George Lyman Duff Memorial Lecture

Atherosclerosis: A Problem of the Biology of Arterial Wall Cells and Their Interactions with Blood Components

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I am most honored to give the Lyman Duff lecture, since Dr. Duff has had such an impact on atherosclerosis research. As many of you may know, one of Dr. Duff's earliest papers, published in 1932, concerned changes in the rabbit aorta related to medial degeneration produced by diphtheria toxin. His first paper on atherosclerosis was his thesis, written in 1935, in which he looked at "Experimental Cholesterol Arteriosclerosis and its Relationship to Human Arteriosclerosis." Together with Gardner McMillan, he published a paper in 1948 on the inhibition of experimental cholesterol atherosclerosis by Alloxan diabetes in the rabbit. In this paper, they examined the mitogenic activity in the rabbit aorta at a time when most investigators considered atherosclerosis to be an entirely degenerative disease process.

Lyman Duff was the Strathcona Professor of Pathology and Dean of the Faculty of Medicine at McGill University. We are still pursuing many of the important questions that were raised by him and his colleagues, and are indebted to them for the foundation they provided in research.

In this lecture, I should like to raise a series of questions concerning the role played by each of the cells of the blood and of the artery wall as they interact with one another, and with components in the plasma in the etiology and pathogenesis of the lesions of atherosclerosis.

The Lesions of Atherosclerosis

Although human atherosclerosis has been studied for over 100 years, it is only in the last two decades that we are beginning to understand more about the nature of the cells involved in the lesions. Lately, we have become particularly interested in the roles played by the platelet, the monocyte/macrophage, and the smooth muscle cell in the development of the human lesion. Together with colleagues in the Department of Surgery at the University of Washington School of Medicine, we have been examining human lesions obtained from peripheral leg arteries removed during bypass surgery in the leg, or during endarterectomy of the carotid arteries. At the time of surgery, some of the vessels that contain lesions can be removed. Those that are, are carefully examined under sterile conditions. Appropriate representative segments of each artery are fixed immediately for light and electron microscopy. The remainder of the tissue is then utilized for culture of the cells from carefully dissected lesion, sublesion (media), and nonlesion (adjacent) tissues. The results of these studies will be reported in detail elsewhere. Observations made in these investigations have demonstrated that many of the foam cells found in the lesions are derived from monocytes that have entered the lesions and become macrophages, as well as from proliferated intimal smooth muscle cells.

Although there have been a number of hypotheses of atherogenesis, including the "lipid insudation hypothesis" and the "monoclonal hypothesis," in this talk I should like to emphasize the "response to injury hypothesis" of atherosclerosis that we have been investigating in a number of our collaborative studies at the University of Washington.

The Response to Injury Hypothesis of Atherosclerosis

The response to injury hypothesis of atherogenesis, originally proposed by Virchow,[1] suggested that injury to the artery wall somehow led to a series of tissue responses that culminated in the lipid-filled lesions of atherosclerosis, originally considered to be largely a degenerative process. Cell and tissue degeneration have commonly been observed in the advanced, complicated lesions of atherosclerosis. In contrast to advanced complicated lesions, however, both the early form of this disease process, the fatty streak, and the lesion most commonly associated with the clinical sequelae of heart attack and stroke, the fibrous plaque, are cellular proliferative responses.[2] The cells in the fatty streak may be largely monocyte/macrophages, whereas the cells in the fibrous plaque are principally smooth muscle cells, together with variable numbers of macrophages.

The response to injury hypothesis of atherosclerosis[3-6] postulates that injury occurs specifically to the lining endothelial cells of the artery. The injury may result from a number of different types of insult, on an interrupted or chronic basis, and may modify the critical balance between cell proliferation and cell destruction that determines whether lesions progress, remain relatively constant, or regress. When cholesterol deposition exceeds removal, lesion formation may progress toward irreversibility and clinical disease may develop.

The response to injury hypothesis has been particularly useful in the formation of testable questions that interrelate the biology of at least two cells from the blood, the platelet and the monocyte, and the principal cells of the artery wall — endothelium and smooth muscle. Data obtained from the resultant studies have stimulated the development of new concepts regarding the growth characteristics of these cells, substances made by the cells, and their metabolic responses to different environmental conditions. These data have demonstrated increasing complexity in the interactions among these different cells, each of which may play a role in maintaining arterial homeostasis, as well as in the initiation, progression, and regression of the lesions of atherosclerosis. We have explored a series of questions that relate to the biology of the cells as they interact in forming the lesions of atherosclerosis and would like to suggest approaches that may be used to extend the study of this problem.

Four biological phenomena are of paramount interest in terms of understanding the cellular alterations that are associated with atherogenesis. These are:

1. Altered endothelial integrity, or endothelial injury;
2. Intimal smooth muscle cell proliferation;
3. Synthesis and deposition by the smooth muscle cells of connective tissue matrix proteins including collagen, elastic fibers, and proteoglycans;
4. Accumulation of lipids within proliferating smooth muscle cells and macrophages, as well as in the newly formed connective tissue matrix.

Figure 1 represents a modified version of the response to injury hypothesis. Endothelial cells normally form a continuous nonreactive layer that line the artery and mediate metabolic exchange through the artery wall.[7-10] Endothelial injury may be diverse in nature and can result from exposure of the cells to mechanical forces,[11-17] chemical agents such as lipoproteins,[6, 18-21] various toxins,[22-24] immunologic injury,[25-29] viruses,[30] and other as yet uncharacterized deleterious substances. These interactions, as shown in figure 1 B, may lead to changes such as separations between endothelial cells, altered endothelial permeability, or frank desquamation of the endothelial cells. If these changes are sufficiently severe, they may lead to platelet adhesion and formation of mural thrombi, and/or to adhesion of blood monocytes, which may subsequently migrate into the tissue. The interactions among substances derived from platelets and monocytes, as well as from the plasma, such as lipoproteins or hormones, may stimulate the migration of smooth muscle cells from the media of the artery into the intima, and induce the proliferation of these migrating cells, as shown in figure 1 C. If the injury is limited, this process may be reversible, and the integrity of the artery wall may be reconstituted as shown in figure 1 D. On the other hand, if the injury is continuous or repeated over a long period of time, as depicted in the inner progression cycle (figure 1 E and F), this may lead to lesion progression due to continuing interactions of components derived from the blood at sites where lesions have already formed. Eventually, blood flow may be compromised by partial narrowing of the arterial lumen and possibly complicated by thrombosis. This, then, sets the stage for the clinical sequelae of atherosclerosis.

This hypothesis poses a series of questions, some of which can be answered now, some of which must await the development of new concepts, approaches, and techniques. In testing this hypothesis, it has been helpful to examine how the artery wall responds to different stimuli.

The sequence of events in the response to injury hypothesis of atherosclerosis focuses on the importance of endothelial injury as the primary event that initiates lesion formation. In testing this hypothesis, the formulation of an adequate definition of endothelial injury has been a major
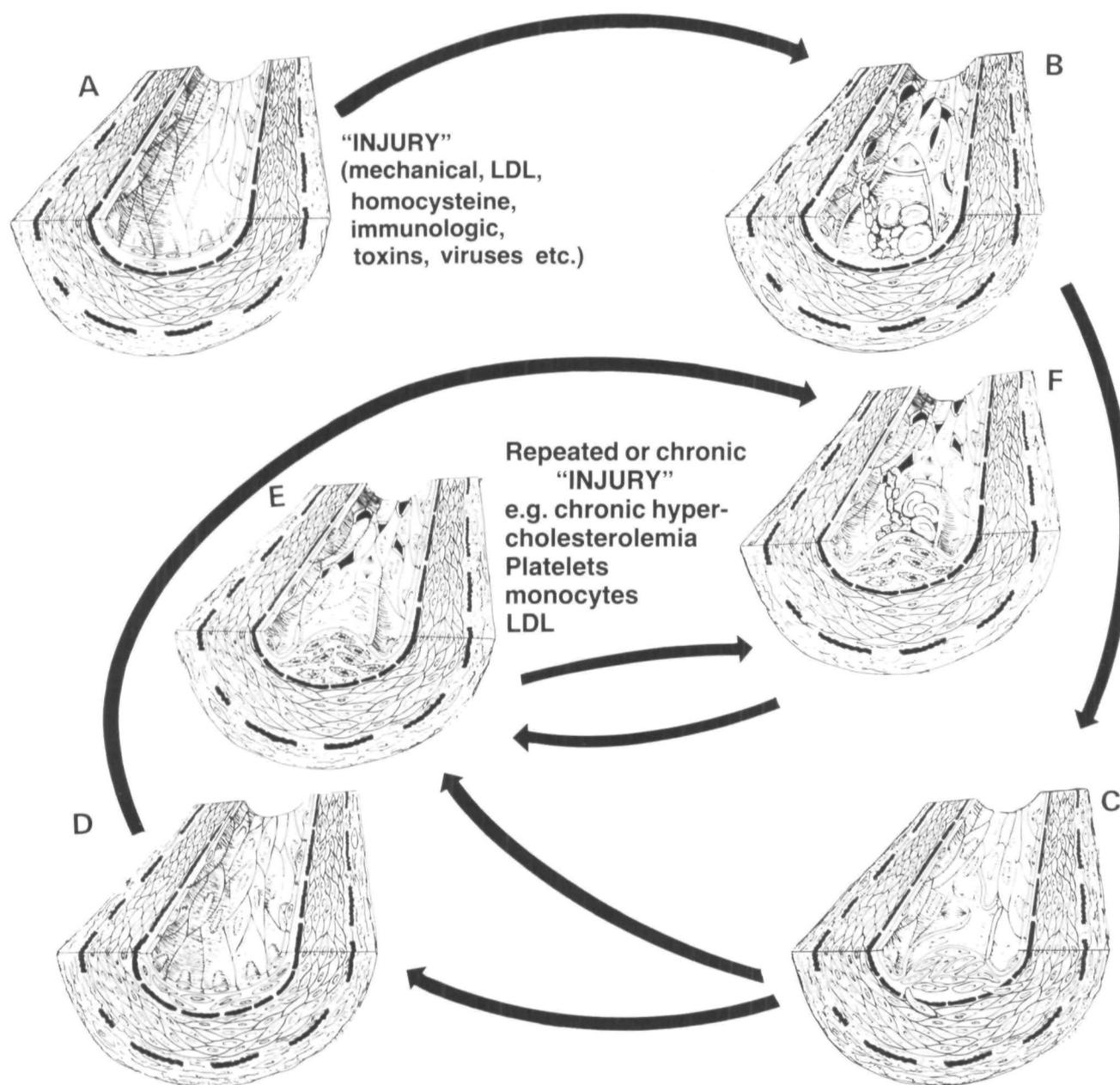
Figure 1. This figure represents a modified version of the Response to Injury Hypothesis of Atherosclerosis. A number of cyclic events may occur. In the outer, or regression, cycle (**A–D**) injury to the endothelium is depicted in **B** by separations between endothelial cells or by frank desquamation of the endothelium in which both adherence of platelets and of monocyte/macrophages may occur. If platelet adherence occurs, aggregation and release of platelet contents may also take place at such sites, whereas monocytes may go on to enter the tissue either at sites of desquamation or between endothelial cells. As shown in **C**, these interactions may be followed by migration of smooth muscle cells from the media into the intima, and by proliferation of these and possible pre-existing intimal smooth muscle cells in response to the released mitogens. In **D**, the end of the regression cycle, if the lesion is a single event and endothelial integrity is restored, the remnant of the proliferative response may simply be manifest as a somewhat thickened intima.

The inner, or progression cycle (**E** and **F**), demonstrates the possible consequences of repeated or chronic endothelial injury as may occur in hyperlipidemia, or after other forms of continuing injury. Both lipid accumulation, as well as continued smooth muscle proliferation, may occur after recurrent sequences of proliferation and regression, and may eventually lead to the development of complicated lesions that may go on to calcify. This continued cyclic progression could eventually produce the clinical sequelae of thrombosis and infarction.

problem that poses a series of important questions. These include:

- By what mechanisms do the different injurious agents act upon the endothelium?
- Are there common underlying modes of response that alter the capacity of the endothelial cells to act as a thromboresistant, blood-containing, permeability barrier?
- What controls cell–cell attachment and endothelial cell-connective tissue attachment?
- Is injury to the endothelium different in the case of hypercholesterolemia compared with cigarette smoking, homocysteinemia, hypertension, or diabetes?
- Does endothelial desquamation occur as an early response to these stimuli, or only later after lesion formation has occurred?
- If these events take place throughout the arterial tree, why do these agents lead to lesions in some areas but not others?

These questions raised by the response to injury hypothesis have led us to examine how specific agents affect endothelial integrity.

Endothelial Integrity

The endothelium serves a number of roles. Endothelial cells actively transfer metabolic substances of varied molecular size between the circulating blood and the surrounding tissues.[10, 31, 32] They form a permeability barrier whose functional state controls the passage of both small and large molecules from the plasma into the artery wall.[33] The cells are polarized, both morphologically and functionally. Their luminal surface forms a nonreactive interface between blood elements, such as platelets and the artery wall. They synthesize and secrete connective tissue proteins, including specific types of collagen (Type III[34] and Type IV[35]) beneath their abluminal surface, whereas their luminal surface exhibits glycoproteins (such as Factor VIII, von Willebrand factor[36]) and proteoglycans. Endothelium is thromboresistant through several mechanisms, including a heparin-like surface proteoglycan, synthesis of prostacyclin (PGI₂),[37] secretion of plasminogen activator, and uptake and clearance of thrombin and vasoactive amines (figure 2).[33]

Endothelial cells as well as smooth muscle cells synthesize relatively large amounts of PGI₂.[37] This particular prostaglandin derivative is a potent vasodilator and antiaggregatory substance for platelets.[38] Although the normal levels of PGI₂ synthesized by arterial endothelium in vivo are not well established, it has been reported that endothelium and smooth muscle produce in-

Endothelial Cell Function
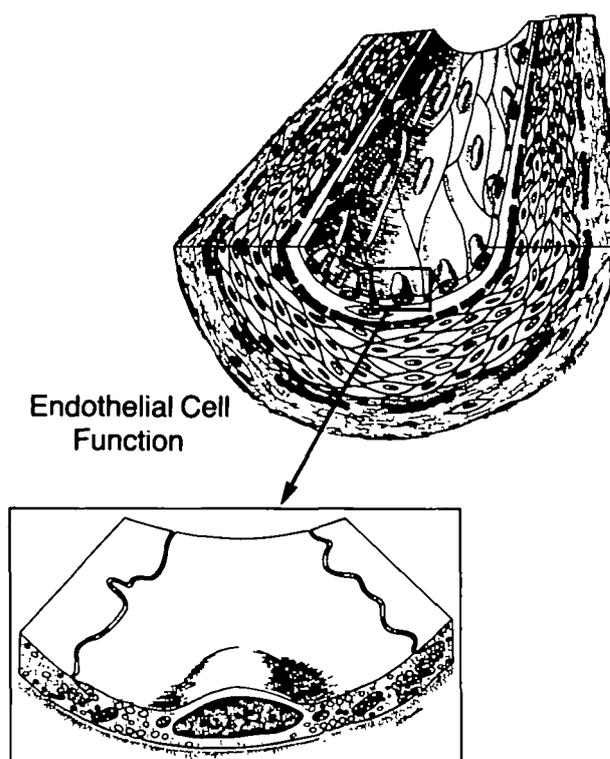
1. Permeability barrier
2. Non-thrombogenic properties
   a) Cell surface
   b) PGI₂
3. Metabolism of vasoactive substances
4. Production of growth factors
5. Connective tissue formation

Figure 2. Diagram of the endothelial cell below which are listed the important functions served by arterial endothelium, ranging from a permeability barrier to the production of growth factors and formation of specific connective tissues unique to endothelium.

creased amounts of PGI₂ in response to injurious stimuli such as thrombin, suggesting that this may represent a protective mechanism preventing further platelet interactions. The importance of PGI₂ as a physiological antiaggregant in vivo remains to be determined. What other mechanisms do endothelial cells possess to protect themselves from injury, to initiate repair and regeneration, or to deal with substances associated with atherogenesis, such as lipoproteins? The special characteristics of the endothelium: growth in a continuous monolayer; capacity to form PGI₂; association with lipoprotein lipase; formation of a factor that can be mitogenic for

smooth muscle cells; ability to form a permeability barrier — all appear to play roles in one way or another in the maintenance of normal endothelial integrity.

Until recently, it has been difficult to accurately characterize the morphology of the normal endothelial cell in vivo. New approaches to fixation of the arterial tissues involve perfusion of the vessel under appropriate pressure with aldehyde fixatives and make critical analysis of endothelial structure possible. Studies have been performed that provide a convincing picture of normal endothelial architecture as seen by scanning and transmission electron microscopy. These studies establish a basis (figure 3) for investigation of the types of changes observed in different situations associated with atherogenesis, such as chronic hypercholesterolemia or immune injury. Endothelial alterations resulting from virus-induced injury,[30] toxins,[24] mechanical injury,[11-17] or chemical injury[6, 22, 23] can now be critically examined at early times, particularly at short intervals after exposure to the insult.

When endothelium is injured by mechanical or immunologic methods, platelets adhere, aggregate, and release their contents. Such platelet interactions are followed within 3 to 5 days by migration of smooth muscle cells from the media into the intima of the artery, followed by proliferation of these cells.[13] Focal endothelial denudation may be produced by cytolytic or detaching processes associated with such disorders as autoimmune injury, homocysteinemia, and endotoxemia. Desquamating injury can be detected directly by the morphology of properly prepared tissues, presence of circulating endothelial cells, and the demonstration of increased endothelial cell turnover. Indirect in vivo indicators of endothelial denudation include the detection of platelet activation (using measurements of platelet survival time and measurements of platelet-specific proteins in plasma) or activation of the coagulation cascade (measuring fibrinopeptide A or protein C). Reliable quantitative measurements of altered endothelial function still need to be developed.
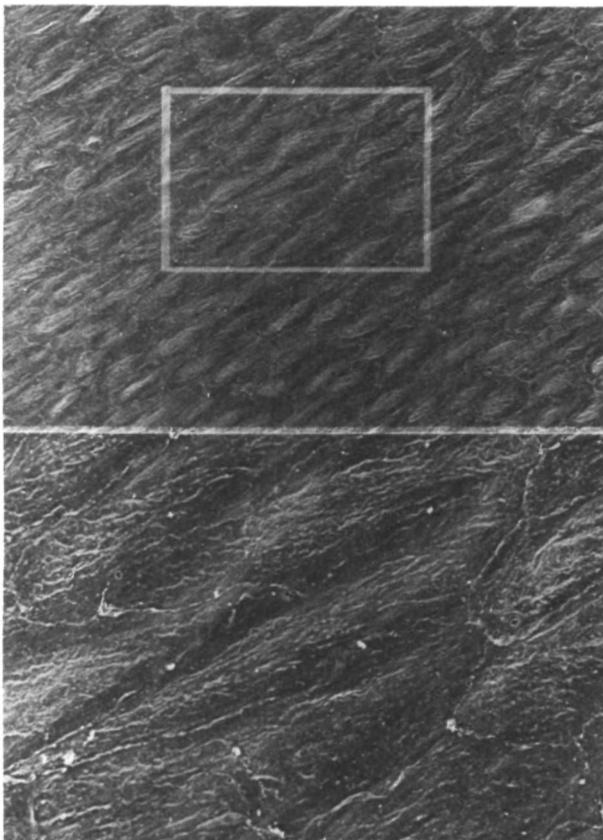

Figure 3.   Scanning electron micrographs of thoracic aorta endothelium from normal monkey (*Macacca nemestrina*). × 540. At somewhat higher magnification, the overlapping folds of endothelial cells can be clearly visualized. The elongated, and elliptical appearance of the endothelium can also be seen. The long axes of the cells appear to be diagonal and are oriented in the main direction of the flow of blood in the artery. × 2100
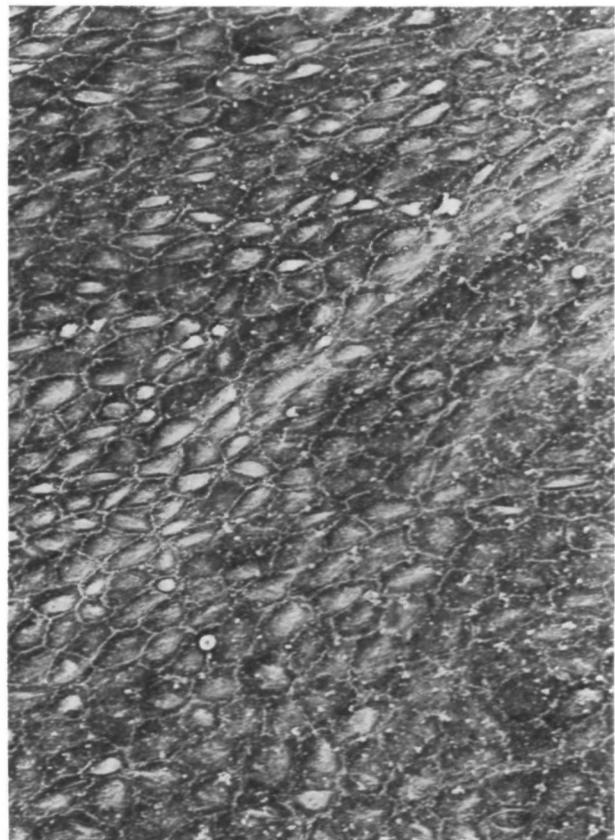

Figure 4.   Scanning electron micrograph demonstrating alterations in the shape and direction of endothelial cells in a hypercholesterolemic monkey (*Macacca nemestrina*). These changes in shape and direction are some of the first signs of alteration seen in a hypercholesterolemic monkey. × 700

In a recent series of studies involving non-human primates fed a high fat, high cholesterol diet for more than 2 years, the arterial tree was perfusion-fixed using normal systolic pressure and buffered glutaraldehyde for 1 hour prior to removing the vessels. The arteries were prepared for scanning and transmission electron microscopy, as well as light microscopy. Numerous regions of altered endothelial cell shape were observed as shown in figure 4. At some sites endothelial injury resulted in loss of cells and exposure of the subendothelial connective tissue, particularly at regions overlying intimal lesion formation (figures 5 and 6). In many of these regions, platelets were found adherent and spread over such sites to form a monolayer of platelets ("pseudoendothelium") (figures 6 and 7). All of the hypercholesterolemic monkeys in

Figure 5. Scanning electron micrograph from a 2-year hypercholesterolemic pigtail monkey showing an area of endothelial desquamation. This animal was carefully perfuse-fixed under normal arterial pressure. The area of desquamation is not an artifact, since the area of exposed subendothelium is covered by at least two layers of platelets which have spread upon the exposed connective tissue. These platelets are seen in higher magnification in figure 6. × 700

this study had significantly shortened platelet survival throughout most of the 2 years.[6] Such chronic increases in platelet utilization suggest that the platelets might play a role not only in the process of lesion initiation, but possibly in lesion progression by delivering the mitogen contained in the platelet alpha granules to the subendothelium at sites of endothelial injury. The platelet-derived growth factor can induce cells such as smooth muscle, but not endothelium, to proliferate in culture. This is discussed further below.

How might chronic hypercholesterolemia "injure" or modify the endothelium and its function? A number of mechanisms are possible. For example, one of the simplest means by which hypercholesterolemia may affect endothelial integrity is through cholesterol exchange from lipoproteins, such as low density lipoprotein (LDL), with the plasma membranes and other membrane compartments of the cells. Such exchange would be rapid and could, hypothetically, alter a number of cell functions including cell-cell and cell-connective tissue attachment.[39] Endothelial cells, like smooth muscle and monocytes, contain specific high affinity receptors for LDL on their suface.[40-43] We need to know the answers to the following questions:

- Are the LDL receptors in endothelium controlled similarly to those in these other cell types?
- What is the role of the chylomicron receptor as compared with the LDL receptor in the endothelium?
- What is the role of the acetyl-LDL receptor in endothelium?
- How does the endothelium utilize these mechanisms to maintain normal lipid metabolism?
- What factors control turnover and normal function of lipoprotein receptors in endothelium?
- When endothelial cells are altered, is their ability to maintain a monolayer in the presence of shear forces affected by the anatomical location of the cells?

Endothelial functions such as attachment can be measured in cell culture as well as in vivo; until recently, however, it has not been possible to observe the effects of shear combined with factors such as hypercholesterolemia in vitro. New approaches (such as instruments that will induce shear forces on cultured endothelial cells recently used by Gimbrone) may permit examination of the interactions between shear stress in vitro, similar to that observed in vivo, combined with traditional risk factors associated with atherosclerosis, such as hypercholesterolemia. Other possible approaches to evaluation of
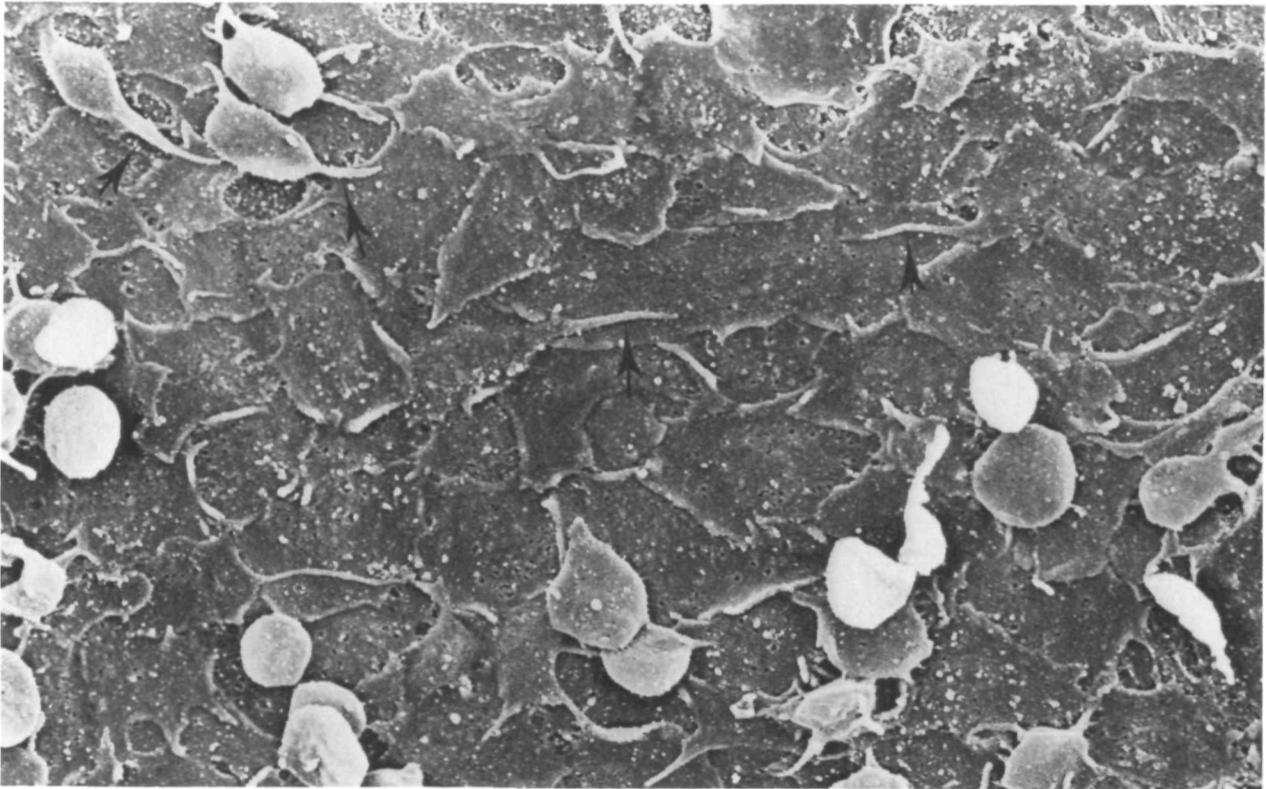
Figure 6. Scanning electron micrograph in which the first layer of platelets most adjacent to the subendothelium can be recognized by the small projections (**arrows**) from a number of the platelets that are identical to those seen in the uppermost layer of platelets that have attached, but have not spread. The first layer of platelets appears to form a type of "pseudoendothelial coverage" upon the exposed connective tissue. In accompanying transmission electron micrographs, such platelets can be seen to have degranulated. X 7000

altered endothelial function might include quantitative assessment of altered morphology, altered synthetic products (PGI$_2$, growth factor, angiotensin-converting enzyme, factor VIII/vWF, plasminogen activation, collagen, proteoglycans, etc.), altered permeability or transport, and altered metabolic pathways. These and other investigations should provide information that will help us to understand the regulatory mechanisms associated with normal endothelial function.

Growth Control of Endothelium and Endothelial Senescence

One aspect of endothelial cell function that may be helpful in understanding the response of these cells to injury is their characteristic growth pattern, which is strikingly different from that of most other cells, including smooth muscle. Endothelial cells grow in a unique monolayer in vivo, as well as in cell culture.[8] When the monolayer is
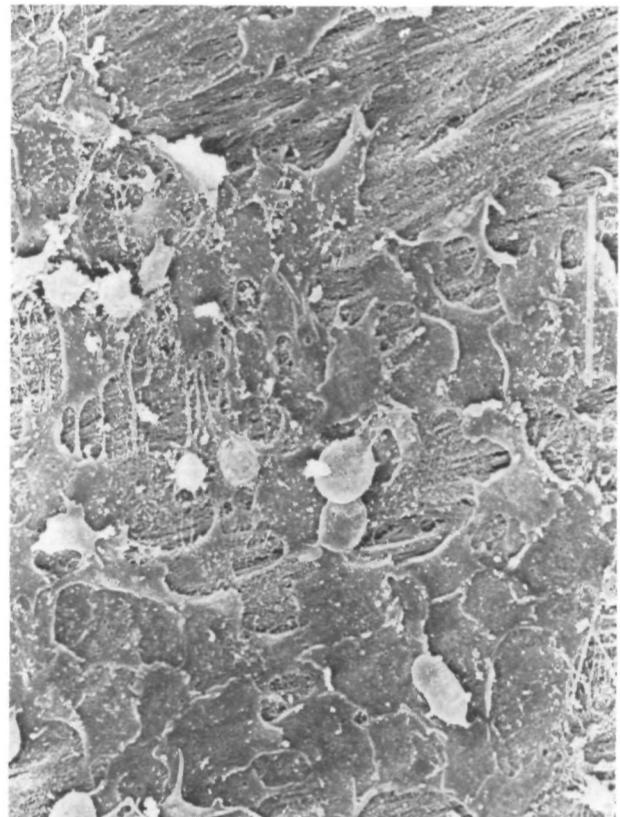

Figure 7. Scanning electron micrograph showing areas of platelet adherance and spreading adjacent to areas of exposed connective tissue lacking any platelet coverage. X 4300

injured by making a wound in the culture, the cells at the wound margin first try to migrate and close the wound while remaining attached to their neighbors.[44] If the wound is too large, they respond by multiplying.[45] Those cells, principally capable of synthesizing DNA and proliferating, are located within a relatively short distance from the wound edge. Most of the cells at greater distances from the wound generally do not synthesize DNA, and do not participate in the proliferative response.[46] These observations raise several questions:[33,46-49]

• Is it possible that, because of cell-cell contact, DNA synthesis is inhibited in endothelium and thus is under a different set of controls than smooth muscle?

• Is it possible to determine at sites of endothelial injury in vivo whether only those cells close to the wound are capable of participating in the proliferative response?

• If the site of injury remains anatomically constant over a period of years, do endothelial cells that participate in the repair of the injury undergo a state of senescence similar to that described for these and other cells in culture?

When the endothelial cells proceed through the limited number of doublings of which they appear to be inherently capable, it is conceivable that such cells could no longer regenerate at sites of continuing injury, whereas cells distal to these sites might retain their potential to proliferate. Senescent endothelial cells at the margin of an injury site would then be unable to aid in the repair process, however, because they remain attached to their neighbors and cannot migrate into the site of injury to provide a continuing source of proliferation. Thus, senescence of endothelial cells might become a potentially important factor over periods of years of chronic injury and repair. This question can be tested in culture and potentially in vivo as well.

It will also be important to understand the differences in the various forms of injury inflicted upon the endothelium by different atherogenic agents, and to determine the capacity of the endothelial cells to respond to each form of insult, both structurally and functionally.

Cell Proliferation — The Role of Growth Factors In Cell Culture and In Vivo

Cell proliferation, particularly by smooth muscle cells, is perhaps the principal cellular response associated with progression of atherosclerosis. There have been many studies of growth of cells in culture in general, and lately specifically of smooth muscle and endothelium. In the process of these studies, numerous growth factors have been discovered that stimulate different cell types to synthesize DNA, initiate cell cycle traverse, and undergo mitosis. These include epidermal growth factor (EGF),[50] fibroblast growth factor (FGF),[51] the family of somatomedins and insulin-like growth factors (IGF),[52] sarcoma growth factor (SGF)[53] platelet-derived growth factor (PDGF),[54-59] endothelial-derived growth factor (EDGF),[60,61] and the monocyte/macrophage-derived growth factor (MDGF).[62-64] The growth factor that has been most completely studied is epidermal growth factor EGF.[50] The role of EGF in embryonic growth and normal development is poorly understood and much research remains to be performed to clarify its role. Since the discovery, isolation, purification, and characterization of EGF, the approaches used in the study of this growth factor have served as an example to study many of the others.[65-67]

Of particular interest in understanding the cells of the artery wall, and the components that may be important in the induction of their proliferation, are the growth factors released by endothelium, platelets, and the monocyte/macrophage.

EDGF is released into culture medium by both proliferating and stationary phase arterial endothelial cells derived from many different species.[60,61] It can be formed in the presence of serum-free culture medium by bovine endothelium and is, therefore, attractive as a candidate to play a role in stimulating smooth muscle proliferation in vivo since it is a potent mitogen for these cells in culture. There is, as yet, no evidence to determine if this factor is formed in vivo. It appears to have a number of differences from the mitogen derived from the platelet, including antigenic characteristics that distinguish it from PDGF (DiCorleto, personal communication). It is tempting to speculate a role for EDGF in embryogenesis and development. Whether it is important in atherogenesis remains to be determined.

Platelets contain a potent mitogen, the PDGF;[54-59] this hormone has been purified to homogeneity by others[59,68] and recently in our laboratory as well. It is present in the alpha granules of the platelets[69-72] and appears to be the principal mitogen present in whole blood serum that is capable of inducing many cells to proliferate in culture, including arterial smooth muscle, and fibroblasts.[54,55,73-75] The PDGF will not induce DNA synthesis by arterial endothelial cells.[76] It is a cationic protein (pI 9.8), is highly stable, and has multiple effects upon susceptible cells like smooth muscle. Exposure to 6 ng/ml of purified PDGF is equivalent to providing 5% whole blood serum to smooth muscle for initiation of DNA synthesis that begins 12–16 hours after binding of the mitogen to a specific high affinity cell surface receptor[77] (Bowen-Pope and

Ross, and Glenn et al., unpublished data). The number of specific receptors on cells such as WI-38 (human fibroblasts) and human smooth muscle ranges from 300,000/cell to 50,000/cell respectively. The potential role of this mitogen in the pathogenesis of the lesions of atherosclerosis is discussed below.

A third growth factor, possibly of equal interest with PDGF in atherosclerosis, was found to be derived from the peritoneal macrophage.[62,63] A presumptively similar factor has been found to be derived from the circulating monocyte, but is only released from monocytes after they have been placed in culture and appropriately stimulated.[64] This monocyte/macrophage-derived growth factor (MDGF) stimulates not only smooth muscle cells and fibroblasts to proliferate, but arterial endothelial cells as well.[63] Since monocytes represent one of the two principal cells present in both the early and late lesions of atherosclerosis[78–81,94,95] (see figure 10), the purification, characterization, and investigation of the role of MDGF will be important in understanding its participation in atherogenesis.

Each of these three factors may be capable of stimulating smooth muscle cell LDL receptor activity (see figure 13). Their discovery, coupled with the observation that platelets, endothelial cells, and the monocyte/macrophage participate in various phases of atherogenesis, suggest that one or any combination of these may be involved in different phases of lesion formation, progression or regression. Their discovery has provided an opportunity to delve further into the roles of each of these three cells, not only in the maintenance of normal arterial wall homeostasis, but in the pathogenesis of the lesions of atherosclerosis as well. There are as yet no data available to establish whether any of these growth factors is released in vivo. Consequently, we do not know if they are active at sites of endothelial injury and smooth muscle proliferation in experimentally induced atherosclerosis, or in the disease as it occurs in man. Pure PDGF is now available in our laboratory together with an antiserum to PDGF. The purification of other factors, together with the development of antibodies to each factor, should provide tools to ask questions concerning their roles in arterial wall biology. Ultimately, such experiments should provide information concerning the role of each factor in the pathogenesis of atherosclerosis in man.

Role of the Platelet

The intact platelet contains a large number of biologically active compounds (figure 8). After endothelial cells have been injured, their capacity to form a thromboresistant barrier is clearly altered, since platelets adhere at such sites,
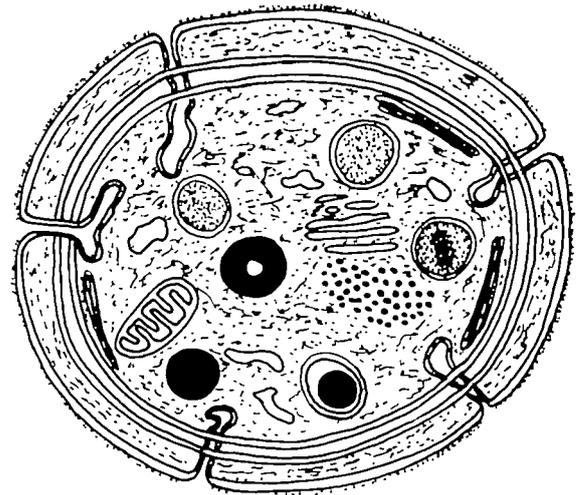
ADP, ATP, serotonin	Phagocytosis
Thromboxane A_2 synthesis	No protein synthesis
β thromboglobulin	Fibrinogen
Platelet factor IV	Glycosidases
Platelet derived growth factor	Proteinases
Ca^{++}	Cationic proteins

Figure 8. Diagram of a platelet demonstrates the principal constituents of the platelet which are listed beneath the figure.

aggregate, and undergo the release action (figures 1,5,6).[13–17,82] In experimentally induced atherosclerosis, if the endothelium has been injured mechanically by using techniques such as intraarterial balloon catheterization, a number of events follow the adherence and degranulation of platelets at the sites of injury. Of particular interest is the observation that medial smooth muscle cells migrate from the media into the intima through fenestrae of the elastic lamina within the first few days after injury.[13] Subsequent to this migration, these intimal smooth muscle cells proliferate and form a sizable intimal fibromusculoelastic hyperlastic lesion.[13,15,16] Such an intimal proliferative response can be prevented if platelet function is inhibited, either by removing platelets from the circulation using an antiplatelet serum,[83,84] by preventing platelet adherence and release with appropriate pharmacologic agents,[23] or by studying animals genetically defective in Factor VIII antigen, a protein required for platelet adherence.[85,86]

The PDGF induces proliferation of arterial smooth muscle cells, fibroblasts, glial cells, and a number of other cell types.[55,57,75] As noted above, this factor has no effect upon endothelium, epithelium, lymphocytes, or some transformed cells. This correlates with the observation made by Heldin et al.[77] and recently in our laboratory that endothelial cells have no receptors for PDGF.

PDGF has multiple effects upon susceptible cells in culture, such as smooth muscle. It will increase their rates of pinocytosis,[76,87,88] cholesterol synthesis,[89,90] binding of low density lipoproteins to specific high affinity cell surface receptors,[90,91] and will increase phospholipid metabolism, leading to the formation of increased amounts of arachidonic acid (Habenicht et al., unpublished data). The latter observation may be related to increased prostaglandin synthesis, such as PGI_2, by stimulated smooth muscle cells. PDGF also stimulates protein synthesis, including collagen and proteoglycan synthesis, by smooth muscle cells.[92] Since all of these events may be important in atherogenesis, it will be necessary to determine whether they occur in vivo during lesion formation, as has been observed in culture.

An observation that may be particularly relevant to understanding the process of atherogenesis was recently made by Grotendorst et al.[93] They examined the ability of a number of purified growth factors and other substances to stimulate chemotactic movement of smooth muscle cells. Among the growth factors that have been isolated and purified, they tested EGF, FGF, PDGF, and insulin. Of these, only PDGF was chemotactic for fibroblasts and for arterial smooth muscle cells. The observations were made in a Boyden chamber system in which both a chemokinetic and a chemotactic effect can be measured after exposure of the different cells to each of the growth factors. These observations, coupled with those in which platelet adherence and release at sites of endothelial injury in vivo leads to migration of smooth muscle cells into the intima,[5,13] suggest that platelet interactions at such sites of injury may be an important early stimulus that establishes conditions for entry of smooth muscle into the intima of the artery and for the subsequent development of the proliferative lesions of atherosclerosis.

A critical question concerning the role of platelets in the response to injury hypothesis of atherosclerosis requires the determination of whether the PDGF is present and active at sites where the endothelium has been either detached or only functionally injured. Additional questions of importance include:

• Does platelet activation induce release of PDGF into the plasma?

• Are there increased levels of PDGF in the circulation in animals in which experimental atherosclerosis has been induced?

• Can PDGF be found, using immunologic techniques, (eg., antibody-colloidal gold, etc.) in the intima after experimental injury, induced either mechanically or by experimental hypercholesterolemia?

With the availability of antibody to PDGF and isotopically labeled pure PDGF, it should be possible to explore these questions. Techniques are now available to explore the localization of many antigens in tissue sections. Such approaches should provide answers to these important problems.

Role of the Monocyte/Macrophage

A third cell type is now also recognized as an important component of the proliferative aspects of atherogenesis, as well as in foam cell formation, the blood monocyte. This cell plays a particularly important role as a phagocytic cell when it enters the tissues, is important in several aspects of the immune response, and carries many biologically important constituents when in the circulation, or is able to synthesize them after appropriate stimulation (figure 9). Once
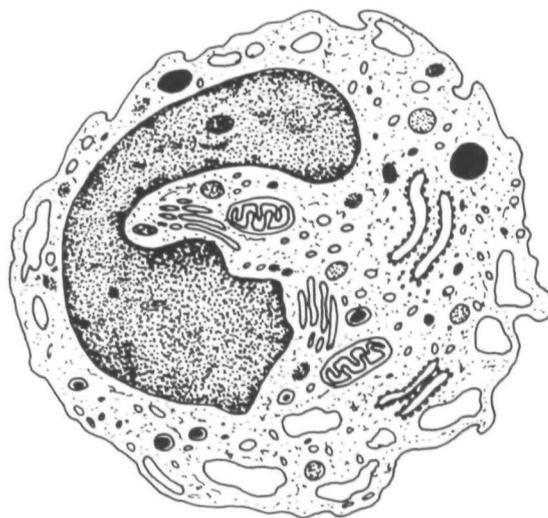
Neutral proteases
Acid hydrolases
Complement
Enzyme inhibitors
Reactive metabolites of O_2
Bioactive lipids
Chemotactic factors
Growth factors
Factors inhibitory to replication of lymphocytes, viruses, and tumors cells
Modified LDL receptors
LDL receptors

Figure 9. Diagram of a monocyte/macrophage cell containing a large number of substances, many of which are not present in the circulating monocyte but are formed after the monocyte leaves the blood and enters a specific tissue. A few of the components that can be found in macrophages are listed beneath the diagram.

monocytes have been appropriately stimulated in vitro, they form increasing amounts of macrophage-derived growth factor (MDGF) over a period of 5 days.[64] Production of MDGF in culture is dependent upon specific stimulation and upon a number of culture conditions, including monocyte density.

In vivo, monocytes appear to enter the atherosclerotic lesions early[94,95] and to be an important source of some of the foam cells in many of the lesions.[96-98] In this regard, the PDGF may be chemotactic, not only for smooth muscle cells, but for monocytes as well. This may explain, in part at least, their attraction at sites of endothelial injury and platelet release. Several

investigators have reported that monocytes have been observed in the process of entering the artery wall at sites of altered endothelial permeability in hypercholesterolemic animals.[94] This apparently takes place at junctional sites between endothelial cells, or possibly where endothelial injury has occurred, and the cells may be missing.

Studies of human atherosclerosis, and recent examination by light and electron microscopy of lesions obtained during surgery, demonstrate identifiable smooth muscle cells and monocyte/macrophages in human fibrous plaques (figure 10). Both the macrophages and the smooth muscle cells can become foam cells.
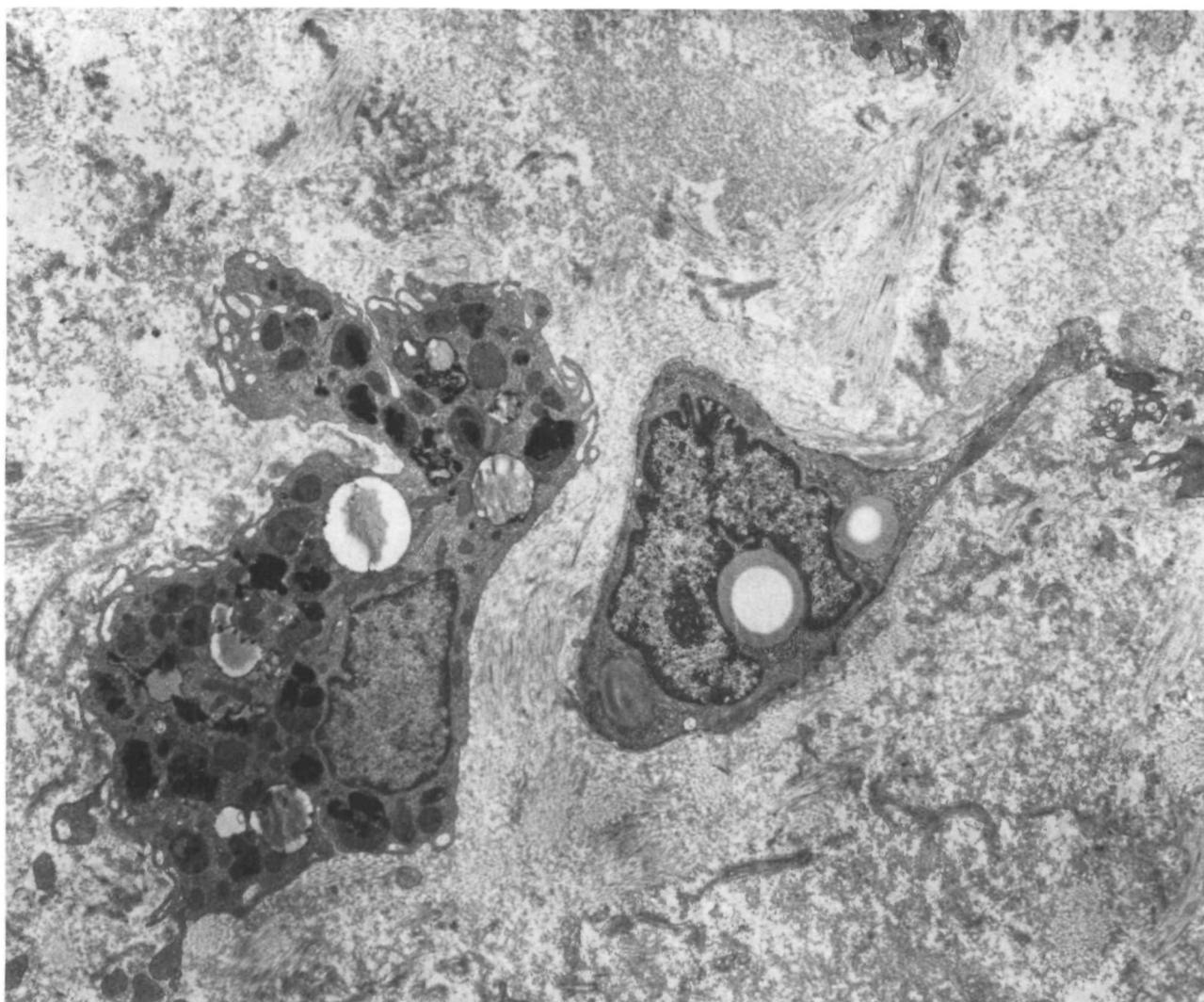

Figure 10. Electron micrograph demonstrating two cells from a human lesion of atherosclerosis, obtained and fixed at the time of surgery. The cell on the left is a macrophage that can be clearly recognized by its fimbriated cytoplasm, as well as its nuclear morphology. The cell contains numerous membrane-bounded secondary lysosomes, as well as nonmembrane-bounded lipid droplets. The cell on the right is a smooth muscle cell, as can be determined by the basement membrane surrounding most of the cell, its nuclear morphology, and its cytoplasmic content of myofilaments. This cell also contains several lipid droplets. The dense connective tissue matrix found in this lesion is characteristic of the fibrous cap overlying the fibrous plaque that represented this particular atherosclerotic lesion. × 8000

When these lesions are appropriately fixed and prepared for electron microscopy, the identification of most macrophages is straightforward and reliable. The macrophage and the smooth muscle cell seen in figure 10 are each readily identifiable by their ultrastructural characteristics. The lipid droplets in each cell have a different appearance and may reflect differences in lipid composition. In many instances however, it is impossible to determine the origin of the foam cell due to alterations in cellular morphology. Monoclonal antibodies are now available in our laboratory against monocytes and smooth muscle cells. Such reagents should permit a quantitative assessment of the proportions of these two cells in foam cell formation in atherosclerosis.

The macrophage plays important roles in inflammation and immunity, including its capacity to phagocytose material, remove effete cells and process antigens. This cell also secretes a potent growth factor that stimulates fibroblasts, smooth muscle, and possibly endothelium to multiply in culture.[62-64] All macrophages found in inflammatory sites, as well as in the lesions of atherosclerosis, are derived from peripheral blood monocytes. Many monocytes accumulate lipid in the lesions and represent part of the population of foam cells.

Human peripheral blood monocytes obtained in pure preparations can be specifically induced in culture to release a potent growth factor (MDGF) for smooth muscle cells. This factor does not appear to be present in an active form in the cells while they are in the circulation.[64] It is not yet clear whether this lack of activity in circulating monocytes is due to presence of inactive precursor or total absence of factor. The latter is believed to be the explanation, since at least 4 hours is required to activate the monocyte after placing it in culture.[64]

Since macrophages produce a growth factor in addition to acting as a phagocytic cell, it is important to determine whether the MDGF is actually formed during atherogenesis. It will also be important to determine:

• What other effects does the growth factor have upon susceptible cells?

• Does MDGF possess chemotactic properties for smooth muscle cells as does PDGF?

• Do macrophages play a role in lesion progression or regression, or possibly even in lesion initiation?

• Is foam cell formation related to stimulation of growth factor production by monocytes after they enter the artery wall?

The purification and characterization of the MDGF and its comparison with the other factors that may participate in atherogenesis will help to probe the role of this cell in atherosclerosis and in inflammation.

Smooth Muscle Cell

The extent of intimal proliferation of smooth muscle cells is one of the critical events that determine whether the lesions of atherosclerosis will lead to the development of clinical sequelae (figure 1). Numerous investigators have studied smooth muscle cells and their capacity to respond to different stimuli in culture and in vivo. For example, Goldberg, et al.[99] developed a method for quantifying smooth muscle cell proliferation in vivo following endothelial injury with an intraarterial balloon catheter. This approach may be useful in evaluating the role of agents such as PDGF and other mitogens in studying smooth muscle cell proliferation in vivo. These same investigators[100] have also shown that a factor derived from the same platelet granule as PDGF, platelet factor 4, can permeate the vessel wall following endothelial injury.

Although a great deal has been learned about the regulation of smooth muscle function by studying these cells in culture (figure 11), recent observations suggest that some of this work needs to be reassessed. Many observations made on smooth muscle cells in culture are dependent upon how the cells were initially obtained for culture and how they were subsequently maintained.[101-103] When smooth muscle cells are derived by what has been termed "primary culture," they manifest markedly different characteristics from smooth muscle cells obtained by classical explant culture techniques. In "primary culture" the cells from the media of a
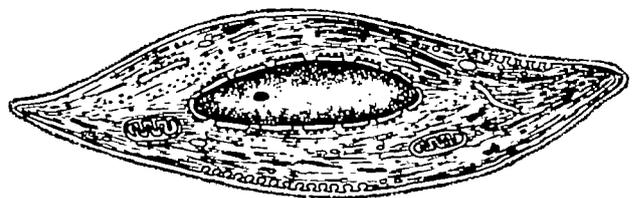
Contractility
Connective tissue synthesis
Lipid metabolism
    LDL binding, internalization, degradation via high affinity receptors
    Cholesterol synthesis
    Phospholipid metabolism → prostaglandin synthesis
Proliferation
    Dependent on state of modulation, contractile vs synthetic
    Induced by:
        PDGF, MDGF, ECDGF, LDL?, PGI_2, Ca^{++} flux

Figure 11. Diagram of a smooth muscle cell, showing many of the phenotypic characteristics of normal smooth muscle, as well as factors that can induce smooth muscle proliferation.

segment of artery are obtained for culture by digestion with collagenase and elastase.[101,102] If the segments of the media of the artery are obtained by explant techniques, the cells require approximately one week to emigrate from the explants and to proliferate in response to serum (or PDGF). Such explant procedures do not sample most of the cells in the tissue because the technique selects for those cells capable of migrating out of the explant and proliferating.

In contrast, the method of primary culture samples a larger percentage of the cells removed by enzyme digestion from the artery wall capable of surviving the enzymatic digestion and of attaching to the culture dish. During the first 5 days in culture, the majority of such cells contract in response to norepinephrine or angiotensin II. If these contractile cells are plated in sparse culture, they lose their capacity to contract over a period of 7 to 9 days, in parallel with the loss of most of their cytoplasmic myosin.[101] Moreover, smooth muscle cells that manifest myosin and contractility do not respond to mitogens such as the PDGF. Conversely, when the cells have lost their myosin and their capacity to contract, they proliferate in response

to PDGF. As the cells lose their contractile capacity and respond to mitogens, they begin to synthesize connective tissue proteins. This change from a contractile to a synthetic state has been termed "modulation" (figure 12).[101-103] It is possible to prevent the modulation from a contractile state by maintaining the smooth muscle cells at high density in culture[103] or by giving the cells cyclic AMP; in contrast, placing the cells in sparse culture promotes the process of modulation (figure 12).

These observations suggest that interpretations of some studies using cultured smooth muscle cells obtained by explant may need to be reevaluated, since these cells may have been in a modulated, synthetic state. If most of the cells in the media of an artery are in a contractile state, refractory to PDGF, a delayed proliferative response following endothelial injury could be explained by their initial unmodulated state.

It is tempting to suggest that the chemotactic properties of the PDGF attracted individual smooth muscle cells to migrate from their density-inhibited, contractile state in the media of the artery, into the intima, where they could then modulate with time to a synthetic state,

CONTRACTILE STATE

Maintained by
↑Density
Endothelial derived
inhibitors

Refractile to PDGF
Responsive to angiotensin
No connective tissue synthesis

Modulation

Stimulated by
↓Density

Responsive to PDGF
Noncontractile
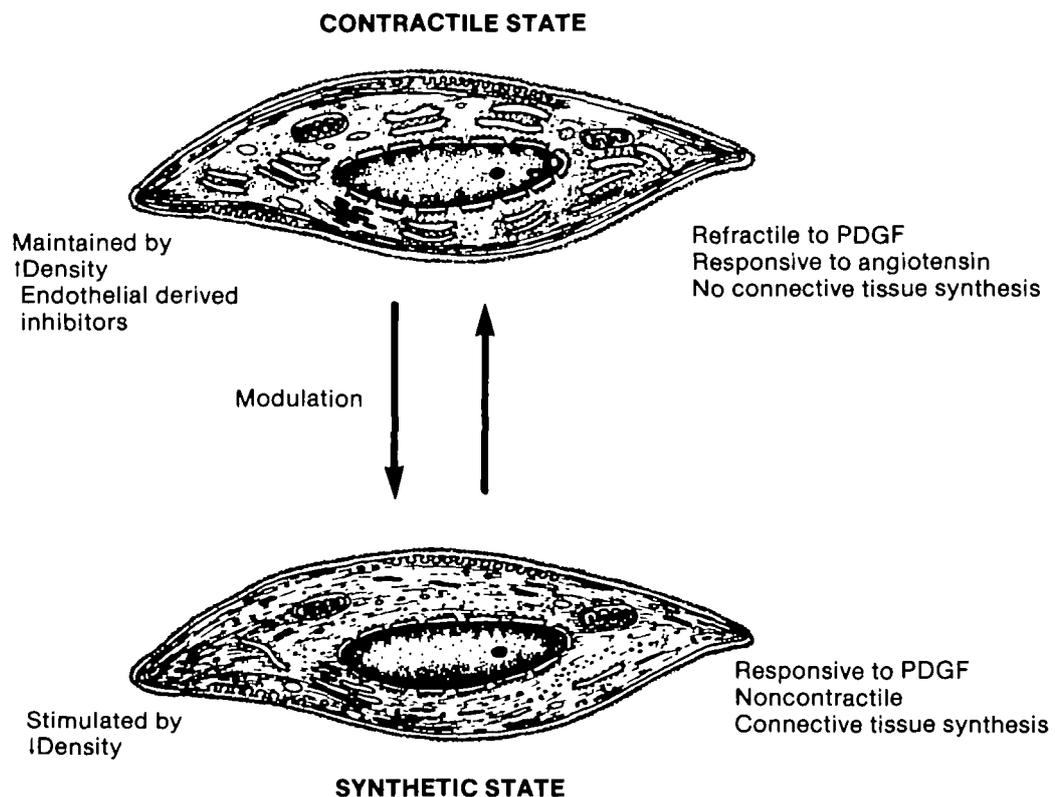Connective tissue synthesis

SYNTHETIC STATE

Figure 12. Diagram illustrating the state of modulation of smooth muscle cells in which the contractile vs the synthetic state are maintained by different sets of factors.

respond to the mitogen, similar to cells observed after seven days in sparse culture. If this were the case, the physiological state of the smooth muscle cells in vivo may be critical to understanding this response. It is important to answer the following questions:

- Is it possible that these two states, contractile vs synthetic, represent quiescent vs cycling cells in general? In other words, do other cell types characteristically manifest different functional characteristics in different parts of the cell cycle?
- Will a comparison of contractile and synthetic state cells show differences in terms of their capacity to bind mitogens, such as PDGF, to metabolize lipids or lipoproteins, or to synthesize prostaglandins?
- Do these two different states of smooth muscle actually exist in vivo, or is this simply an artifact of cell culture?
- What is the state of modulation of cells obtained from human lesions vs those obtained from the underlying media?

These observations provide a hypothetical explanation for the delay observed in smooth muscle proliferation following endothelial injury in vivo, and emphasize the chemotactic role of PDGF in inducing the cells to leave their density-inhibited, mitogen-refractory state in the media of the artery. Much work remains to be done to clarify the importance of these phenomena in relation to our ability to understand the smooth muscle cell and its role in atherosclerosis.

Role of Lipoproteins

Low density lipoproteins (LDL), the principal class of lipoproteins associated with atherogenesis, appear to play several roles in this disease process. LDL may cause functional endothelial injury, as evidenced by both morphologic and physiologic alterations in the endothelium[4-6] Furthermore, Fless et al.[104] and Fischer-Dzoga et al.,[105] colleagues of Wissler, have reported that a special category of LDL obtained from hyperlipemic monkeys is uniquely atherogenic.[104,105] This lipoprotein, entitled LDL_1, is also obtained from normal monkey plasma,[106] and appears to be mitogenic for smooth muscle cells. The potential importance of this lipoprotein or equivalent lipoproteins, if they exist in man, and their relationship to other mitogens remains to be clarified.

Another role for LDL has been suggested by the observations of Chait et al.[90] They demonstrated that exposure to mitogens such as PDGF induces smooth muscle cells to increase their binding and degradation of LDL, as well as to increase uptake of LDL via bulk phase endocytosis. Chait et al. (unpublished observations) ob-

served that PDGF enhancement of LDL uptake and degradation occurs even in the presence of a large excess of the lipoprotein in the culture medium. Dependent upon the relative capacities of the cells to degrade the cholesteryl ester that forms as a result of increased uptake of LDL, such stimulation could result in the accumulation of cholesterol and cholesteryl esters in the smooth muscle cells, and thus in the formation of foam cells.[107]

It is not yet clear why low density lipoproteins are atherogenic. It will be important to determine:

- Does LDL act directly on both endothelium and smooth muscle, beyond their capacity to bind to cell surface receptors and modify cholesterol synthesis?
- Do other lipoproteins such as VLDL, chylomicron remnants, and HDL interfere with normal healing processes?
- Is internalization of the lipoproteins necessary for them to exert their effect in vivo?
- What part of the lipoprotein moiety is responsible for their effects?
- How does the interaction of lipoproteins with cells affect the uptake of ions such as calcium?
- How do lipoproteins modify the capacity of the cells to metabolize phospholipids from arachidonic acid, and, thus, form prostaglandins?

Much remains to be learned concerning the role of these particles and the apparent inverse relationship of HDL in its so-called "protective" capacity in atherogenesis.

It is of interest that macrophages bind both normal and altered LDL by separate high affinity receptors.[108,110] Uptake of altered LDL (malonated, for example, after exposure to platelets[109]) and normal LDL may occur at lesion sites. Such reactions could underlie the formation of foam cells by macrophages,[110] as well as by smooth muscle, thereby explaining the two types of foam cells seen in human atherosclerosis. They also may provide information concerning the effects of monocytes on atherogenesis.[111]

Regression

Although I have not attempted to discuss the issue of regression in this lecture, there are many important questions concerning lesion regression that require further research:

- Does regression occur in man with symptomatic vascular disease?
- If so, under what circumstances?
- Can this be accurately and reliably measured in patients?

- What is the cellular basis for regression of atherosclerosis?
- Regression of atherosclerosis may have some features in common with the final phases of wound repair in which the wound decreases in cellularity and the connective tissue is remodeled to make a thinner scar. The factors that cause cells to either necrose or migrate from an injury

site are not known. Are these two phenomena similar?
- What determines whether regression is possible? If so, to what extent?

The biology of this phenomenon is little known and represents an important area for further study.
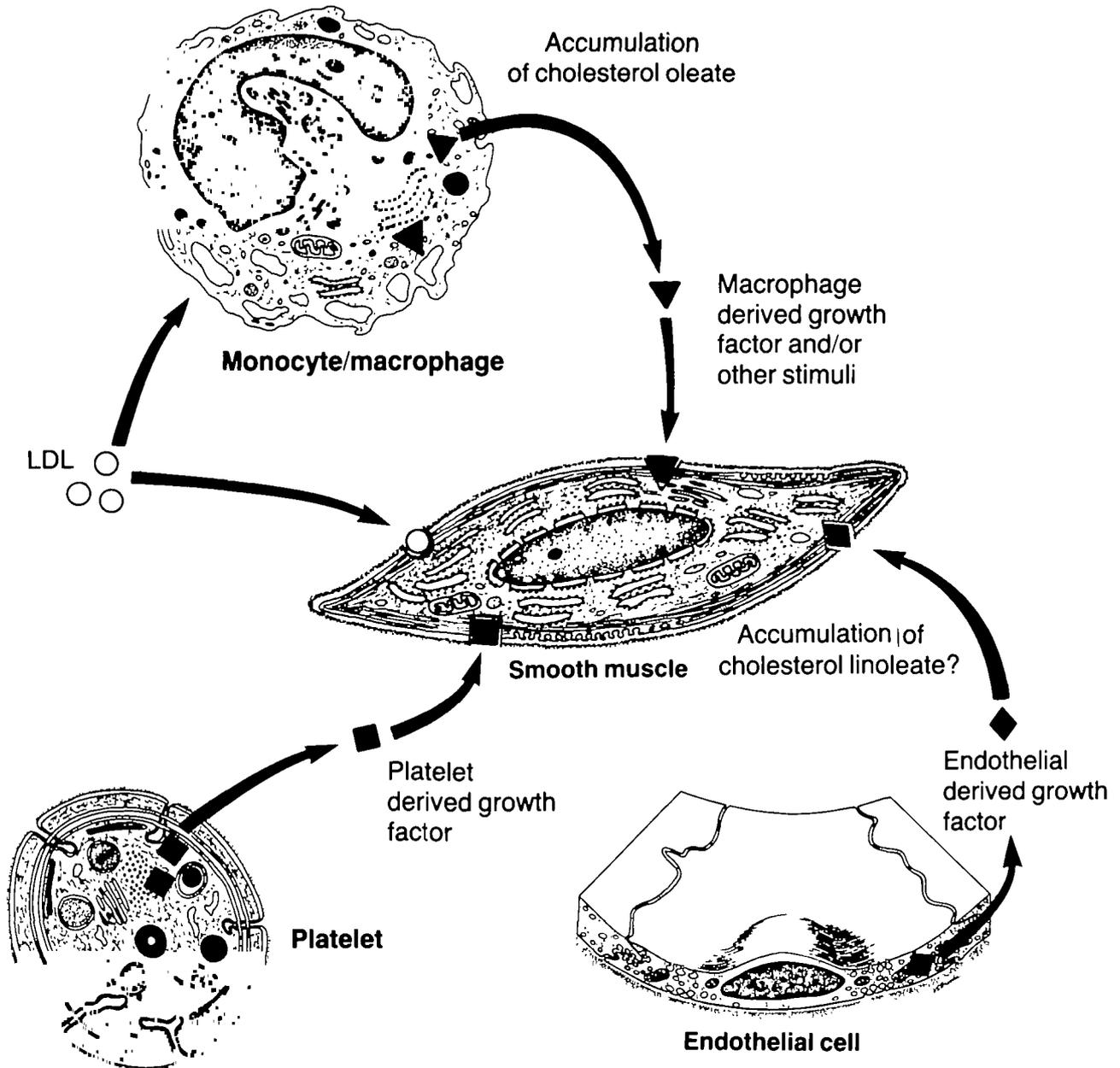
Figure 13. Diagram proposing that smooth muscle cells are susceptible to at least four potentially stimulatory growth factors, derived from the platelet (PDGF), the endothelial cell (EDGF), the monocyte/macrophage (MDGF), and low density lipoprotein (LDL-l). The particular importance of each of these as a source of smooth muscle proliferation in the genesis of the lesions of atherosclerosis needs to be better defined for each special set of circumstances, and provides important opportunities for further research.

Conclusions

The cellular response to vascular injury involves the participation of at least four different cells, as well as constituents in the plasma, with respect to the proliferative lesions of atherosclerosis. Endothelium, platelets, and the monocyte/macrophage can each produce growth factors that may participate in smooth muscle proliferation and thus in lesion initiation or progression. Low density lipoproteins from hypercholesterolemic animals may also be mitogenic (figure 13). These different mitogens, coupled with endothelial alterations resulting from a number of different risk factor-associated processes as diverse as hypercholesterolemia, hypertension, diabetes, cigarette smoking, immune or viral injury, provide a complicated background in which cellular interactions and materials formed and released by the cells may participate in the intimal proliferative smooth muscle response known as atherosclerosis.

Platelets may be important in lesion initiation in some circumstances, but not in others, dependent upon the type and extent of endothelial injury. The capacity of platelets or monocytes to participate in lesion progression and regression needs to be clarified and the role of each cell better defined under each set of conditions. The state of responsiveness of the smooth muscle cell to stimulating factors should also be considered, since it is apparent that the cells may be in different states of susceptibility to agents such as growth factors when the cells are in the media, as compared to cells in lesions in the intima. A great deal of new data concerning the biology of each of these cell types, together with new observations in man, have provided information to demonstrate that the formation of the lesions of atherosclerosis is quite complex. The ability to utilize the tools of modern cell and molecular biology to probe this problem will undoubtedly lead to the development of new diagnostic tools and of new means for both intervening and potentially for preventing this most important killer of man in the Western World.

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Index Terms: atherosclerosis   ·   platelets   ·   smooth muscle cells   ·
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