

The lesions of atherosclerosis¹

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The speaker describes the formation of the lesions of atherosclerosis as a response to tissue injury and the resulting interaction between the endothelium, smooth muscle, monocyte/macrophages, and the platelet. When the nature of the lesions of atherosclerosis is better understood, prevention of sequelae such as thrombosis or infarction will be improved.

Index terms: Arteriosclerosis, pathology • Irvine H. Page lectures

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It is a great pleasure to have the opportunity to present the Irvine Page lecture. I am a long-time admirer of Irvine Page and am deeply honored to have been invited to present this lecture during his lifetime. Few individuals have contributed as much as has Dr. Page to cardiovascular medicine. Of particular importance is his ability to stimulate research by young people, and perhaps most significantly, his capacity to support the infusion of new ideas into the mainstream of research so that they stand a chance of being tested.

Atherosclerosis, as a disease process, has been known for many years and remains the principal cause of death in the United States and Western Europe.¹ Despite this, our understanding of the cellular interactions that may take place during the development of the lesions of atherosclerosis has, until recently, grown extraordinarily slowly. In fact, until the last decade, this disease process was considered primarily to be a degenerative process. Now, it is clear

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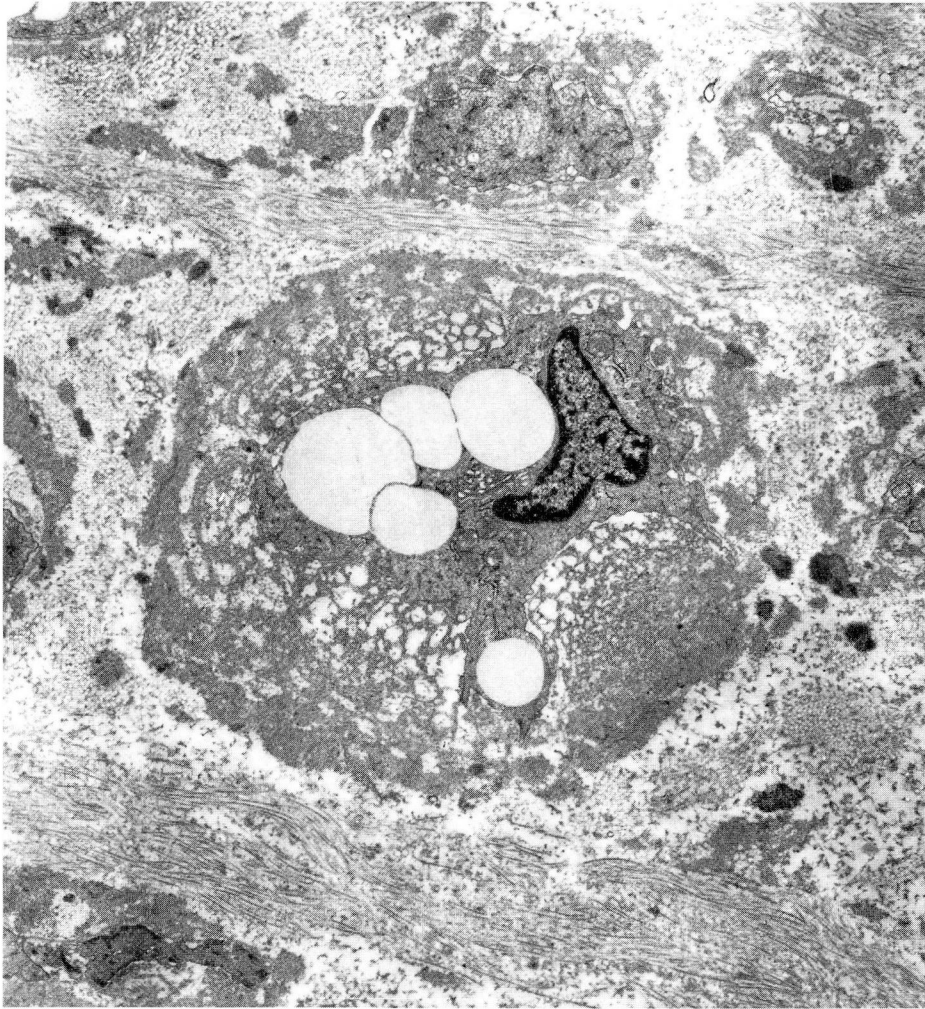


Fig. 1. This lipid-laden smooth muscle cell was present in the abluminal portion of the fibrous cap of a fibrous plaque present in the superficial femoral artery of a 58-year-old white man. The dense connective tissue matrix and the thickened basement membrane around each of the smooth muscle cells are typical of this portion of the lesion in both human and nonhuman-primate atherosclerosis ($\times 15,000$).

that during their formation, the lesions of atherosclerosis develop mainly by the accumulation of two cell types within the intima of the artery wall: smooth muscle and monocyte/macrophages. The smooth muscle cell accumulation, that is the *sine qua non* of the disease, appears to be the result of proliferation within the intima derived principally from the media of the artery. These cells synthesize new connective tissue and, together with the connective tissue, accumulate lipid to form the lesions of atherosclerosis.

The past two decades have witnessed the remarkable development of the disciplines of cell and molecular biology. This has permitted us to

study in isolation, and in various combinations, arterial endothelial cells, smooth muscle cells, monocytes and macrophages, and platelets and to begin to probe more deeply into the roles each of these cells plays when arterial homeostasis is disturbed and the lesions of atherosclerosis begin to form.

Lesions of atherosclerosis

If we are to understand how the lesions of atherosclerosis develop and if we are to study atherosclerosis in appropriate animal models, we must have a clear understanding of the constituents of the lesions as they occur in man. This

understanding has been difficult in the past because most published observations have relied on autopsy material in which the tissue is relatively poorly preserved. A few reports of human atherosclerosis have demonstrated the paramount role of the smooth muscle cell.²⁻⁴ Yet, most studies have used formalin-fixed, paraffin-embedded tissues in which cellular detail and, in fact, specific cell recognition are often impossible.

We are in the process of studying human atherosclerosis in segments of the superficial femoral artery, as well as in endarterectomy specimens of carotid arteries, obtained during surgery from patients with occlusive disease of either of these two vessels. This presents an opportunity to obtain representative sections of the artery, to fix them immediately in the operating room, and to take the remainder of the specimen to the cell culture laboratory to obtain cells derived from the lesions and, in the case of the superficial femoral artery, from the underlying media as well. After the cells have grown out in culture from the explants, they can be passaged, and we can examine their metabolic and growth properties in culture. These studies have yielded some surprising and exciting new data and present a splendid opportunity to explore firsthand the nature of the cells of the human lesion and to compare their behavior with cells derived from different animal models, including the nonhuman primate, swine, and rabbit. Thus far, the studies have demonstrated that human fibrous plaques and complicated lesions are covered by a well-known fibrous cap and that this cap consists of dense connective tissue and cells, principally smooth muscle, each of which is situated in a lacunar-like space (*Fig. 1*). These lacunar spaces are filled with connective tissue, consisting of alternate lamellas of basement membranes, proteoglycans, and small collagen fibrils. Occasionally, these smooth muscle cells contain lipid deposits. One of the principal characteristics of this part of the lesion is the extreme density of the connective tissue in the fibrous cap. Beneath the fibrous cap, there is a mixture of two cell types, both of which are often rich in lipid deposits. These deposits sometimes distort the cell so that recognition of cell type becomes virtually impossible using routine morphologic criteria. In most cases, the two cells in the lesion can be unambiguously shown to be smooth muscle and macrophages. Macrophages in all tissues derive from circulating blood monocytes.⁵

Monoclonal antibodies are now available against specific cell-surface antigens found on smooth muscle cells, fibroblasts (Gown, unpublished data), and monocytes and macrophages. These antibodies should be useful for recognition and quantitation of the different cells in the lesions of atherosclerosis in different arteries from different individuals. Such data should help us understand more about the genesis of these lesions.

Cell culture studies of smooth muscle cells derived from lesions versus the underlying media of a given artery have demonstrated that in most cases the lesion cells behave like senescent cells as compared with the underlying medial cells obtained from the same artery.⁶ Although the reason for this difference in behavior is not yet clear, the simplest interpretation of this data suggests that this is a reflection of the increased number of cell doublings undergone by the lesion smooth muscle cells as compared with the underlying medial smooth muscle cells. Much work remains to be performed involving numerous aspects of the metabolic capacity of lesion versus normal cells in the same artery, including the responsiveness to mitogens, capacity to metabolize lipids, capacity to synthesize connective tissue, as well as other responses related to atherogenesis.

Response to injury hypothesis

The suggestion that atherosclerosis represents a response to "injury" in the tissue is quite old. Virchow suggested this possibility in 1856.⁷ During the past 10 years, my colleagues, John Glomset and Laurence Harker, and I have developed a hypothesis based partially on Virchow's original ideas in an attempt to understand the roles played by each of the cells in the artery in the development of the lesions of atherosclerosis and to identify factors that may be important in this development.⁷⁻¹²

The hypothesis, which has been modified several times, suggests that, at least in some individuals, atherosclerosis may develop as a result of various types of injury to the endothelial cells of a given artery. This injury may be detectable morphologically or may be measured only in functional terms. The endothelium normally serves not only as a blood container, but also as a nonthrombogenic surface, a permeability barrier, and a vasoactive surface.¹³ Injury to the endothelium may alter these and other functional

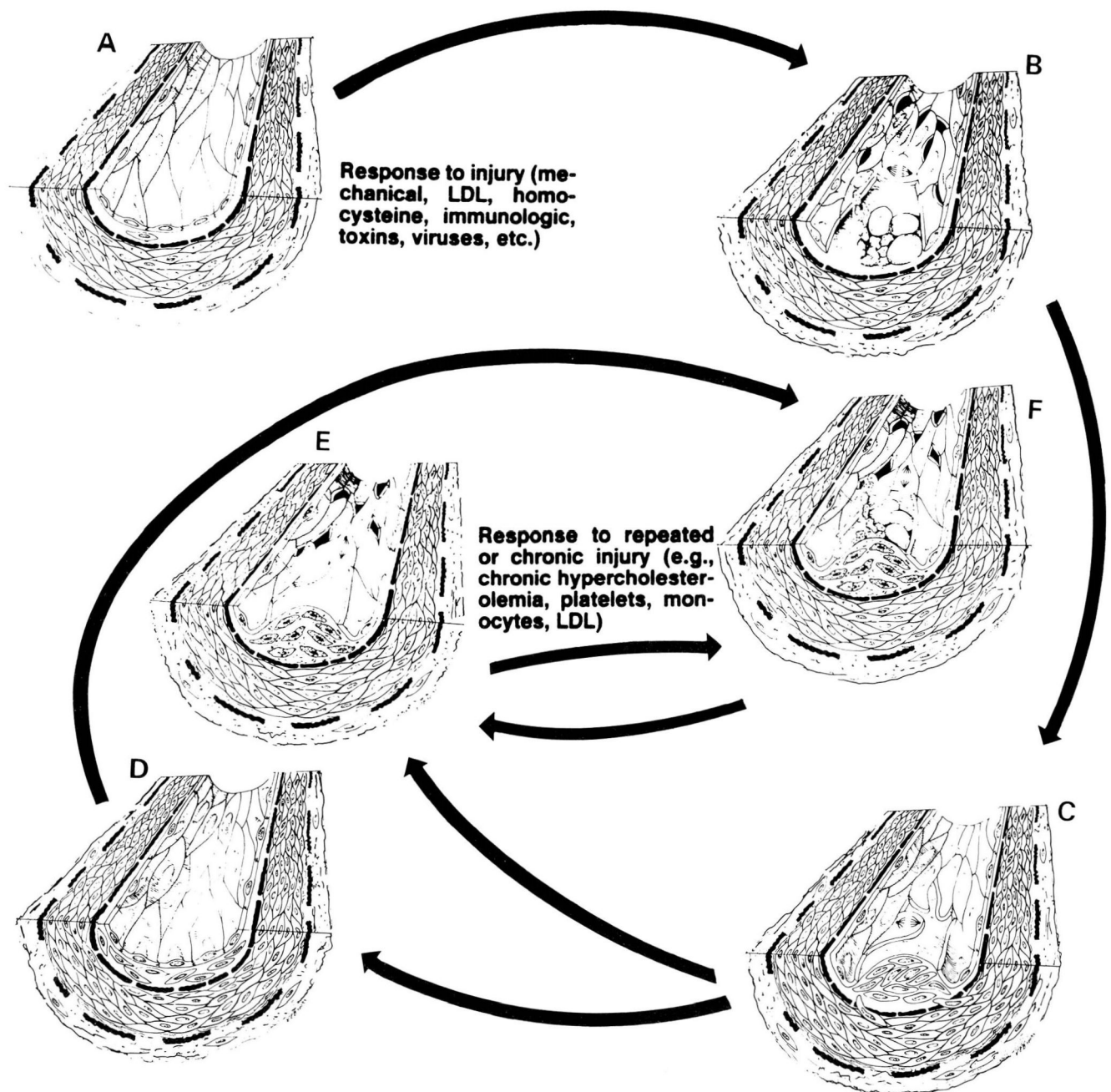


Fig. 2. Modified version of the Response to Injury Hypothesis of Atherosclerosis. A number of cyclic events may occur.

In the outer, or regression, cycle (A–D), injury to the endothelium is depicted (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 subsequently enter the tissue either at sites of desquamation or between endothelial cells. These interactions may be followed by migration of smooth muscle cells in response to the released mitogens (C). At the end of the regression cycle (D), if formation of the lesion is a single event and endothelial integrity is restored, the remnant of the proliferative response may simply 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 with 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 later calcify. This continued cyclic progression could eventually produce the clinical sequelae of thrombosis and infarction.

capacities, leaving no morphologic sign of the injury. Alternatively, the injury may be sufficiently extreme and lead to endothelial cell-cell detachment, or cell-connective tissue detachment, possibly resulting in desquamation of endothelial cells from the underlying subendothelial connective tissue into the bloodstream.

Such endothelial injury could permit interactions with at least two cells in the circulation: the platelet and the monocyte. An early functional injury resulting in accumulations of lipid in the intima may lead to migration of monocytes between the junctions of endothelial cells so that macrophages appear in the subendothelial space. Such changes have been noted within one month after induction of hypercholesterolemia in non-human primates.^{14,15} This type of response could represent the earliest lesion of atherosclerosis (fatty streak), which in man consists largely of intimal, lipid-laden macrophages. Subendothelial deposition of macrophages could result in further endothelial alterations and could also be important in stimulating smooth muscle proliferation.

If the nonthrombogenic character of the endothelium is altered, platelets may interact directly with the endothelial cells, or if there is cell-cell or cell-connective tissue detachment, platelets may then interact directly with the exposed subendothelial connective tissue. In either case, platelet adhesion and aggregation, followed by release of platelet contents at such sites of injury, could occur. The combined reactions of platelets and monocyte/macrophages could result in the release of biologically active substances, such as growth factors derived from either platelets or macrophages, into the intimal connective tissue. Each of these biologically active substances may stimulate chemotactic migration of smooth muscle cells from the media into the intima at injury sites and could further stimulate the smooth muscle cells to proliferate, to synthesize new connective tissue, and perhaps to accumulate lipid.

The hypothesis suggests that if the injury is a transient affair, the proliferative event may regress and would therefore be clinically silent. On the other hand, if the patient is exposed to a series of risk factors (e.g., cigarette smoking, hypercholesterolemia, hypertension, or diabetes), the resultant injury might be recurrent or chronic, and after many years, could progress slowly until sufficient vascular occlusion compromises the blood supply, leading to thrombosis and possibly infarction (*Fig. 2*).

This hypothesis has led to a number of ques-

tions concerning the biological reactivity of the endothelium, smooth muscle, monocyte/macrophages, and the platelet and to a better understanding of the interactions of these cells during the genesis of atherosclerosis.

Maintenance of endothelial integrity

Perhaps one of the keys to understanding the development and prevention of atherosclerosis would be the determination of the factors critical to the protection and maintenance of endothelial integrity. Endothelial cells maintain their nonthrombogenic character on the basis of their cell-surface coat of proteoglycan, particularly heparan sulfate,¹⁶ together with their capacity to synthesize antiaggregatory substances, such as prostacyclin (PGI₂).¹⁷ The cells characteristically grow in a continuous monolayer. Not only do they form prostacyclin, but they also appear to maintain reasonably tight control over the passage of molecules from the plasma into the underlying tissue.¹⁸⁻²⁰ In addition to these characteristics, the endothelial cells are capable of synthesizing various connective tissue macromolecules,^{21,22} as well as mitogens that can stimulate smooth muscle proliferation—the endothelial-derived growth factor (EDGF)²³ and the platelet-derived growth factor (PDGF).²⁴

The use of tissue culture to evaluate the maintenance of endothelial integrity has allowed studies of arterial endothelium from many different species. Using *in vivo* approaches, investigators have examined the endothelial alterations that result from toxins,²⁵ viruses,²⁶ mechanical injury,²⁷⁻³⁰ chemical injury,³¹⁻³³ and immunologic injury.³⁴ These studies can then be correlated with *in vitro* investigations in which the injury can be studied at the cellular level. For example, the endothelium of a monkey can be injured mechanically with an intraarterial balloon catheter²⁷ or chemically by inducing dietary hypercholesterolemia.^{14,15,23} The events that occur can be studied within minutes, days, months, or years after induction of such injury. A mechanical injury with an intraarterial catheter rapidly leads to platelet adherence and degranulation at sites of exposed subendothelium. Within three to five days after platelet adherence, smooth muscle cells migrate from the media into the intima, proliferate, and deposit large amounts of connective tissue. In a normocholesterolemic monkey, such an intimal smooth muscle proliferative response is maximal three months after injury and regresses within another three

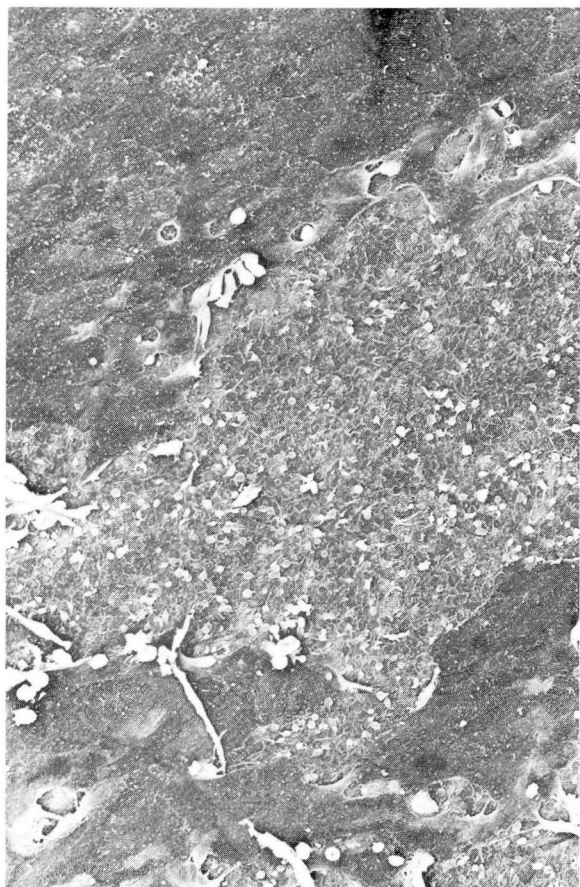


Fig. 3. Scanning electron micrograph of a two-year-old hypercholesterolemic pigtail monkey, showing an area of endothelial desquamation. This animal was carefully perfuse-fixed under normal arterial pressure. The area of desquamation is not an artifact since the area of exposed subendothelium is covered by at least two layers of platelets which have spread on the exposed connective tissue ($\times 700$).

months. If the monkey is on a high-fat, high-cholesterol diet, the smooth muscle proliferative response, instead of regressing, reaches a maximum after three months, but is maintained at this level and continues to accumulate lipids so that the lesion takes on the appearance of an early atherosclerotic plaque.³⁵ Several investigators have shown that the cell proliferation that occurs after such mechanically induced injury can be completely prevented if the experimental animal is made thrombocytopenic^{36,37} or if interference of platelet function is caused either by pharmacologic agents²² or by using animals that genetically lack the factor VIII antigen (as in the case of swine that are homozygous for von Willebrand's disease³⁸). All of these studies suggest

that platelet-artery wall interactions may be important in the induction of smooth muscle migration and proliferation within the intima. Such investigations can also be correlated with studies dealing with the possible role of the PDGF in this process.

A different sequence of events results when studying hypercholesterolemic monkeys with no mechanical injury. If nonhuman primates are on a high-fat, high-cholesterol diet, cholesterol levels of 300–500 mg/dL can be induced. Within one to two months on the diet, no discontinuities of the endothelium are evident. Foam cells which can be recognized as lipid-laden macrophages, however, are found within the subendothelium immediately beneath the intact endothelium as early as one month on the diet. After three to four months on such a regimen, not only are lipid-rich macrophages present, but smooth muscle cells also begin to accumulate in relatively small numbers in the intima. The cells also have lipid deposits within them, although much more lipid is present in the macrophages. Faggiotto and Ross^{14,15} have described the sequence of events that occurs in chronic hypercholesterolemic monkeys from 12 days to 13 months at monthly intervals.

If the examination of the hypercholesterolemic monkey arteries continues after the monkey has been on a hypercholesterolemic regimen for two years, patchy areas of missing endothelium will be evident; platelets will form mural thrombi at the sites where the endothelial cells have been lost (*Fig. 3*). These mural thrombi are usually found at the same anatomic sites that later are found to consist of intimal proliferated smooth muscle cells that contain lipid, together with varying numbers of macrophages—many of which are also lipid-laden. The endothelial desquamation and platelet interactions begin to occur in this hypercholesterolemic nonhuman primate model in the iliac arteries at 600–1,000 mg/dL cholesterol between four to five months on the diet. These observations suggest that one of the earliest events in hypercholesterolemia is the attachment and influx of monocytes which may lead to early smooth muscle accumulation in the intima and slightly later may be associated with more obvious endothelial injury and platelet interactions. This poses a potential role for a relatively newly discovered growth factor—one derived from the monocyte/macrophage.

Smooth muscle proliferation: the role of platelets and monocytes

Since intimal smooth muscle proliferation is one of the key events involved with atherosclerosis, the basis of this cellular proliferative response must be understood and the factors responsible for its occurrence must be delineated. At least three cell types associated with the lesions of atherosclerosis are capable of providing growth factors in the artery wall: PDGF, monocyte/macrophage-derived growth factor (MDGF), and EDGF.

Platelet-derived growth factor

Whole blood serum has long been known to be necessary for the multiplication of cells such as fibroblasts or smooth muscle in a culture. In 1974, the principal mitogen, which is present in whole blood serum and lacking in cell-free, plasma-derived serum, was found to be contained in the alpha granules of the platelets and was thus named "platelet-derived growth factor." This factor was proposed to be released at sites where platelet adherence, aggregation, and granule release occurred.^{39,40}

PDGF is a highly cationic protein (PI 9.8) (approximately 30,000 mol wt) which consists of two polypeptide chains (approximately 17,000 and 14,000 mol wt) that are crosslinked by disulfide bonds necessary for retention of biological activity. Purification of PDGF has been accomplished in a number of laboratories.⁴¹⁻⁴⁴ Purified PDGF has been radioiodinated so that it retains all of its biological activity.⁴⁵ I-125-PDGF binds to a specific high-affinity cell-surface receptor on cells such as fibroblasts and smooth muscle. This receptor is unique for PDGF, and I-125-PDGF cannot be competed for by other presently purified growth factors, such as epidermal growth factor, insulin, or fibroblast growth factor.⁴⁵ The apparent dissociation constant (K_d) for I-125-PDGF is 10^{-11} M, demonstrating an extremely high affinity of the ligand for its receptor. Binding occurs rapidly and is saturable, permitting an analysis of receptor numbers on each cell type. Arterial smooth muscle cells from the nonhuman primate and man contain 80,000 and 40,000 high-affinity receptors, respectively, whereas Swiss 3T3 cells contain receptor numbers ranging from (a) 600,000 receptors in a specific high-receptor line to (b) 130,000 receptors representative of most 3T3 cells to (c) a line of 3T3 cells

that has been mutated and selected to contain fewer than 2,500 receptors per cell.⁴⁵ The specific high-affinity receptor for PDGF has been further analyzed and has been found to consist of a cell-surface protein of approximately 164,000 mol wt.⁴⁶ Research is underway to further characterize this receptor and to determine its mechanism of interaction with PDGF and, it is hoped, the means by which it controls cell proliferation.

In addition to its role as a mitogen, PDGF has a number of effects on smooth muscle cells that occur shortly after it binds to its receptor. These include a marked increase in the rate of endocytosis⁴⁷; increased binding of low-density lipoprotein (LDL) to its high-affinity receptor on the surface of smooth muscle cells or fibroblasts due to an increase in the number of receptors^{48,49}; increased phospholipid metabolism⁵⁰; and most interestingly, chemotaxis. Grotendorst et al⁵¹ have demonstrated that of all the growth factors that have thus far been purified and analyzed, only PDGF is chemotactic for cells such as smooth muscle and fibroblasts. This observation may help to explain why smooth muscle cells migrate from the media into the intima after platelet adherence and degranulation at sites of endothelial injury.

Monocyte/macrophage-derived growth factor

As indicated previously, not only do platelets contain a potent growth factor, but macrophages derived from monocytes that are attracted into tissues at injury sites also release a growth factor after appropriate stimulation.⁵²⁻⁵⁴ The formation of the macrophage-derived growth factor was first demonstrated in a culture medium containing plasma-derived serum in which PDGF was absent.⁵² Since that time, several laboratory researchers have shown that macrophages from a number of different sources release a potent growth factor after appropriate stimulation with substances such as zymosan (yeast cell wall), endotoxin, or lectins such as concanavalin A.

We have asked whether monocytes in the circulation contain growth factor in storage granules prior to entering into the tissue. Our studies of purified monocytes maintained in suspension after purification by counterflow centrifugation demonstrated no active growth factor. These cells had to be plated in culture and specifically stimulated after they had become macrophages.

After approximately four hours of stimulation, they began to release MDGF into the culture medium in increasing amounts for approximately 22 hours.⁵⁰ Thus, it would appear that the macrophage does not travel through the circulation containing a growth factor, but rather, it is specifically attracted into the tissue and stimulated prior to synthesis and release of the growth factor into the adjacent tissue. The purification of this factor is in its early stages; consequently, little information is now available concerning its nature. Some observations have been made to suggest that at least part of the mitogenic activity is due to secretion of PDGF by the monocytes (Shimokado et al, unpublished observations). Thus, there may be multiple sources of PDGF available to the arterial tissue at selected sites in the artery wall.

Conclusions

We have come a long way in understanding the nature of the lesions of atherosclerosis, although we have much to do before we will be able to understand the specific biological aspects of all of the cells involved in lesion formation. More importantly, such understanding could have a major impact in terms of the development of new diagnostic tools and of the means for intervention and prevention of the lesions of atherosclerosis. I am heartened by the fact that the day when prevention and treatment may be possible is not too far away. With the rate of advance of knowledge in this field in the last decade, this could become possible within the foreseeable future. For example, methods have already been developed to inhibit platelet interactions at potential sites of endothelial injury. Brown et al⁵⁵ have shown that atherosclerosis developing at anastomatic sites of a coronary bypass graft can be stopped with pharmacologic inhibitors of platelet function. Now that PDGF has been purified, it should be possible to develop inhibitors of this growth factor or to find means of preventing its interaction with and binding to cell surfaces.

I hope that progress in understanding the role of the monocyte/macrophage and its growth factor and the role of the endothelial cell and its growth factor in the process of atherogenesis will also accelerate at a rapid pace. Furthermore, the role of low-density lipoprotein in injuring the endothelium and potentially in serving as a stimulus of the proliferative response should also be

clarified in the near future. Thanks to the development of cell and molecular biology, tools are at hand which will allow us to ask most of the pertinent questions related to the understanding of the etiology and pathogenesis of atherosclerosis. Therefore, the future is bright indeed. This is particularly true because of the conducive research environment that has been established in the field of cardiovascular disease due to a few individuals such as Irvine Page. Once again, I am most indebted and honored to have been asked to give this lecture. I thank you for the invitation.

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