Leaky Vessels, Fibrin Deposition, and Fibrosis: A Sequence of Events Common to Solid Tumors and to Many Other Types of Disease[1-3]

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SUMMARY Solid tumors must induce new blood vessels if they are to grow beyond minimal size. As an initial step in this process, tumors secrete a vascular permeability factor that renders the local microvasculature hyperpermeable to fibrinogen and to other plasma proteins. Extravasated fibrinogen is rapidly clotted to crosslinked fibrin gel. Over time, this gel is invaded by macrophages, fibroblasts, and endothelial cells and undergoes "organization," such that it is replaced by vascularized granulation tissue and finally by mature connective tissue. This sequence of events is not unique to tumors but occurs in wound-healing and in a wide variety of other disease processes, including some that prominently affect the lung. AM REV RESPIR DIS 1989; 140:1104-1107

Introduction

Fibrin is deposited in the lungs and in other tissues during the course of many types of diseases with widely different etiologies (1). These include infections (e.g., pneumonia), collagen diseases, neoplasia (e.g., carcinoma of the lung), trauma, aberrant effects of drugs (e.g., bleomycin toxicity), hyaline membrane disease, adult respiratory distress syndrome, and both immediate and delayed types of hypersensitivity (1, 2). In all of these entities, fibrin forms a three-dimensional gel matrix in which small amounts of insoluble protein (crosslinked fibrin) trap and immobilize a substantially larger quantity of plasma exudate.

Fibrin deposition in tissues is important because of its sequelae. Rarely, deposited fibrin may resolve completely without leaving stigmata of any sort. Much more commonly, however, fibrin deposition has long-lasting consequences, particularly neovascularization and scarring, that result in impaired function (in the lungs, diminished gas exchange).

Our interest in extravascular fibrin deposition was kindled nearly a decade ago by the observation that fibrin deposits represented a regular, early morphologic event following the transplantation of syngeneic guinea pig carcinomas (3, 4). In such tumors, fibrin provided a provisional matrix that was gradually replaced by granulation tissue and then by mature stroma. This sequence of events mimicked the process of normal wound healing, so much so that we have suggested that normal wound healing is a useful paradigm for understanding tumor stroma generation (5). Subsequent studies have confirmed and extended these observations to many other types of tumors, transplantable and autochthonous, animal and human (5-7).

These observations raise a number of questions. How does fibrin form in tissues? What is the nature of the "fibrin" found in tissues? What are the consequences of fibrin deposition in tissues? What relevance do these events have for physicians involved in the treatment of pulmonary disease?

Generation of Fibrin Deposits in Tissues

As far as we know, fibrin deposition in tissues always results from the extravasation and extravascular coagulation of plasma fibrinogen (5, 8). Because of its large size (340 kDa) and oblong shape, fibrinogen escapes from normal blood vessels less readily than smaller, more globular plasma proteins such as albumin. For fibrinogen to leak from the blood into tissues to any significant extent, the vasculature must undergo substantial changes in permeability.

Hyperpermeability of Tumor and Wound Blood Vessels to Macromolecular Tracers

In fact, the microvasculature of solid tumors is hyperpermeable to circulating macromolecules (5-10). Radiolabeled fibrinogen and other plasma protein tracers enter and accumulate in experimental tumors several times more rapidly than they enter comparable control tissues (6-8) (table 1). Up to about 4 days after wounding, the vessels of healing skin wounds are comparably hyperpermeable to macromolecules (6) (table 1).

The primary leaky vessels in transplantable animal carcinomas are well-differentiated small veins and venules lined by a continuous endothelium (11). Leaky vessels were identified in tumors by their extravasation of labeled macromolecules; they were found in bands of stroma interposed between individual tumor nodules, but they were present in highest concentration at the tumor-host interface at the edge of the tumor. Vessel leakiness could not be attributed to structural defects, to vessel immaturity, to vessel injury, or to the formation of an inherently leaky type of endothelium (e.g., a fenestrated endothelium such as that lining renal glomeruli).

Characteristically, interendothelial junctions of leaky vessels were closed, as judged by electron microscopy, and did not contain deposits of colloidal carbon tracer, suggesting that macromolecular leakage did not occur through open intercellular junctions. However, carbon was found in vacuoles and deep invaginations in endothelial cells consistent with a transcellular route of transport. Therefore, although much more work needs to be done, we hypothesize that tumor vessel hyperpermeability is attributable not to open interendothelial cell junctions but to increased levels of transendothelial transport, either by way of vesicular or vacuolar shuttling or by the formation of transendothelial channels (11, 12).

Molecular Events Responsible for Tumor Vessel Hyperpermeability

That only mature tumor vessels were leaky suggested that the hyperpermeability of tumor vessels might be attributable to a tumor-secreted mediator, i.e., a tumor product that interacts with specific cell surface receptors present on well-differentiated endothelial cells. Our laboratory has, in fact, demonstrated that many animal and human tumor cells secrete a factor (vascular permeability factor or VPF) that at low nanomolar concentrations renders normal endothelium hyperpermeable (3, 13-15). VPF is a peptide with a M_r of 34,000 to 43,000 that is $\geq$ 50,000 times as active as histamine in enhancing microvascular permeability. Concentrations of VPF are particularly high in tumor ascites, suggesting that it also has an important role in malignant fluid accumulation in body cavities.

VPF probably acts directly on endothelial cells. It apparently does not cause the release of histamine from basophils or mast cells or induce the synthesis of other classic mediators of increased vascular permeability such as kinins or arachidonic acid metabolites. Like histamine, VPF is able to induce the reversible contractile separation of the endothelial cells that line venules and small veins without injuring these cells. However, as noted above, leaky tumor vessels we have studied showed no evidence of interendothelial cell gap formation. Therefore, leakage of tracers apparently took place by some other mechanism such as by transendothelial transport. VPF may have a role in modulating such transport. Other mediators have also been

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TABLE 1

INFLUX OF ^{125}I-FIBRINOGEN INTO GUINEA PIG TUMORS AND WOUNDS*

Tumor or Wound	Days after Implant/ Wounding	^{125}I-Fibrinogen (Urea Insoluble) Influx† ($\mu l/g$)
Line 1	4	17.2 ± 1.9
	7	24.2 ± 2.5
Line 10	4	15.4 ± 1.9
	7	20.3 ± 4.4
Skin wounds	1	16.5 ± 3.7
	2	13.6 ± 2.2
	4	15.1 ± 6.4
	7	5.9 ± 1.1
Normal skin		4.6 ± 0.7

* Adapted from reference 6.
† Values are mean ± SE.

claimed to be responsible for the increased permeability of the tumor vasculature, but with less experimental support (5, 13).

Coagulation and Lysis of Extravasated Plasma Fibrinogen

Fibrinogen is a heterodimer composed of paired Aα, Bβ, and γ chains (16). In normal clotting, the A and B fibrinopeptides are cleaved by thrombin to form fibrin monomers; these monomers polymerize spontaneously to form noncrosslinked fibrin. Crosslinking then follows by the action of clotting factor XIII, a calcium-dependent plasma transglutaminase that is itself activated by thrombin. Factor XIIIa introduces covalent bonds into polymerized fibrin, recognizable on reduced SDS-PAGE as γ-γ dimers and polymerized α chains (17).

Much of the labeled fibrinogen tracer that enters tumors and healing wounds is rapidly clotted (5–8) and transformed to crosslinked fibrin, as attested by its reactivity with monoclonal antibodies specific for fibrin (18), by its insolubility in urea (19), and by its characteristic appearance on reduced SDS electrophoretic gels. Tumor cells express procoagulant activities that may figure importantly in the coagulation process (20–22). The best characterized tumor procoagulant is tissue factor, a phospholipid-protein complex that initiates the extrinsic pathway of the coagulation cascade (23). Both living tumor cells and tumor cell homogenates express substantial tissue factor activity. Also, many tumor cells shed nanometer-sized vesicles from their plasma membranes into tumor ascites or into culture fluid that express tissue factor activity (20, 21).

Many tumor cells and their shed membrane-bounded vesicles also express a second procoagulant activity that initiates prothrombinase generation at a later step in the coagulation cascade (24). Prothrombinase is an enzyme complex that cleaves prothrombin to thrombin. It is made up of clotting factors Va, Xa, and prothrombin and an appropriate phospholipid surface; calcium ions are also required (25). For the complex to function properly, the phospholipid surface must bind factor Va with high affinity. The tumor cells that have been measured, as well as their shed vesicles, provide an appropriate surface, binding factor Va avidly ($K_d = 4 \times 10^{-10}$ M), and thus promoting efficient prothrombinase assembly (24). This step is critical because activation of the coagulation pathway earlier in the cascade, for example by tissue factor, would be unproductive in the absence of a functional prothrombinase, able to generate thrombin from prothrombin and, thence, fibrin from fibrinogen. Additional procoagulant activities have been ascribed to tumor cells, and infiltrating host inflammatory cells, particularly macrophages, may also elaborate procoagulants (22, 26).

Tumor procoagulants have attracted considerable interest, but the capacity to effect coagulation in tissues, outside of the vasculature, is not a unique property of malignant cells or of inflammatory cells. In several normal tissues, increased microvascular permeability, sufficient to cause extravasation of fibrinogen and other plasma proteins, leads inevitably to the same rapid extravascular deposition of crosslinked fibrin that occurs in tumors (27). It seems likely, therefore, that tumor procoagulants are not newly acquired activities of malignant cells, as many have thought, but rather are activities shared by many normal cells that are simply retained after malignant transformation.

The catabolism of fibrin deposits in tumors has been little studied, but it apparently proceeds at more than 90% of the rate of fibrinogen influx (8). Such rapid kinetics could not have been predicted from morphologic studies, which depict net fibrin deposits at a single moment of time and which provide no clues as to the relative rates of fibrinogen influx, clotting, and fibrinolysis.

To summarize, it appears that the extent of microvascular permeability, rather than the availability of tissue procoagulants, is the rate-limiting step that governs extravascular coagulation in both normal tissues and tumors. Fibrinolysis also contributes importantly to the extent of net fibrin accumulation. Fibrinogen influx and initial clotting rates have been found to be very similar in experimental tumors that express significantly different amounts of net fibrin accumulation (8); in these tumors, the large differences in observed fibrin content are primarily attributable to differences in rates of plasminogen activator release and consequent fibrinolysis (5). The factors regulating fibrin dissolution in other types of disorders (e.g., wound healing) have not yet been investigated, but the expression/secretion of plasminogen activator and other proteases by inflammatory and other host cells may play an important role.

Consequences of Fibrin Deposition in Tissues

What is the significance of the tissue fibrin deposition that occurs in malignancy and in other disease processes? If fibrin deposition is only an epiphenomenon, there may be little reason to investigate it further. However, accumulating evidence suggests that fibrin deposits may have considerable importance for tumor biology by contributing significantly to the initial structure of solid tumors and by promoting angiogenesis and mature stroma generation.

Imposition of Initial Structure

Fibrin is deposited in tumors within minutes of transplantation, forming a three-dimensional gel that encompasses individual tumor cells and separates them from other nearby tumor cells/cell clumps (4). As a result, and despite continuing fibrin turnover and new fibrin deposition, tumors grow in the form of discrete nodules that remain separated from each other by bands of fibrin gel matrix. Over time, the fibrin gel that envelops tumors and that separates individual tumor nodules is transformed progressively into immature and finally into mature vascularized connective tissue (4, 5, 28). Thus, the elementary structure imposed by fibrin within a few minutes of tumor transplant persists as tumor cells remain compartmentalized, initially by fibrin gel and later by mature stroma.

Indirect evidence that fibrin defines solid tumor structure comes from the study of ascites tumors growing in body cavities (29). Ascites tumors generally lack overt fibrin deposits and grow as a suspension in a plasma exudate. The reasons for the lack of fibrin accumulation in ascites tumors, despite hyperpermeability of lining vessels equivalent to that of solid tumors, have not been fully elucidated.

Induction of Angiogenesis and Mature Stroma

The fibrin gel initially deposited in tumors undergoes a series of reproducible changes that lead to its gradual replacement by connective tissue stroma. Central among these changes is the coordinated immigration of macrophages, fibroblasts, and new blood vessels, followed by local synthesis and deposition of interstitial collagens (4, 28). The factors regulating these events are not yet well understood, although a variety of chemoattractants and growth factors has been implicated (30, 31). A critical question to be addressed is whether fibrin itself has an essential role, and, if so, what that role might be. Some years ago we demonstrated that fibrin gels, planted in the subcutaneous space of experimental animals, were invaded by fibroblasts and new blood vessels and were gradually replaced by vascularized connective tissue (4). Thus, fibrin gels themselves seemed to elicit an angiogenic and connective tissue response similar to that induced by tumors.

We recently developed an improved assay for extending these findings (32). Plexiglas® chambers, enclosed except for multiple small pores on one face, may be filled with varying contents and implanted in the subcutaneous

space of guinea pigs or mice. When chambers were filled with purified homologous fibrin, at or near plasma concentrations, new blood vessels and accompanying stromal elements grew into the chambers through the small pores on one face and flared out radially. The angiogenesis in these chambers developed with kinetics similar to those of new blood vessel formation in solid tumors and in healing wounds. The vessels that entered fibrin-filled chambers, like those of developing tumor stroma, were accompanied by macrophages and fibroblasts; presumably, fibroblasts are responsible for the synthesis and secretion of interstitial collagens that inevitably attend stroma formation.

Accepted at face value, these findings suggested that fibrin deposits in tissues are themselves sufficient to induce angiogenesis. If this is the case, then fibrin gels must be able to perform at least two functions: (*1*) provide a three-dimensional matrix that supports cell migration, and (*2*) express selective chemotactic and/or chemokinetic activity such that endothelial cells, fibroblasts, and macrophages migrate into fibrin-filled chambers.

That fibrin gels might provide a three-dimensional matrix capable of supporting cell migration is not surprising. That fibrin gels might also impart information to cells, stimulating and directing their migration, is unexpected, though perhaps not unprecedented (33, 34). Most of the chemotactic or chemokinetic agents described to date have been soluble molecules, not insoluble structural proteins such as fibrin. Therefore, consideration must be given to another possibility, namely, that a contaminant and not fibrin itself is the moiety responsible for inducing vessel ingrowth. Fibrinogen preparations often contain small amounts of fibronectin, clotting factor XIII, fibrinogen, or fibrin degradation products, and perhaps other, as yet unrecognized, contaminants; in addition, of course, thrombin is added to clot fibrinogen, and fibrinopeptides are liberated from fibrinogen during clotting. Possible candidate mediators of cell migration, therefore, include the fibrin gel itself, soluble products associated with fibrin gelation, crosslinking and degradation, and contaminants. Thrombin and fibrinopeptides seem not to be responsible (32), and we are currently sorting out the other possibilities.

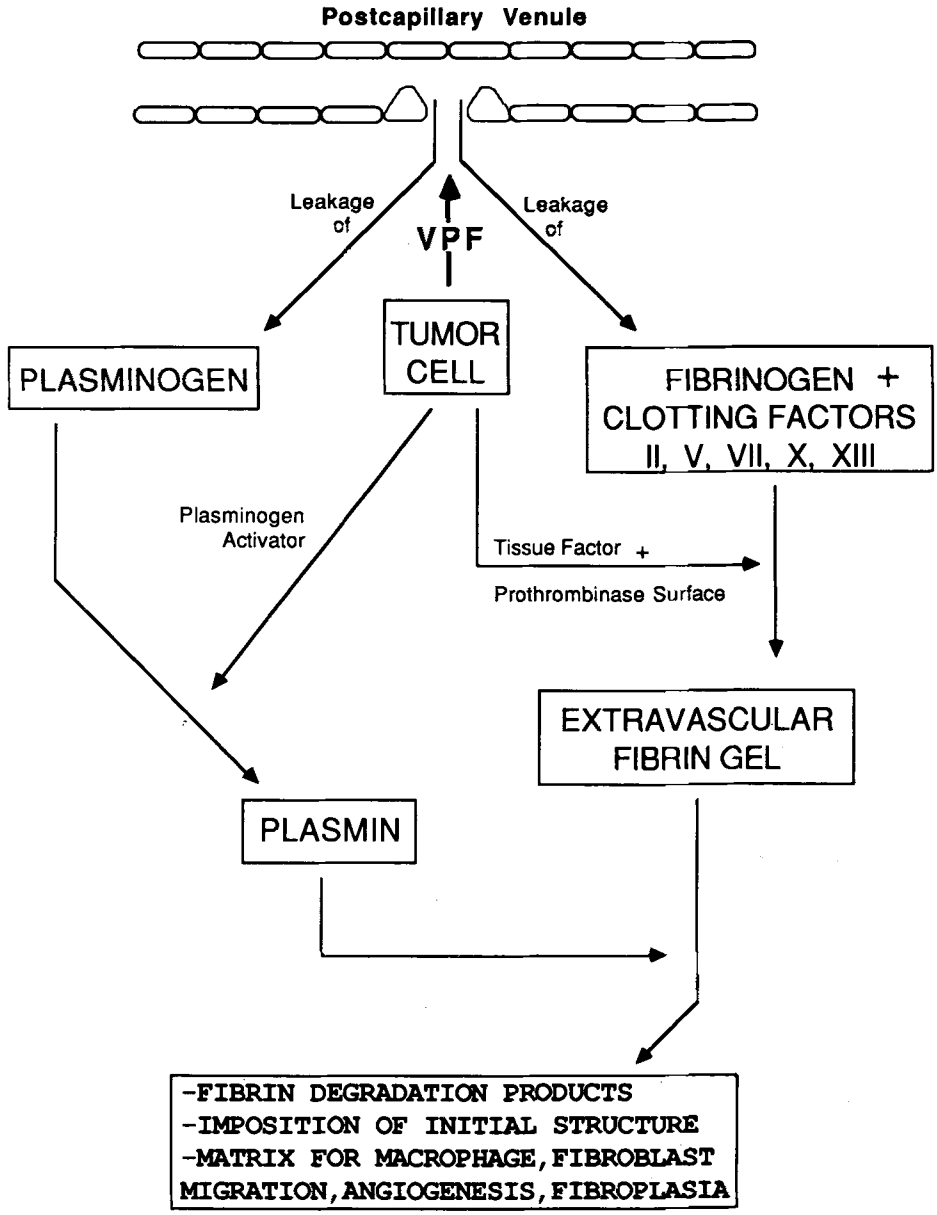

Fig. 1. Schematic diagram summarizing the events that regulate vascular hyperpermeability, fibrinogen extravasation, fibrin deposition, and stroma formation in tumors. Modified from reference 5.

Relevance to Pulmonologists

Our findings, summarized schematically in figure 1, are likely to be of practical interest to pulmonary physicians. Although of obvious application to traumatic lung injury and to cancers that arise in or metastasize to the lung, the findings are also likely to be relevant to a wide variety of pulmonary diseases, including ARDS, drug- and hypersensitivity-mediated diseases, etc. In all of these entities, blood vessels become leaky, resulting in extravasation of fibrinogen, fibrin deposition (either in the lung interstitium or within alveolar spaces), and finally neovascularization and fibrosis with impaired gas exchange.

However, studies in the lung also indicate that extravascular fibrin deposition does not always lead to irreversible tissue damage. Thus, in some babies with hyaline membrane disease, the process is reversible and alveolar fibrin deposits clear without evidence of scar formation. Similarly, in lobar pneumonia, alveolar fibrin deposition and inflammatory cell infiltration are extensive; nonetheless, if antibiotic treatment is initiated in time, these changes may be reversed completely and need not proceed to neovascularization or fibrosis. The lung therefore affords a useful model for studying the consequences of fibrin deposition and its sequelae and may provide unique insights that will be of use in understanding the pathogenesis of angiogenesis.

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