphate/heparin affects smooth muscle phenotypic expression by this mechanism is unknown; however, the observation that high $V_v$myo smooth muscle cells constantly internalise and degrade their own cell surface heparan sulphate suggests that this may be the case (Campbell et al., 1991).

The inhibitory effect of heparin on smooth muscle proliferation was first shown in vivo by Clowes and Karnovsky (1977). Upon heparin treatment, the smooth muscle proliferation usually seen after endothelial cell denudation (see sections II.C and III.B) was almost completely abolished. Non-anticoagulant heparin was as effective as anticoagulant heparin, indicating that its effect was independent of the antithrombin, anticoagulant activity of heparin (Guyton et al., 1980). Heparin inhibits both the cell proliferation and the migration of nondividing smooth muscle cells (Clowes and Clowes, 1986). It does not prevent early (after 24 hours) changes in actin isoform expression by the vascular smooth muscle cells, but it does cause a reinduction of smooth muscle α-actin mRNA after 5 days (Clowes et al., 1988).

The ED_{50} for the antiproliferative activity of heparin is in the 1- to 10-μg/ml range (Hoover et al., 1990). Smooth muscle cells that are quiescent (i.e., in G_0) before exposure to growth medium containing heparin, are 50- to 100-fold more sensitive to heparin than smooth muscle cells that are exponentially growing at the time they are exposed (Castellot et al., 1981). Other glycosaminoglycans, such as dermatan sulphate, chondroitin sulphates A and C, and hyaluronic acid, require at least 500-μg/ml concentrations to inhibit smooth muscle growth by 50%. The smallest heparin fragment with antiproliferative activity is a pentamer, whereas di- and tetrasaccharide fractions are inactive. The maximum antiproliferative activity is obtained with dodecamer and larger fragments (Oosta et al., 1981; Castellot et al., 1986). O-Sulphation, or at least a negative charge at the O position, is necessary for the antiproliferative activity (Castellot et al., 1984, 1986). Heparan sulphate derived from endothelial cells is also a potent inhibitor of smooth muscle growth, exhibiting activity approximately 1000-fold greater than heparin itself (Benitz et al., 1990; Castellot et al., 1981).

The heparin-sensitive point in the cell cycle of vascular smooth muscle is in the middle to late G_1 phase, approximately 4 hours before the S-phase (Castellot et al., 1985a,b; Majesky et al., 1987). The $G_0 \rightarrow S$ transit functionally can be divided into two phases: the competence phase, in which factors such as PDGF stimulate cells to leave the G_0 state and become "competent" to traverse the rest of G_1, and the progressive phase, in which competent cells move through the rest of G_1 and enter S-phase (Pledger et al., 1977).

Heparin also has other effects in vascular smooth muscle. It induces the synthesis of a novel 60-kDa collagen in the cell fraction and the secretion of 35- and 37-kDa proteins into the culture medium (Majack and Bornstein, 1984, 1985; Cochran et al., 1985). The release of another protein of approximately 37 kDa, which shares antigenic determinants with major excreted protein, is decreased by heparin (Cochran et al., 1988). It also changes the regional distribution of elastin, collagen, and proteoglycans in blood vessels following experimental

injury in a specific and differential manner (Snow et al., 1990).

Heparin is chemotactic for endothelial cells (Terranova et al., 1985) and is a modulator of the biological activity of endothelial cell growth factor (Maciag et al., 1984), specifically by increasing the activity of the mitogen by a mechanism involving endothelial cell growth factor receptor occupancy (Schreiber et al., 1985). The heparan sulphate chains produced by endothelial cells contain heparin sequences (Nader et al., 1987). Endothelial cells grown in the combined presence of heparin and endothelial cell growth factor exhibit profound alterations in the synthesis of extracellular matrix proteins. Specifically, the expression of types I and IV collagens, fibronectin, and decorin proteoglycan genes are markedly inhibited, whereas versican proteoglycan and β-actin genes are unaffected.

Heparan sulphates can also modulate cell growth by influencing the availability of growth factors with the vessel wall. It has been known for some time that heparan sulphate proteoglycan binds bFGF (Burgess and Maciag, 1989). This appears to protect bFGF from degradation as well as provide a matrix-bound or cell surface-bound reservoir of the growth factor (Folkman et al., 1988). From this reservoir, active bFGF-heparan sulphate complexes can be generated following proteolysis of the proteoglycan core protein or partial degradation of the glycosaminoglycan (Saksela and Rifkin, 1990). The binding of bFGF to its receptor requires prior binding either to the heparan sulphate side chains of membrane-bound sulphate proteoglycan or to free heparan sulphate or heparin chains (Yayon et al., 1991). This may be due to the glycosaminoglycan changing the conformation of bFGF, such that it gains the ability to bind to the receptor. Alternatively, binding to heparan sulphate may affect the oligomerisation of the receptors (Rapraeger et al., 1991), a phenomenon considered necessary for signal transduction by other receptor systems linked to the tyrosine kinases (Ullrich and Schlessinger, 1990).

Other vascular cell growth factors that bind to heparin and heparan sulphate proteoglycan include PDGF and TGF-β. PDGF binds to the glycosaminoglycan section of the molecule, specifically via the -Lys^{115}-Lys^{116}-Arg^{117}-Lys^{118}- of the long PDGF A-chain isoform. This results in a reversible inactivation of this mitogen (Fager et al., 1990). On the other hand, TGF-β binds to cell surface heparan sulphate proteoglycan via the core protein, with subsequent delivery to the signal transduction receptors (Andres et al., 1989). TGF-β also binds to the protein core of an extracellular matrix proteoglycan, decorin (Yamaguchi et al., 1990). The protein core of decorin is leucine rich and is associated with type I collagen fibrils in tissues. Decorin antagonises the activity of TGF-β by competing with the TGF-β receptors for the same or adjacent binding sites in TGF-β. The synthesis of decorin is stimulated by TGF-β, and, therefore, decorin may be an effector molecule in a negative feedback loop that regulates TGF-β activity. The binding of TGF-β to decorin is reversible, and thus decorin-TGF-β complexes in the extracellular matrix may act as a local reservoir of the growth factor (Ruoslahti and Yamaguchi, 1991).

VI. Pharmacological Manipulation of Growth Factor Activity

A. Atherosclerosis

A wide variety of agents have been examined for their ability to inhibit experimentally induced atherosclerosis, mainly in rabbits. However, there are substantial differences between the type of atheromatous lesions found in rabbits fed a diet rich in cholesterol (total plasma cholesterol $\leq$ 50 mmol/liter) and those seen in humans. Advanced lesions in humans contain abundant fibrous tissue and calcium deposits as well as lipids (see section III.A; Fagiotto et al., 1984; Ross and Glomset, 1976).

1. Calcium channel blockers. Several lines of evidence now indicate that antagonists of voltage-operated calcium channels attenuate the development of atherosclerosis. Ginsburg et al. (1983) demonstrated that lanthanum, diltiazem, and flunarizine reduce atherosclerotic lesions in the aorta of rabbits fed a high cholesterol diet for 10 weeks. Verapamil, as well as the dihydropyridine analogues such as nifedipine, also has a preventative effect on the development of atherosclerosis in cholesterol-fed rabbits (Sievers et al., 1986; Henry and Bentley, 1981; Schmitz et al., 1991). In humans, there is also angiographic evidence that calcium antagonists retard the progression of atherosclerotic lesions (Lichthen et al., 1990).

Multiple mechanisms appear to account for the antiatherosclerotic effects of the calcium antagonists. They inhibit vascular smooth muscle cell proliferation and migration, which are key events in the formation of the vascular lesions. Most calcium antagonists, including verapamil and the dihydropyridine analogues nifedipine and nicardipine, reduce serum-stimulated [^{3}H]thymidine incorporation into the DNA of cultured human aortic subendothelial intimal cells; total intracellular cholesterol is also reduced (Orekhov et al., 1988). Similar effects on [^{3}H]thymidine incorporation have been observed in vascular smooth muscle from other species (Tomita et al., 1987; Thyberg and Palmberg, 1987). Dihydropyridine analogues are the most potent of the calcium antagonists in inhibiting [^{3}H]thymidine incorporation, exerting their effect via the salvage pathway for thymidilate biosynthesis (Agrotis et al., 1992). Despite being potent inhibitors of this pathway, calcium antagonists are relatively weak inhibitors of smooth muscle cell proliferation (Agrotis et al., 1992; Nomoto et al., 1987), although inhibitory effects are observed at high concentrations (Nomoto et al., 1987).

The dihydropyridine analogues nicardipine and nivaldipine are also potent inhibitors of chemotaxis and

PDGF-mediated smooth muscle cell migration (Nakao et al., 1982; Nomoto et al., 1988). These agents are effective in vivo, reducing silylastic cuff-induced intimal thickening of the rabbit carotid artery, presumably by preventing smooth muscle cell migration (Nomoto et al., 1987). The effects of the calcium antagonists on migration are readily apparent at concentrations 100-fold less than those that affect smooth muscle cell proliferation. Although the calcium antagonists can influence the function of many cells within the blood vessel wall, including the reduction of extracellular matrix biosynthesis, the activity of the nucleoside transporter, and the metabolism of lipids (for details see Schmitz et al., 1991), their ability to affect cytokine- and growth factor-induced smooth muscle cell migration appears to be the most important mechanism through which they retard the progression of atherosclerotic lesions. However, their full potential as antiatherosclerotic agents has yet to be elucidated.

2. Hydroxymethylglutaryl-coenzyme A reductase inhibitors. Recently, a number of inhibitors of cholesterol biosynthesis have been introduced to treat hypercholesterolemia (Shepherd and Packard, 1988). Lovastatin and simvastatin, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, suppress the synthesis of mevalonic acid, a key intermediate in the cellular production of cholesterol. However, because mevalonic acid is also utilised in pathways responsible for the posttranslational modification of proteins that attach to cell membranes via thioether-linked prenyl groups (isoprenylations) such as farnesylation, these agents also inhibit these modifications.

Lovastatin and its related analogues have the potential to inhibit cell proliferation through a variety of mechanisms by preventing the posttranslational modification of nuclear lamins and small molecular weight G proteins, such as the *ras* oncogene-encoded protein ($p21^{ras}$), and their subsequent attachment to the nuclear and cell membranes (Mumby et al., 1990; Yamane et al., 1991). Recently, lovastatin has been reported to arrest cells in the G_1 phase of the mitotic cell cycle, also prolonging, and in some cases arresting, cells in the G_2 phase of the cell cycle (Jakóbisiak et al., 1991). Indeed, it has been suggested that the G_1 arrest is a consequence of the loss of the signal transduction capacity of the $p21^{ras}$ altering its intracellular location.

Other mechanisms that could contribute to the inhibitory effects of lovastatin on cell proliferation include the prevention of adenosine modification by isopentyl groups in some tRNAs (Goldstein and Brown, 1990). Lovastatin also attenuates the induction of c-*fos* mRNA by EGF, insulin, and IGF-I but not by bFGF or PDGF (Vincent et al., 1991). The mechanism by which lovastatin selectively inhibits the transcriptional activation of the c-*fos* gene by some, but not other, activators of tyrosine kinase-dependent growth factors is unclear. Despite this, there appears no doubt that 3-hydroxy-3-methylglutaryl-coenzyme A reductase can attenuate activation of cells within the vessel wall. Further studies are required to elucidate the importance of this property in the prevention of atherosclerotic lesions.

3. Miscellaneous. Orekhov et al. (1988) examined the antiatherosclerotic potential of a wide variety of agents which included those activating cyclic AMP, stable prostacyclin analogues, prostaglandin E_2 and D_2, the phenothiazines chlorpromazine and trifluoperazine, dihydroergotamine and reserpine, and the antioxidants α-tocopherol and butylated hydroxytoluene. The substances that increased cyclic AMP, reserpine, and the prostacyclin analogues inhibited aortic smooth muscle cell proliferation and reduced cellular triglyceride and cholesterol ester levels.

Butylhydroxytoluene and α-tocopherol also inhibit growth factor-induced smooth muscle cell proliferation. More recently, Boscoboinik et al. (1991) confirmed this inhibitory effect of α-tocopherol which protects against free radical damage. α-Tocopherol and probucol also inhibit IL-1β expression (Akeson et al., 1991). Although antioxidants including probucol exert their antiatherosclerotic effect by preventing the oxidation of low-density lipoproteins, which induce the vascular endothelium to produce a variety of growth factors (see section IV.D.1.b), their ability to directly inhibit growth factor biosynthesis as well as smooth muscle cell proliferation is a complimentary property that may contribute to their antiatherosclerotic effects.

B. Vascular Restenosis

As mentioned previously (see section III.B), restenosis following percutaneous transluminal coronary angioplasty, endarterectomy, and saphenous vein grafts continues to be a major clinical problem. To date, no drugs have been shown to be clinically effective in preventing either the proliferation of smooth muscle cells or the intimal fibrous lesion responsible for the ensuing restenosis. Aspirin and oral anticoagulants are efficient in preventing early aortocoronary bypass occlusion but do not influence the late vascular changes associated with restenosis. Heparin inhibits the smooth muscle cell proliferation induced by balloon catheter vessel injury (Clowes and Karnovsky, 1977); however, this agent is relatively ineffective in preventing venous graft thickening.

From a theoretical point of view, a pharmacologically effective agent for preventing restenosis of a mechanically injured vessel would be expected to possess the following properties: (*a*) induce rapid reendothelialisation of the injured vessel; (*b*) prevent any phenotypic change in the medial smooth muscle cells, their replication within the media, and subsequent migration; and (*c*) inhibit any neointimal smooth muscle cell proliferation and extracellular matrix biosynthesis.

ACE inhibitors and the products of growth factor genes fused to modified bacterial toxin genes attenuate the rate of restenosis in experimental animals rather than facilitate healing of the damaged vessel.

1. Angiotensin-converting enzyme inhibitors. Powell et al. (1989) originally reported that antihypertensive doses of the ACE inhibitor cilazapril greatly attenuated development of the neointima following balloon catheterisation of the carotid artery in the rat. Both the volume of neointima and the area of the internal elastic lamina covered by the neointima (approximately 80%) was reduced by continuously administering the ACE inhibitor throughout the period when the response of the vessel to injury was most marked (see section III.B). This property appears to be a general characteristic of the ACE inhibitors.

Prevention of restenosis by the ACE inhibitors does not appear to be due to any direct inhibitory effect on vascular smooth muscle cell proliferation (Powell et al. 1989). Rather, the major mechanism responsible for preventing the neointimal thickening appears to be the consequence of the ACE inhibitor attenuating smooth muscle cell migration from the media (Prescott et al., 1991). Whether this effect is primarily the consequence of the smooth muscle cells being unable to alter their phenotype or migration out of the media still requires further study. The recent finding that the angiotensin type I receptor antagonist DuP753 also attenuates the migratory ability of smooth muscle cells suggests that angiotensin II participates in this early response to vascular injury.

In cell culture, angiotensin II promotes smooth muscle cell migration (Bell and Madri, 1990). However, in contrast to the ACE inhibitors, which do not appear to reduce smooth muscle cell replication following balloon-induced injury to a vessel, the angiotensin type I receptor antagonist DuP753 inhibits medial smooth muscle cell replication (Prescott et al., 1991). This inhibition is not the consequence of any direct inhibitory effect of DuP753 on vascular smooth muscle because DuP753 does not affect serum-stimulated vascular smooth muscle cell proliferation in culture. In contrast to their inhibitory effects on vascular smooth muscle migration in cell culture, ACE inhibitors promote endothelial cell migration. For example, lisinopril increases endothelial cell migration, as does the angiotensin II antagonist saralasin (Bell and Madri, 1990); this stimulatory effect can be abolished by angiotensin II.

Despite these observations in vitro, there is little evidence that ACE inhibition facilitates reendothelialisation of a damaged vessel. There is also little evidence to indicate that ACE inhibition reduces the rate at which luminal smooth muscle cells forming the pseudoendothelium proliferate. ACE inhibitors also prevent the intimal thickening of aortic allografts (Plissonnier et al., 1991) as a consequence of reductions in neointimal extracellular matrix biosynthesis. However, ACE inhibition does not prevent adventitial inflammatory infiltration or smooth muscle cell proliferation. ACE inhibitors also appear to be effective in preventing the early structural changes associated with venous graft thickening (Roux et al., 1991). Virtually all of these effects on vascular structure have been described in rats. Clinical trials will no doubt determine whether these properties of the ACE inhibitors are also therapeutically beneficial to subjects undergoing vascular reconstruction or repairs.

2. Recombinant chimeric toxins

Recently, recombinant chimeric toxins have been proposed as potential therapeutic adjuncts for the prevention of restenosis following percutaneous transluminal coronary angioplasty (Epstein et al., 1991). This therapeutic strategy is based on the findings that cancer cells (undergoing rapid proliferation) often express high levels of receptors that may be either absent or present only at very low densities on normal cells (Xu et al., 1984; Yamamoto et al., 1986; Merlino et al., 1985). Targeting of these proliferating cells is being achieved by coupling toxins to specific ligands, cytokines, or growth factors for which the cells possess high quantities of receptors (Simpson et al., 1982; Batra et al., 1989).

Recombinant *Pseudomonas* exotoxin (PE40) containing only domains II and III have, in the main, been coupled to cytokines or growth factors (Debenski et al., 1991; Chaudhary et al., 1991); domain I is normally replaced with the targeting peptide (fig. 9). Domains II and III of this exotoxin are vital for cytotoxicity because they are responsible for translocation of the exotoxin across the cell membrane and they then catalyse the transfer of the ADP-ribosyl moiety of oxidised nicotinamide-adenine dinucleotide onto the elongation factor (Middlebrook and Dorland, 1984). Because elongation factor is required for the addition of amino acids onto

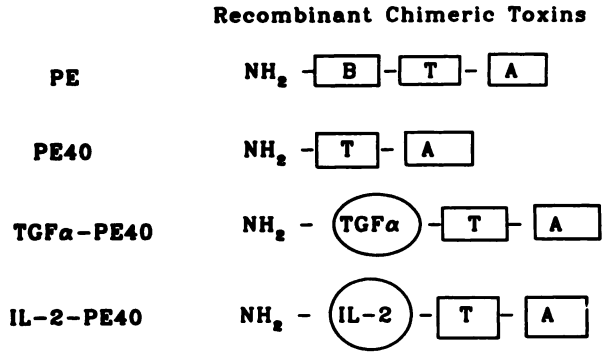

FIG. 9. Recombinant chimeric toxins. Domains of *Pseudomonas* exotoxin (PE) fusion proteins. B, binding domain; T, translocating domain; A, ADP-ribosylating domain. TGF-α, IL-2, or other cytokine of interest may be attached to the amino terminus of PE40 to produce the TGF-α-PE40 or IL-2-PE40 chimeric molecule. Although these chimeric molecules have been examined for their ability to inhibit neointimal smooth muscle cell proliferation, they may be more suitable as angiostatic agents if coupled to the appropriate growth factor/cytokine.

developing protein chains during mRNA translocation, its inactivation results in cell death. The chimeric toxin TGF-α-PE40, a cytotoxin for cells expressing EGF receptors, is toxic to rapidly proliferating cultured vascular smooth muscle cells (Epstein et al., 1991). The cytotoxicity of TGF-α-PE40 is 30-fold less on quiescent cultured vascular smooth muscle. Toxicity is directly related to its ability to inhibit protein synthesis and the expression of receptors binding EGF. The product of the chemical conjugate of bFGF to saporin, a ribosome-inactivating protein from the seeds of *Saponaria officinalis*, has recently been shown to decrease the number of viable smooth muscle cells in the arterial wall after balloon injury by more than 50% (Lindner et al., 1991).

Although highly promising, the successful use of this approach to prevent neointimal thickening and subsequent vessel restenosis clinically will depend on binding toxins to ligands highly specific for proliferating vascular smooth muscle cells. bFGF is unlikely to be a suitable choice because both vascular smooth muscle cells and endothelial cells are likely to express receptors for this growth factor. Any impairment of endothelial cell function, particularly an attenuation of their ability to migrate and proliferate, following balloon catheter or other surgical trauma to the arterial wall, would prevent rather than promote healing of the lesion.

3. Miscellaneous. Myointimal proliferation of vascular smooth muscle after balloon catheter injury of vessels has also been shown to be attenuated by calcium antagonists (Jackson et al., 1988). Nifedipine, verapamil, and diltiazem all reduced [^{3}H]thymidine incorporation into medial smooth muscle after balloon catheterisation of the aorta. Nifedipine also reduced neointimal thickening. The mechanism(s) by which these agents exert their effect is unclear. Dihydropyridine-derived calcium antagonists, such as nifedipine, are potent inhibitors of the "salvage pathway" for thymidilate biosynthesis, whereas verapamil and diltiazem are relatively weak inhibitors of this pathway (Agrotis et al., 1993). In cell culture, all are relatively weak inhibitors of vascular smooth cell proliferation.

The finding that lanthanum, minoxidil, and prazosin also attenuate [^{3}H]thymidine incorporation into the medial smooth muscle early after balloon angioplasty suggests that these agents exert their antiproliferative effects by multiple mechanisms, including a relaxation of the damaged vessel. In addition, the presence of lanthanum in the medium bathing cultured vascular smooth muscle induces them to express morphological characteristics similar to those of cells forming a pseudoendothelium. Such a mechanism might also contribute to the mechanisms by which calcium antagonists attenuate the size of the neointima following balloon angioplasty (Kuzuya et al., 1983).

Trapidil, a purported coronary vasodilator pharmacologically related to dipyridamole, has also been shown to reduce myointimal smooth muscle cell hyperplasia in experimental animals (Tiek et al., 1983). As with the other agents, the mechanisms by which trapidil exerts its antiproliferative effect is obscure. In cell culture, trapidil only attenuates vascular smooth muscle proliferation after a lengthly incubation period prior to the addition of a growth factor; this suggests an intracellular site of action. Recently, lovastatin (see section VI.B.2) has also been shown to suppress neointima formation following balloon injury (Gellman et al., 1991). As with the other agents being currently evaluated for their ability to prevent myointimal thickening, there is no evidence to indicate that trapidil promotes reendothelialisation of damaged vessels.

Despite the fact that many structurally and pharmacologically unrelated agents reduce myointimal thickening in experimental animals, albeit at relatively high doses, their potential role in preventing or at least delaying restenosis of human vessels still remains to be elucidated.

C. Hypertension

1. Antihypertensive agents. The mechanisms by which different pharmacological agents may influence the development of vascular hypertrophy in hypertension have been investigated in relatively few studies. Adams et al. (1990) demonstrated that early treatment of SHR with the ACE inhibitor enalapril prevented the development of vascular hypertrophy and, to a large extent, the hypertension. Twenty weeks after withdrawal of the drug, vascular structure apparently remained close to normal, as did blood pressure. Both effects were associated with a reduction in left ventricular hypertrophy. Christensen et al. (1989) also observed a similar long-term antihypertensive effect of the ACE inhibitors. In these studies, mesenteric resistance vessel structure, measured as vessel media to lumen ratio, was reduced by the ACE inhibitors to values midway between those of untreated SHR and normotensive Wistar-Kyoto rats. At present it is uncertain whether this effect on vascular structure is due solely to the trophic effects of angiotensin II. Angiotensin II can, in addition to exerting a direct trophic effect on vascular smooth muscle cells (Geisterfer et al., 1988), also stimulate TGF-β_1 gene transcription (Gibbons et al., 1990) and increase their sensitivity to PDGF-BB (Bobik et al., 1990a). Treatment of young SHR also reduces the incidence of tetraploid smooth muscle cells later in life (Black et al., 1989). At present it is uncertain whether this reduction in the percentage of tetraploid cell is the consequence of the ACE inhibitor attenuating vascular TGF-β_1 production.

Short-term treatment of adult SHR with enalapril also reduces the proportion of 4N smooth muscle cells in the aorta, perhaps as a consequence of cell death during enalapril treatment (Black et al., 1989). ACE inhibition is known to increase vascular kinin levels, which in turn

could increase endothelial cell-derived nitric oxide. Nitric oxide, in addition to being a vasodilator, also inhibits cell growth (Schulz et al., 1991) and at high concentration is cytotoxic. It should be pointed out that support for such a hypothesis is circumstantial and based on the following findings: (*a*) treatment of young SHR with the angiotensin II type I receptor antagonist DuP753 is less efficacious in its long-term effects on hypertension than ACE inhibition (Oddie et al., 1992; Adams et al., 1990); (*b*) a component of the rapid decrease in blood pressure following administration of captopril is attributed to increased bradykinin levels; and (*c*) other antihypertensive agents including the vasodilator hydralazine and the calcium antagonist isradipine, but not β-adrenoceptor-blocking agents, have been shown to retard the development of vascular hypertrophy in growing SHR (Christensen et al., 1989). It remains to be determined whether these agents are reducing vascular smooth muscle growth or the production of extracellular matrix in hypertension.

There is good experimental evidence to suggest that antihypertensive agents that retard the development of vascular hypertrophy may be beneficial in treating young persons with borderline hypertension, by reducing the full development of hypertension as occurs in the SHR. Antihypertensive agents that possess antiproliferative properties for vascular smooth muscle may also be beneficial in preventing the progression of cardiovascular atherosclerosis in persons with hypertension. Reductions in blood pressure alone by agents that have no affect on vascular smooth muscle proliferation have little effect on the incidence of atherosclerotic complications, particularly coronary artery disease, in persons with hypertension (Mathison et al., 1969; Kannel et al., 1984).

D. Angiogenesis

During the past 10 years, a variety of inhibitors of angiogenesis have been described that, although not suitable for clinical use due to either excessive toxicity, limited efficacy, or availability, have provided new insights as to how pathological angiogenesis may be controlled in, for example, diabetic retinopathy vascularisation of the retina and the vascularisation of solid tumours.

1. Angiostatic steroids. Folkman et al. (1983), in an attempt to visualise more clearly the heparin-induced enhancement of tumour angiogenesis on the chorioallantoic membrane, added cortisone to suppress background inflammation; surprisingly, this combination inhibited angiogenesis. Alone, cortisone has little or no effect on angiogenesis. The heparin-cortisone combination also inhibits vascularisation of rabbit corneas stimulated by deepithelialisation as well as by autograph transplant (Folkman et al., 1983). Heparin fragments which do not possess anticoagulant activity are equally effective with cortisone. Results of structure-activity studies with a variety of steroids indicate that neither glucocorticoid nor mineralocorticoid activity is required for steroids to possess antiangiogenic activity (fig. 10) (Crum et al., 1985). 11α-Epihydrocotisol, 17α-hydroxyprogesterone, and tetrahydrosteroid derivatives all exhibit potent antiangiogenic activity. Antiangiogenic activity of these steroids appears associated with the pregnane structure and is primarily determined by structural elements on the D-ring (fig. 10).

At present little is known about how these angiostatic steroids interact with heparin to inhibit angiogenesis. In cell culture, heparin plus cortisone inhibits endothelial cell proliferation (Sakamoto et al., 1986). Others have suggested that these steroids could inhibit angiogenesis by inducing basement membrane breakdown, leading to capillary regression (Ingber et al., 1986). Inhibition of collagen fibril formation and deposition by a variety of proline analogues that are inhibitors of proxyl hydroxylase leads to the regression of growing capillaries (Ingber et al., 1988). Recently, the angiostatic steroid, medroxyprogesterone, has been shown to suppress the synthesis by endothelial cells of a novel laminin-related polypeptide (A′ subunit) while stimulating the synthesis of the A subunit (Tokida et al., 1990). Because laminin is a major glycoprotein of basement membranes which influences cell adhesion, growth, differentiation, and the mi-

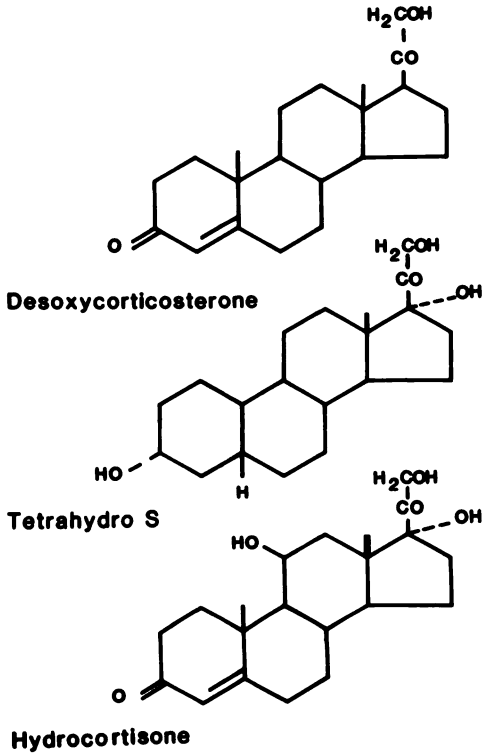

FIG. 10. Structural characteristics of angiostatic steroids and their relationship to hydrocortisone and desoxycorticosterone. Desoxycorticosterone possesses mineralocorticoid activity and has weak antiangiogenic activity; Tetrahydro S possesses no glucocortocoid or mineralocortocoid activity but possesses potent angiostatic activity; hydrocortisone possesses approximately 50% of the angiostatic potency of tetrahydro S and also has glucocortocoid and mineralocortocoid properties.

gration of cells (for review, see Martin and Timpl, 1987), it is possible that multiple effects of these steroids on the composition of the extracellular matrix contribute to their angiostatic activity in the presence of heparin or its analogues.

2. Interferons. Potential roles for IFNs as antiangiogenic agents are indicated by the findings that α-IFN is effective in the treatment of hemangioendotheliomas (White et al., 1989). γ-IFN has also been shown to inhibit capillary formation in vitro (Tsuruoka et al., 1988). γ-IFN has also been shown to inhibit aFGF- and IL-2-stimulated endothelial cell growth (Friesel et al., 1987; Hicks et al., 1989). The former inhibitory response was associated with a reduction in the binding of aFGF to the endothelial cell surface. The question as to whether these inhibitory responses of the IFNs on growth are mediated through modulation of cell membrane growth factor receptors needs investigation, as does their potential as angiostatic factors.

3. Protamine. Protamine, a polycationic protein that binds avidly to heparin, inhibits angiogenesis in a wide variety of experimental models (Taylor and Folkman, 1982). Protamine prevents the early vascularisation of the chorioallantoic membrane and the neovascularisation of a V_2 carcinoma in the rabbit ear. It also appears to inhibit inflammatory mediated angiogenesis. Protamine inhibits vascularisation of the embryonic rat heart, reducing final capillary density (Rakusan and Turek, 1985). Because protamine binds to heparin, it is possible that some of its effects are mediated by impairing the binding of heparin-binding growth factors, in particular the FGFs, to their membrane receptors (for details, see section IV.B). Protamine sulphate has been shown to inhibit the mitogenic activity of both matrix-associated and soluble FGF (Neufeld and Gospodarowicz, 1987).

4. Tissue-derived inhibitors. A number of tissue extracts from avascular tissues in the body have been shown to inhibit angiogenesis. Recently, Moses et al. (1990) demonstrated that a 34-kDa protein derived from cartilage inhibits the proliferation and migration of capillary endothelial cells in vitro and angiogenesis in vivo. This cartilage-derived inhibitor inhibits aFGF-stimulated bovine adrenal cortex capillary endothelial cell proliferation without affecting the proliferation of aortic smooth muscle or other nonendothelial cell types; cartilage-derived inhibitor also inhibits mammalian collagenase activity. These two effects could not be mimicked by other peptidase inhibitors, such as trypsin ovoinhibitor, pancreatic trypsin inhibitor, or the collagenase inhibitor α_2-macroglobulin. Cartilage-derived inhibitor also inhibits angiogenesis in the chick chorioallantoic membrane. It is more potent than either protamine or heparin plus cortisone. Similar inhibitory factors are known to be present in other avascular tissues, such as the vitreous and the lens of the eye (Preis et al., 1977; Williams et al., 1984), but their chemical nature has not been elucidated.

Recently, human recombinant platelet factor-4, a 28-kDa tetrameric protein that has a high affinity for heparin, has been shown to be a potent inhibitor of angiogenesis (Maione et al., 1990). Human recombinant platelet factor-4 inhibits angiogenesis in the chick chorioallantoic membrane system in a dose-dependent manner; its potency is similar to that of protamine. Heparin attenuates its angiostatic effect, and the results of structure-activity studies using small peptide fragments indicate that its angiostatic activity is associated with the heparin-binding domain. Human recombinant platelet factor-4 appears to exert a relatively specific inhibitory effect on the proliferation of venous endothelial cells; neither the proliferation of human dermal fibroblasts nor keratinocytes is unaffected. Although human recombinant platelet factor-4 binds avidly to heparin, it does not appear to interfere with the ability of bFGF to bind to its receptor. Rather, Moses et al. (1990) suggested that its collagenase inhibitory activity is responsible for its ability to inhibit neovascularisation.

E. Conclusions

During the past 10 years, there has been a tremendous increase in our knowledge about the tissue-derived factors that contribute to the development of vascular lesions and growth abnormalities that occur in atherosclerosis, postangioplasty restenosis, posttransplantation of organs, and hypertension. Many of the growth factors that contribute to these often dramatic changes in arterial vessel wall structure are vessel wall derived. They may also participate in pathological angiogenesis, as occurs in diabetic retinopathy and solid tumour neovascularisation. PDGFs, FGFs, TGF-βs, and IGFs, to mention a few, can be expressed in a variety of different isoforms to interact with at least an equal number of receptor subtypes, which may be present on the same cell or adjacent cells. Glycoaminoglycans on the cell surface or within the extracellular matrix can also have a profound modulatory effect on these multifunctional factors.

We are only now commencing to learn how the different receptor subtypes for growth factors account for the multifunctional properties of the growth factors. In normal vascular development, the growth and differentiation of smooth muscle and endothelial cells in the larger vessels and pericytes and endothelial cells in the capillaries are regulated by growth factors in a highly coordinated manner. Because the same growth factors are also expressed during the development of vascular abnormalities, we suggest that these are initially the consequence of a mismatch in the expression of growth factors and/or their receptor subtypes. Other factors derived from nonvascular cells, such as monocytes, macrophages, and tumours, may also contribute to this mis-

match. The identification of these mismatches and the definition of their consequences of vascular growth will enable us to identify specific pharmacological targets to prevent vascular lesions and growth abnormalities.

The challenge to pharmacologists will be to produce appropriate drugs. The challenge is great, but the benefits will also be equally rewarding, as indicated by a number of recent studies. For example, in experimental hypertension, amelioration of the vascular changes during the development of hypertension reduces the severity of the hypertension later in life. This effect is maintained long after withdrawal of the converting enzyme inhibitor (Adams et al., 1990). There is also evidence that calcium antagonists may attenuate the development of atherosclerotic lesions in humans, an effect that might be partially due to their ability to reduce the migratory responses of vascular smooth muscle (Nomoto et al., 1988). Recently, White et al. (1989) demonstrated that IFN-α2a was effective in pulmonary capillary hemangiomatosis, a usually fatal disease in which there is excessive growth of capillary blood vessels in the lung. Its effectiveness may be a consequence of its ability to downregulate FGF receptors on endothelial cells.

Toxicity and poor efficacy greatly limit the use of currently developed angiostatic agents to prevent angiogenesis in other situations (Kusaka et al., 1991). The real challenge for pharmacologists in the future will be to develop appropriate drugs targeted at correcting the specific defect in the various vascular growth disorders. In some instances, these may serve as primary drugs, e.g., to prevent angiogenesis, or, in the case of hypertension or atherosclerosis, as adjunctive therapy to blood pressure and cholesterol lowering agents.

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