Blood Vessel Wall and Thrombosis

Volume II

Raymund Machovich

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PREFACE

Thrombosis is one of the most serious medical problems of our time. The mechanisms by which thrombosis develops have been studied extensively for many decades. Attention has always been centered on the role of the blood vessel wall and its interaction with the platelets, the coagulation factors, and their inhibitors. More details are known about the intricate interactions among the multiple factors operative in thrombus formation and lysis. We have, as yet, been unsuccessful in finding the crucial determinant for effective prevention and treatment of this number one killer in the developed world.

The purpose of these multiauthor volumes is to present the latest, authoritative information on the regulatory role of the blood vessel wall on the process of hemostasis in health and disease. From this point of view, blood vessel wall and circulating blood can be considered as an indivisible system. Such an interpretation is also supported by the fact that pathological changes in hemostasis are not only involved in intravascular thrombus formation and bleeding from the injured vessel wall, but also contribute to various pathological injuries of the vessel wall e.g., in atherosclerosis, hypertension, diabetic angiopathy, immunopathology alterations, and tumor metastasis.

The regulation of hemostatic interactions involves a delicate balance of pro- and anticoagulant forces. Our knowledge of the structure and function of the blood vessel wall as well as of cellular and humoral factors of blood involved in hemostasis has logarithmically increased during the past few years. For specialists and nonspecialists these two volumes provide an easier survey of the vast range of the accumulated knowledge in this fascinating field of biomedicine.

Susan R. Hollán, M.D.
May 1986

THE EDITOR

Raymund Machovich received his M.D. degree from Semmelweis University Medical School (Budapest) in 1961 and subsequently specialized in internal medicine. He obtained the Ph.D. degree in 1972 for his work in the laboratory of Professor B. F. Straub on the biosynthesis of pancreatic enzymes. His research on thrombosis and hemostasis began in 1973, and he was awarded the D.Sc. degree in 1978 for his thesis, "Regulation of Thrombin Inactivation".

He spent 1 year (1970) in the laboratory of Professor E. Knox (Harvard Medical School) working with Dr. O. Greengard on the activation of thymidine kinase in tumor cells. Eight years later, he received the Canadian Heart Foundation Award and attended McMaster University for 1 year studying the role of heparin in hemostasis. In 1982, he spent 6 months at the Centre National de Transfusion Sanguine in Paris.

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Dr. Machovich has published about 70 papers and has edited a two-volume book *The Thrombin*, (CRC Press, 1984). He was an editor of *Thrombosis Research* from 1981 to 1985 and is currently a member of the editorial board of *Excerpta Medica*.

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Chapter 1

THROMBOTIC PROCESSES IN ATHEROGENESIS

John R. Guyton and Joel L. Moake

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I. INTRODUCTION

The presentation of a review on the thrombogenic theory of atherosclerosis and related topics brings to mind the proverb "Don't whip a dead horse!" Indeed, it is not the purpose of this chapter to champion a general causative role of thrombosis in atherogenesis. Decisive studies and alternative theories will be reviewed, yet there are undisputed associations between thrombosis and atherogenesis. Can we also infer causation, whereby components of the humoral or platelet clotting system may help to induce or exacerbate atherogenesis? A modified and limited role for thrombotic atherogenesis can be supported by available evidence, although exact mechanisms and the extent of the thrombotic role are not yet fully known.

Clinical interest in this topic is heightened by recent success in partially preventing thromboembolic phenomena in the setting of atherosclerotic disease, using inexpensive and almost innocuous drugs such as aspirin.[1,2] Could aspirin also be expected to prevent the development of atherosclerotic lesions? Further interest relates to invasive therapies for atherosclerotic disease — bypass grafting, endarterectomy, and transluminal balloon angioplasty. Particularly for angioplasty, a major limiting factor is recurrent stenosis, the mechanism of which could involve some form of thrombogenic or platelet-induced intimal thickening. Recent preliminary data indicate that carotid artery disease recurring after endarterectomy may be largely due to ongoing thrombogenesis and thrombus organization.[3] Thus, a review related to the thrombogenic theory of atherogenesis is timely.

There are recent reviews[4-6] of this topic that assume viewpoints somewhat different from those presented here. Two books and a series of papers from a recent symposium have appeared dealing with generally related topics.[7-11]

II. PLATELET-FIBRIN THROMBOSIS AND INTIMAL ENLARGEMENT

Particularly since Duguid[12] published his influential work in 1946, it has been recognized that mural thrombosis provides a growth mechanism at least for mature atherosclerotic plaques. The reality of this process has never been disputed, but its frequency and therefore its significance are unclear. Grossly evident, nonembolic thrombus in a large human artery is almost always associated with a preexistent atherosclerotic lesion.[5] Morphologically the arterial thrombus is characterized by alternating layers or regions of platelet masses and fibrin, comprising the lines of Zahn. Red blood cells are entrapped in the fibrin meshwork, especially in the distal red tail of the thrombus, but the size of the red tail and the numbers of red blood cells are far less than in venous thrombi.[13]

A. Frequency of and Setting for Arterial Thrombosis

The frequency with which arterial thrombosis associated with atherosclerosis may be found post-mortem is highly dependent upon the clinical situation. Coronary thrombosis at the site of an atherosclerotic plaque is now accepted as the usual cause of transmural myocardial infarction. By performing coronary angiography in patients with acute myocardial infarction, DeWood and associates[14] demonstrated complete occlusion in 86% of cases observed within 6 hr after onset of symptoms and in 68% of cases 6 to 12 hr after onset. In 66% of patients undergoing emergency surgical revascularization, fresh thrombus was removed from the involved coronary artery. The data thus demonstrated the pathogenic role of the thrombus in almost all cases and the rapidity of spontaneous lysis in some cases. Intracoronary or intravenous thrombolytic therapy, if given early enough, appears to limit cardiac damage in many cases of acute myocardial infarction.[15] Similarly, the clinical presentation of cerebrovascular disease is often linked to thrombosis or embolism arising at the site of an atherosclerotic lesion,[16] and anticoagulant or antiplatelet therapy can be effective in certain cases.[17]

Clinically evident thrombosis is fortunately an uncommon event in any individual's span of life. Therefore, unless mural thrombosis happens subclinically with reasonable frequency, we cannot expect it to play a very great role in the enlargement of atherosclerotic plaques. Subclinical mural thrombosis is most easily demonstrated for aortic atherosclerosis; in fact, patchy fibrinous encrustation or thrombus is seen regularly in cases of advanced, ulcerated aortic atherosclerosis. In the coronary arteries, the *incidental* finding of mural thrombosis is apparently much less common. In 35 cases of fatal coronary atherosclerosis without myocardial infarction, Virmani and Roberts[18] saw no intraluminal thrombus. Further data on the frequency of subclinical thrombosis in these and other atherosclerotic vessels are needed.

Some investigators have suggested that microscopic foci of thrombosis are present on the arterial luminal surface much more often than has been suspected, and that these may occur even on the surface of normal intima devoid of atherosclerotic involvement.[19,20] However, it is difficult to accept these suggestions, which are based solely on the study of human autopsy material. The difficulties in making accurate observations of the arterial luminal surface have become increasingly appreciated. Even brief interruption of the arterial circulation, especially if pressure is allowed to drop, can compromise the integrity of the endothelial lining. Bhawan and co-workers[21] studied simple occlusion of the normal rat carotid artery with double ligatures. During a 24-hr period, blebbing of the luminal surface due to subendothelial edema was seen, followed by disappearance of the endothelium. A surprising finding was specific localization of platelets against the denuded luminal surface, despite the lack of blood flow. The potential for microscopic post-mortem artifact in the usual clinical human autopsy specimen is obvious. In animals the ability to perform perfusion fixation, with careful attention given to pressure and duration of fixation, greatly aids ultrastructural study of the arterial luminal surface.[22] In animal studies utilizing scanning electron microscopy, with few exceptions, microthrombi are remarkably absent from normal arteries and from arteries with early atherosclerosis. This topic is discussed in greater detail in Section III, where the role of platelets in atherogenesis is covered.

B. Organization of Arterial Thrombus

If mural thrombus is incorporated quickly into growing atherosclerotic plaques, then this mechanism for plaque growth could be common, even while the unequivocal microscopic appearance of luminal thrombus is rare at any single point in time. In animal experiments, the time required for mural thrombus to be covered by neointimal tissue has been about 2 to 4 weeks.[23-25] One must note, however, that these were relatively large thrombi; the incorporation of smaller ones is likely faster. On the other hand, if the capacity of the underlying arterial tissue to repair the site of thrombosis is limited, as in advanced aortic atherosclerosis, then the thrombus may never be resolved.[26]

The overall process by which plasma enzymes and cells involved in tissue repair — leukocytes, endothelial cells, and smooth muscle cells — modify and grow over and into a thrombus is called organization of the thrombus. Component processes of organization and their approximate time scales are listed in Table 1.

Shrinkage of the original thrombus during the first hours to 1 week after its formation can be profound. Fibrinolysis is a part of this process; by electron microscopy disintegration of aggregated platelets is also seen between 7 and 24 hr.[25] Platelets appear to be disposed of more readily than fibrin, leading to condensation of the latter. Polymorphonuclear leukocytes within the thrombus undergo lysis, but monocytes become actively phagocytic and appear to digest the debris. The recruitment of additional monocytes through the surface of the thrombus seems likely. Foam cells, perhaps derived from monocytes ingesting the lipid-rich platelet debris, can eventually be found near the base of a proliferative lesion. Calcification is sometimes seen late in the process, perhaps in areas of remaining, undigested platelet debris.[24]

Table 1
COMPONENT PROCESSES IN ORGANIZATION OF THROMBUS

Process	Time scale
Lysis and shrinkage of thrombus	Hours to days
Reendothelialization	Few days to few weeks
Smooth muscle migration and proliferation	Few days to few weeks
Fibrous tissue synthesis	Days to weeks
Involution of fibromuscular tissue	Weeks to months

Growth of endothelium over the thrombotic surface presumably occurs from existing endothelium at the margins of the lesion.[27] Flattened luminal cells, seen within the first 3 days after onset of mural thrombosis, could have been either spreading monocytes or endothelial cells.[28] Endothelial cells, recognizable as flattened cells with distinct intercellular junctional complexes and underlying basement membrane, appear to partially cover the thrombus as early as 6 days.[29] The formation of vascular channels in a thrombus, called recanalization, occurs regularly in occlusive thrombi and may also occur in large mural thrombi. By electron microscopy these channels have been found lined by endothelium, but it is not known whether this is true at the earliest stages when the channels are being formed. Of historical note, it was the finding of multiple lumens in a coronary artery, which possessed typical atherosclerosis merging with apparent organized thrombus, that launched Duguid's[12] on his investigation and advocacy of the thrombogenic theory of atherosclerosis. On occasion atherosclerotic plaques are found to contain capillary-like vessels arising from the lumen. The vessels are endothelialized, but are surrounded by little supporting tissue relative to their luminal diameter, and thus may have the appearance of giant capillaries.[30] It has been advocated that the presence of these vessels constitutes evidence of a thrombotic origin for an atherosclerotic plaque. Since no other mechanism for the growth of capillaries directly from the endothelium of large arteries has been discovered, this particular argument for thromboatherogenesis remains untarnished. Nevertheless, it must be noted that by far the majority of capillaries within atherosclerotic plaques arise from the adventitia or outer media rather than from the arterial lumen. The frequency with which luminal capillaries occur is unknown. Geiringer[30] noted they were much more common in aortic atherosclerosis than in coronary atherosclerosis.

The migration and proliferation of smooth muscle cells into and over the mural thrombus appears to be very similar to the activity of these cells following selective de-endothelialization of arteries. That experimental situation is characterized in an early stage by the adherence of a monolayer of platelets to the exposed subendothelial surface, without true thrombus formation.[27,31] The likely role of platelets in inducing smooth muscle proliferation is discussed later. In an experiment in which mural thrombus was formed in a single event and not repetitively, the growth of a thick cap of multiple smooth muscle cell layers covering and burying the original thrombus was more impressive than the growth of cells into and replacing the thrombus, although both modes of growth were found.[23]

Some of the component processes of thrombus organization may compete with each other. For example, rapid lysis of the thrombus and/or rapid overgrowth by endothelium could reduce the stimulus for smooth muscle growth. Such competition has been described in detail for endothelial regrowth and smooth muscle proliferation following mechanical de-endothelialization of arteries.[32] Presumably a similar race between endothelial and smooth muscle cells could occur in the setting of mural thrombus, but the controlling factors that might allow an informed wager on the results are not known.

The final slow process of involution of the organized thrombus, with the presumptive goal of reforming a normal arterial wall, also has its counterpart in experiments performed by selective endothelial denudation.[27] Either the inability of endothelium to completely reinvest the lesion,[33] repeated endothelial injury,[34] or other factors such as hypercholesterolemia[35] may cause continuing stimulation to smooth muscle growth and thus a persistently enlarged lesion. In the absence of such continued pathologic stimulus, complete involution of the lesion or involution to the appearance of a simple reparative scar will result.

Another factor which might intervene in the successful resolution of luminal thrombus is genetic change in one or more of the proliferating smooth muscle cells. Following the work of Benditt and Benditt[36] on fibrous plaques, Pearson and co-workers have demonstrated monoclonal characteristics of organizing thrombus or thromboembolus in human aorta and pulmonary arteries.[37] The technique was the same as that used previously to determine the monoclonal nature of fibroid tumors of the human uterus. It depended upon electrophoretic analysis for the A and B isozymes of glucose-6-phosphate dehydrogenase, an enzyme coded by the X chromosome. This was performed on autopsy tissues from women heterozygous for the two isozymes. Since one or the other X chromosome is inactivated in every cell in the female body, each cell will produce either the A or B isozyme, but not both. X chromosomes are randomly selected for inactivation during embryogenesis; subsequently all the progeny of a given embryonic cell have the same inactivated X chromosome. Practical assays for isozymes include enough cells that the A and B forms are present in approximately equal amounts in normal tissue, but in neoplastic tissue, derived from a single cell, one or the other isozyme predominates. The authors found that organizing thrombi showed a significant tendency toward a predominance of either the A or B isozyme and that the strength of this tendency paralleled the histologic stage of organization of the thrombus. Since the developing monoclonality of organizing thrombi was similar to that found in atherosclerotic fibrous plaques, the investigators felt that their work favored the thrombogenic hypothesis for atherosclerosis. An argument based on the similarity of one trait hardly proves the hypothesis. Nevertheless, the data suggest that one or a few smooth muscle cells in an organizing thrombus do gain some growth advantage that can be inherited by daughter cells, and the most facile explanation is some form of somatic mutation. The same investigators later studied fatty streaks, which also represent possible precursor lesions to fibrous plaques. While fatty streaks were rarely found to be monoclonal, one fourth of the lesions studied were considered to have intermediate clonal characteristics, based on isozyme percentages.[38] Thus evidence based on clonal characteristics is not unique to the thrombogenic pathway, but can also be used to support fatty streak transformation as an alternative derivation for the fibrous plaque.

C. Markers of Thrombotic Origin in Atherosclerotic Plaques

In order to know whether thrombosis plays a substantial role in atherogenesis, it would be very helpful to know whether an individual plaque can be identified as having thrombogenic origin on the basis of some particular lesion feature. The feature may be a morphologic pattern or a substance left behind by the organized thrombus (Table 2).

One lesion feature really appears to be distinctive for thrombogenic lesions. This is the presence of vascular channels — either a multichannel lumen or capillaries entering the lesion surface from the lumen. These are found in organized thrombus, as already discussed, and no other mechanism for their formation is known. (Theoretically, growth of plaque in the center of an artery with a single, slit-like lumen could cause the formation of two lumens, but the resulting appearance should not cause confusion.) Roberts[39] pointed out that a multichannel lumen is not uncommon in coronary arteries with advanced atherosclerosis. In such arteries, preexistent severe stenosis or an ulcerated or ruptured plaque could set the stage for luminal thrombosis, followed by recanalization and organization. A multichannel

Table 2
MARKERS OF THROMBOTIC ORIGIN OF LESIONS

Proposed marker	Current status
Multichannel lumen or "giant" luminal capillaries	Reliable if present
Layered appearance of plaque	Not specific
Fibrin/fibrinogen	Insudation vs. thrombosis
Platelet antigens	Unreliable
Iron	Possibly useful

lumen has been documented also in an atherosclerotic cerebral artery,[40] but little notion of its frequency in cerebral or peripheral vessels is available. In certain cases with clear reasons for preceding thrombosis in nonatherosclerotic arteries, multichannel lumens have been found in a setting of extensive intimal fibrocellular proliferation. These lesions did not, however, possess the lipid deposits characteristic of atherosclerosis. One case involved apparent organized thrombus in a poststenotic aneurysm of the subclavian artery just distal to external impingement in a cervical rib syndrome.[41] Another case showed many vascular channels running through a fibrosed coronary artery aneurysm in a 17-year-old patient who had had symptoms compatible with Kawasaki disease at age 3 years. The aneurysm and apparent organized thrombus were thought to be remnants of old arteritis.[42]

A layered appearance of many atherosclerotic plaques has been noted frequently in the coronary arteries[5,39] and elsewhere,[40] and cited as evidence for a thrombotic origin of the lesion. The layers, easily demonstrated by routine or connective tissue stains, form crescents roughly parallel to the luminal surface, leaving a distinct impression that they represent previous locations for the luminal contour. However, layering of an atherosclerotic plaque provides much weaker evidence for thrombotic origin than does the multichannel lumen. Essentially, the layering phenomenon constitutes evidence for recurrent episodes of some kind of process that conforms to the lumen contour. A pathologic process may produce this pattern if it can propagate laterally in a close relationship to the arterial endothelium or luminal surface. Besides thrombosis, one can speculate about a number of other processes that, at least potentially, might do so. Desquamation or dysfunction of endothelium might under certain conditions become a laterally propagating phenomenon. Rapidly dividing progeny of a mutated smooth muscle cell could migrate laterally beneath the endothelium. The recruitment of monocytes into a fatty subendothelial lesion is known to have a pattern of lateral spread,[43] and the repeated formation of such lesions might, under certain conditions, provide an episodic stimulus to growth of underlying smooth muscle cells.

The presence of material with the histochemical staining characteristics of fibrin has long been noted in many atherosclerotic plaques. Often this material can lie in a midintimal location beneath or within the fibrous cap of the lesion. The presence of fibrinogen and/or fibrin within the atherosclerotic lesion is important enough to warrant a special section in this review. In that section, which follows, evidence is summarized indicating that much of the fibrinoid staining is probably due to insudation and trapping of fibrinogen within the lesion rather than thrombotic organization.

Antisera raised against human platelets have been used for immunochemical staining of atherosclerotic lesions. Woolf and Carstairs[44] found positive staining for apparent platelet antigens in about one third of a series of human fibrous plaques of varying sizes, but not in fatty streaks. The location of the antiplatelet staining, when present, corresponded with the location of staining with antifibrin-fibrinogen serum and took the form of one or more

coarse bands of immunologically positive material a short distance below and parallel to the intimal surface. These findings must be interpreted in the light of later results from the same laboratory on the survival time of platelet antigens in a pig model of aortic mural thrombosis.[45] By 1 month, only 40% of the organized thrombi showed specific antiplatelet fluorescence, and by 6 months, only 15% of lesions showed small foci of specific fluorescence. The relatively rapid loss of platelet antigens thus demonstrated is somewhat incongruous with the substantial prevalence of antiplatelet staining in human lesions that presumably develop over years or decades.

Contradictory results have been reported. Walton and Williamson[46] found no immunochemical staining of atherosclerotic lesions with an antiplatelet serum, except in cases of advanced, ulcerated lesions with obvious surface thrombosis.

Even if platelet antigens could be demonstrated unequivocally within human atherosclerotic lesions, the results would still require careful interpretation. It was shown by immunofluorescence that platelet factor 4, released from the platelet alpha granules when platelets attached to a de-endothelialized arterial surface, diffused deeply into the arterial wall.[47] Thus, antiserum to whole platelets could potentially react with releasable platelet antigens diffusing into atherosclerotic lesions. Finally, a number of platelet antigens, mostly related to cytoskeletal components, are cross-reactive with antigenic determinants found in many tissue cells, including vascular smooth muscle.[48]

More consideration should perhaps be given to iron as an archaeologic marker of atherosclerotic lesion development. While iron is found in small amounts in all cells (e.g., as a co-factor for cytochrome *c*) and in tissue fluid, it is concentrated greatly in red blood cells. To the knowledge of these reviewers, it is unknown whether enough red blood cells are trapped in arterial mural thrombi in arteries to leave long-lasting iron deposits at sites of thrombotic organization. Histochemical staining for the ferric ion is fairly sensitive and specific. Moreover, iron concentrations could conceivably be mapped out by X-ray analytical techniques in the scanning electron microscope.

Because iron-carrying red blood cells tend to be excluded from arterial thrombi, relative to their numeric density in blood, the finding of iron deposits in the arterial wall has generally been ascribed to old intramural hematoma rather than old thrombosis. Virmani and Roberts[18] noted recent intramural hematoma ("extravasated red blood cells") in the coronary arteries of 48 of 52 patients dying with coronary atherosclerosis and iron deposits stainable with Prussian blue in 32 of the same cases. Paterson and co-workers found Prussian blue-stainable deposits in aortic fatty streaks as well as more advanced lesions. Although these investigators considered the deposits likely to have resulted from blood leakage from intimal capillaries, they did consider the possibility of mural thrombosis as a source of iron.[49] Capillaries are in fact remarkably absent from normal aortic intima and fatty streaks.[30]

The discussion thus far has exhibited a generally negative viewpoint toward the role of thrombosis in the development of the uncomplicated atherosclerotic lesion. Is it worth pursuing further research on the details of thrombogenic lesion markers? The answer is yes, because these same issues must be faced again in more limited settings. As pointed out already, thrombosis could play a major role in the enlargement of advanced atherosclerotic lesions. Invasive arterial reconstructive procedures — i.e., bypass grafts, endarterectomies, and balloon angioplasties — are other settings in which thrombogenic atherosclerosis and/or fibrous intimal thickening remain reasonable hypotheses.[50]

D. Fibrinogen and Fibrin

Plasma fibrinogen levels have recently been shown to correlate very significantly with the development of cardiovascular disease.[51,52] In one study the association of fibrinogen with cardiovascular death appeared at least as strong as the association between cholesterol and cardiovascular death.[51] It was noted that fibrinogen levels correlate with other, better-