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# COMMENTARY

## REGULATION OF ANGIOGENESIS: A NEW FUNCTION OF HEPARIN

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A new function of heparin has been discovered recently. It has been found that heparin, or specific fragments of heparin, can govern the proliferation of capillary blood vessels and regulate angiogenesis. How was this activity revealed? What are its biological implications? What are its potential pharmacological and clinical applications?

### *A study of mast cells in angiogenesis*

Tissue mast cells generally reside in the neighborhood of mature capillaries and venules. However, increased populations of mast cells are often associated with rapidly growing capillaries such as occur in chronic inflammation or in the most vascularized areas of tumors [1, 2]. These relationships led to the notion that mast cells might in some way be associated with the growth of new capillaries, although the nature of such an association remained a mystery [3, 4].

A study of mast cells in angiogenesis was begun in our laboratory in 1975, because of a paradox that we encountered during the *in vitro* culture of vascular endothelial cells from human umbilical vein and from bovine aorta. Tumor fractions with angiogenesis activity that stimulated endothelial mitosis *in vivo* failed to do so *in vitro*. We considered three possible explanations: confluent cultures of vascular endothelial cells could be refractory to further growth stimulation (in contrast to 3T3 cells), or, (ii) endothelial cells from large vessels (umbilical vein and aorta) might be the wrong type of cells (perhaps only capillary endothelial cells would respond), or, (iii) an intermediate cell was missing *in vitro*—perhaps mast cells were needed to process the putative angiogenesis factor from tumor before the factor could stimulate endothelial cells *in vitro*.

We pursued all three possibilities. In retrospect, it is of interest that the first supposition led to the finding that confluent vascular endothelial cells are refractory to further growth stimulation [5]. The second hypothesis led to the isolation and cloning of capillary endothelial cells [6]. The third hypothesis led to an experiment which quantified the accumulation of mast cells during the initiation of tumor angiogenesis [7].

In the latter experiment, tumor extract was implanted on the chorioallantoic membrane of the chick embryo. There was a 40-fold increase in the density of mast cells surrounding the pellet of tumor extract. These cells appeared in the neighborhood of the tumor pellet about 24 hr ahead of capillary sprouts that were converging upon the tumor pellet. Mast cells alone, purified to 90% on a Ficoll gradient, did not induce angiogenesis when added to the chorioallantoic membrane as a separate pellet of up to $10^6$ cells. One implication of this experiment was that mast cells facilitated capillary growth, although they seemed unable to initiate it.

How mast cells could facilitate capillary growth was unknown. At that time little was understood about the separate events required for the growth of a capillary sprout, except that endothelial proliferation seemed to be necessary.

### *Stimulation by mast cells of migration of capillary endothelial cells* in vitro

By 1980, it had become feasible to re-examine the concept that mast cells could enhance capillary growth. Directional migration of endothelial cells had been found to be one of the earliest events in the formation of a capillary sprout [8]. Capillary endothelial cells had been cloned and were now being grown in long-term culture [6]. Zetter [9] had developed a method to measure the rate of migration (chemokinesis) of these cells on plates coated with colloidal gold. Therefore, Azizkhan *et al.* [10] incubated capillary endothelial cells with mast cell lysates and with mast cell-conditioned medium. Endothelial cell migration was increased significantly by both materials [10]. Tumor-conditioned medium also stimulated endothelial migration (as well as proliferation), but no other non-neoplastic cells, except mast cells, could stimulate endothelial migration.

### *The effect of heparin, a mast cell product, on endothelial migration*

When mast cell products were tested for their ability to stimulate endothelial migration, only heparin caused significant migration. Increased endothelial migration was observed at a heparin concentration of 10 μg/ml. A concentration of 50 μg/ml was equivalent to 100% mast cell-conditioned medium [10]. Other mast cell products could not stimulate endothelial migration, even in log

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increments from 10 ng/ml to 1 mg/ml. These inactive compounds included histamine, eosinophilic chemotactic factor-A, chondroitin sulfates, 5-hydroxytryptamine, trypsin and chymotrypsin. Furthermore, the endothelial migration activity of heparin and of mast cell-conditioned medium was abolished completely by specific inhibitors of heparin, protamine and heparinase, but not by chondroitinases, or by trypsin. These results warranted an *in vivo* test of the effect of heparin on angiogenesis induced by a tumor.

*Heparin promotion of tumor angiogenesis* in vivo

Taylor and Folkman [11] showed that heparin could enhance angiogenesis induced by tumor extracts implanted in the chick embryo. A partially purified angiogenic fraction was prepared from human hepatoma. When this material was implanted on the chorioallantoic membrane of the chick embryo in a methylcellulose pellet, 25 μg of the extract elicited angiogenesis in 30% of embryos at 48 hr; 100 μg of the extract elicited angiogenesis in 90–100% of embryos in the same time period. However, in the presence of heparin (6 μg, i.e. 1 unit), the sensitivity of the assay was increased markedly. Only 25 μg of the tumor extract was required to stimulate angiogenesis in 100% of the embryos. Furthermore, new capillaries appeared 24 hr earlier with heparin than without it. Non-anticoagulant heparin [12] could substitute for whole heparin as an enhancer of tumor angiogensis. Heparin by itself did not stimulate angiogenesis. It has subsequently been shown that angiogenesis induced by non-neoplastic cells, such as adipocytes, can also be enhanced by heparin [13]. Protamine prevented heparin from enhancing tumor angiogenesis [11]. In fact, higher concentrations of protamine inhibited tumor angiogenesis as well as inflammatory and immune angiogenesis. Systemically adminstered protamine inhibited the growth of lung metastases in mice [11]. In a recent report, systemically administered protamine also inhibited tumor-induced angiogenesis in mice and this effect was abolished by heparin [14].

While heparin itself does not initiate angiogenesis, it appears to gain this capacity when bound to copper ions. McAuslan [15] was the first to suggest that copper might play a role in mediating angiogenesis. Subsequently, Raju *et al.* [16] and Alessandri *et al.* [17] showed that heparin, when bound to copper, became angiogenic. Copper-free molecules were not angiogenic.

Thus, the data that were assembled by 1982 suggested a new function for heparin as a positive regulator of angiogenesis, independent of its anticoagulant activity. This conceptual framework soon had to be refashioned to accommodate additional results demonstrating that, under certain conditions, heparin could also become a negative regulator of angiogenesis [18].

*Inhibition of angiogenesis when heparin is administered in the presence of corticosteroids*

The finding that heparin could inhibit angiogenesis, under appropriate conditions, came about in the following way. In our laboratory heparin was being used routinely to improve the sensitivity of the chick embryo assay for testing angiogenic activity. A recurrent problem with this assay is that egg shell dust occasionally falls on the chorioallantoic membrane and produces background inflammation which confuses interpretation of the assay. We decided to improve this assay by suppressing the background inflammation with cortisone. Would this make the tumor angiogenesis more conspicuous? Heparin alone enhanced tumor angiogenesis as expected. Cortisone alone suppressed background inflammation as expected and made it easier to interpret the ensuing angiogenic reaction induced by tumor. However, when heparin and cortisone were applied together, the result was unexpected! All angiogenesis was inhibited [18]. Not only was tumor angiogenesis prevented, but a large avascular zone appeared in the young chorioallantoic membrane as growing embryonic vessels began to regress. Growing vessels in the yolk sac of very young embryos could also be inhibited. By contrast, mature, non-growing vessels in older chorioallantoic membranes (10 days or more) were not affected. This anti-angiogenic effect was independent of anticoagulant activity.

The non-anticoagulant fraction of heparin had a lower molecular weight than whole heparin. We wondered if smaller heparin fragments would still retain anti-angiogenic activity. Therefore, heparinase was obtained from bacterial fermentation and the enzyme purified [19]. When heparin was degraded with this enzyme, and the resulting heparin fragments were tested with cortisone for anti-angiogenic activity, a hexasaccharide fragment was found to be the most potent. Tetrasaccharide and disaccharide fragments were inactive. The hexasaccharide fragment had a molecular weight of approximately 1600. It did not cause anticoagulation. When either this fragment or heparin was mixed with cortisone in a sustained-release polymer pellet [20], tumor angiogenesis was suppressed completely in the rabbit cornea. Again, neither the heparin fragment alone nor cortisone alone could prevent new blood vessels from growing into a cornea containing a tumor implant. Furthermore, when the hexasaccharide fragment and cortisone were injected into mice bearing palpable tumors (200–300 $mm^3$), such as reticulum cell sarcoma, there was rapid tumor regression. However, it was not feasible to produce sufficient hexasaccharide to treat more than a few mice. While whole heparin injected subcutaneously with cortisone also inhibited angiogenesis and brought about tumor regression, anticoagulation and bleeding were dangerous side effects and limited the dose that could be achieved.

*Degradation of orally administered heparin into small fragments*

An unresolved problem was how to deliver the appropriate heparin fragment without causing anticoagulation. In other words, is there a simple way to neutralize the anticoagulant activity of heparin? Heparin taken orally does not usually cause anticoagulation. Therefore, the following questions were posed: Would orally administered heparin be degraded by the intestine into fragments of at least the size of a hexasaccharide? Would these fragments be absorbed into the bloodstream?

It was soon found that heparin adminstered orally to mice released heparin fragments into the bloodstream, which in the presence of cortisone or hydrocortisone demonstrated similar anti-angiogenic effects as when hexasaccharide was administered parenterally. There was no anticoagulation. Subcutaneous tumors of reticulum cell sarcoma, B16 melanoma, Lewis lung carcinoma and bladder carcinoma regressed in animals which drank heparin and received cortisone injections, but not in animals treated with either heparin or cortisone alone. It was possible to eradicate tumors completely in more than 50% of mice. In these mice, tumors did not recur after treatment was discontinued. The number of lung metastases in all mice was reduced to 0.1% of controls.

Curiously, some tumors were non-responders. There were at least four murine tumors for which neither angiogenesis nor tumor growth was inhibited by what seemed to be optimal doses of heparin and cortisone. These were sarcoma 1509a, Meth A sarcoma, glioma 26 and glioma 261B.

Another curious observation was that, for tumors that were responsive to heparin and cortisone, 200 units of heparin/ml of drinking water was generally the minimum effective dose, and tumor regression was more rapid as the dose was increased up to 1000 units/ml. However, when the heparin dose was increased further, i.e. to 2000–5000 units/ml, rapid tumor growth resumed. This result provided the first hint that activities of heparin responsible for positive and negative regulation of angiogenesis might possibly be assigned to separate heparin fragments.

It was vexing that heparins manufactured by different processes and different companies revealed completely different anti-angiogenic activities, despite similar anticoagulant activities. Thus, when heparin was used locally (with cortisone) in the chick embryo or in the rabbit cornea, heparins from many manufacturers were anti-angiogenic, although some heparins had to be applied at eight to ten times the concentration of the most effective heparin. However, when heparin was given orally, only heparin from two manufacturers could bring about tumor regression [18], while other heparins were ineffective.

Another puzzling finding was that, while cortisone and hydrocortisone were about equally as effective at inhibiting angiogenesis in the presence of heparin, dexamethasone had no effect over a wide range of concentrations, though the glucocorticoid activity of dexamethasone is thirty times greater than hydrocortisone. Sandor Szabo* raised the possibility that the anti-angiogenic effect of hydrocortisone was independent of its glucocorticoid effect. Therefore, in the chick embryo assay, 11α-epicortisol (Upjohn) was substituted for hydrocortisone because it is known that if the 11-hydroxyl group, which in hydrocortisone is in the beta position, is moved to the alpha position, the compound loses its glucocorticoid and mineralocorticoid activities. The compound, 11α-epicortisol, retained its anti-angiogenic activity with heparin at approximately the same concentration as hydrocortisone. Subsequent experiments in our laboratory have shown that there is a group of steroids which have little or no glucocorticoid or mineralocorticoid activity, and for which the ability to inhibit angiogenesis in the presence of heparin is the principal activity [21]. We have begun to use the term "angiotropic steroids" to categorize these compounds.

*Angiogenesis inhibition by synthetic heparin fragments in the presence of steroids*

Recent studies [21] have demonstrated that a synthetic pentasaccharide (Choay Institute) [22] can inhibit angiogenesis in the chick embryo in the presence of either hydrocortisone or a corticosteroid without glucocorticoid activity. This synthetic fragment is not as active as enzymatically derived hexasaccharide, but it is equivalent to the activity of the best whole heparin. Only milligram quantities are available, and the pentasaccharide has not been tested in tumor-bearing animals.

*Binding of endothelial cell growth factors to heparin*

The ability of heparin or an appropriate fragment to act as an angiogenesis regulator may depend partly on another property of heparin, reported recently [23, 24]. Heparin has been found to have a very high binding affinity for endothelial cell growth factors, some of which are also angiogenic.

This new property of heparin was uncovered during attempts to purify a tumor-derived endothelial mitogen that was angiogenic. The isolation and purification of tumor-derived angiogenic factors have been a long-term effort in our laboratory. Yuen Shing and Michael Klagsbrun began to use heparin–Sepharose chromatography in this purification strategy, because of our previous experience that heparin could promote endothelial migration and angiogenesis, and because heparin inhibitors such as protamine and platelet factor 4 were also angiogenesis inhibitors [11]. (In fact, Joanne Murray in our laboratory had attempted previously to purify an angiogenesis inhibitor from cartilage by heparin affinity.)

Heparin affinity greatly facilitated the purification to homogeneity of a tumor-derived endothelial mitogen which is angiogenic. The factor, derived from a rat chondrosarcoma, was purified about 500,000-fold to a single band preparation on a silver-stained sodium dodecyl sulfate gel by a two-step procedure that utilized cationic exchange on Biorex 70, followed by heparin-affinity chromatography [23, 24]. The purified factor is a cationic polypeptide with an isoelectric point of 9.8 and a molecular weight of about 18,000. It stimulates capillary endothelial cell proliferation *in vitro* half-maximally at 1 ng/ml and strongly stimulates angiogenesis on the chorioallantoic membrane of the chick embryo at a dose of 120 ng. Histologic sections reveal that neovascularization occurs in the virtual absence of inflammatory cells.

This tumor-derived angiogenic factor was eluted from the heparin–Sepharose column at about 1.5 M NaCl. Its strong affinity for heparin was not shared by other growth factors such as epidermal growth

* Dr. Sandor Szabo, Department of Pathology, Brigham and Women's Hospital, Boston, MA, personal communication.

factor (EGF) and platelet-derived growth factor (PDGF) that do not stimulate endothelial cell proliferation. EGF did not bind at all. PDGF bound but eluted at about 0.5 M NaCl. PDGF is of a charge similar to the tumor-derived angiogenic factor (isoelectric points between 9.5 and 10). From the similarities in positive charge, it might be expected that the two growth factors would bind equally well to heparin, a highly anionic glycosaminoglycan. The much stronger binding of the tumor-derived angiogenic factor suggests that it has a specific affinity for heparin. Additional evidence is that this growth factor does not bind to a column of chondroitin sulfate–Sepharose. Furthermore, an angiogenic factor obtained from retina, which is anionic, also binds tightly to heparin, and is eluted from heparin–Sepharose by 1 M NaCl [25].

*Speculation*

*Biological implications.* Could the fact that endothelial cell growth factors, having a strong affinity for heparin, underlie the ability of heparin to function as a regulator of angiogenesis? Could heparin be involved in the normal mechanisms of endothelial cell proliferation and growth control? Exogenous heparin binds avidly to vascular endothelial cells [26, 27]. While heparan sulfate is the major species of glycosaminoglycan on the endothelial cell surface [28], it is structurally and, at high concentrations, biologically similar to heparin [29, 30]. Thus, it is theoretically possible that vascular endothelial cells could bind specifically those growth factors that have a high affinity for heparin. This binding could potentiate growth factor activity. For example, it has been demonstrated that the proliferative effect of an endothelial cell growth factor on human umbilical vein endothelial cells is greatly potentiated by heparin but not by other glycosaminoglycans such as chondroitin sulfate [31]. There may be some type of three-way interaction between capillary endothelial cells, heparin, and endothelial cell growth factors (and angiogenic factors) with high affinity for heparin. One could speculate that capillary endothelial cells might be exposed to at least two classes of endothelial growth factors: (i) low level "maintenance" factors in the circulation (heparin or a heparan sulfate on the luminal surface of the endothelial cell might function to capture these factors from the circulating plasma) and (ii) potent angiogenic factors elaborated from tumor cells [23], activated macrophages [32], or sensitized T-lymphocytes [33]. These cells usually form a focus external to a resting capillary. Their secreted angiogenic factors would have to traverse an intact basement membrane before reaching the endothelial cell. Perhaps heparin or heparan sulfate within the basement membrane would serve as a specific channel for such angiogenic factors.

*Pharmacological implications.* These newly recognized activities of heparin suggest several important questions for future pharmacologic research.

(i) Can the angiogenesis promoting function and the angiogenesis inhibiting function of heparin be assigned to separate fragments of the molecule, or to a specific isomer of such a fragment? For example, Long and Williamson [34] have hypothesized that heparin preparations rich in 3-*O*-sulfated glucosamine content might be more likely to promote angiogenesis.

(ii) If specific heparin structures are responsible for specific angiogenic functions of heparin, or for its capacity to bind endothelial growth factors, can these structures be isolated and purified?

(iii) Is it possible that certain isomers of heparin could interfere with the angiogenic promoting or inhibiting functions of other heparin components?

(iv) What is the mechanism by which heparin–copper complexes might stimulate angiogenesis? An appealing idea, put forward by Long and Williamson [34], is that Cu(II)-heparin is a strong water-destructuring complex, and such a complex might enhance migration of capillary endothelial cells through the extracellular matrix.

(v) What are the pharmacokinetics of the putative heparin fragments responsible for various angiogenic functions? Are they active if administered orally? Is there a heparinase in the intestinal mucosa that degrades heparin itself when it is administered orally?

(vi) How could the angiogenesis inhibitory effect that results from the synergism of heparin and cortisone administered together be used as a pharmacologic agent? Cortisone has the side effects of immunosuppression and increased vulnerability to infection. Therefore, one might envision the use of a steroid that has anti-angiogenic activity but lacks glucocorticoid (and mineralocorticoid) activity. The major problem with heparin (after its anticoagulant activity has been eliminated) is that it contains both positive and negative angiogenesis regulatory activities. Ideally, a specific heparin fragment having only angiogenesis inhibitory activity would be administered with the appropriate steroid.

These questions indicate that there is a need for a better understanding of the structure–function relationships of small components of the heparin molecule. This need extends beyond the angiogenesis-regulating function of heparin. Other newly discovered functions of heparin should also be amenable to such an experimental approach. One example is the demonstration by Clowes and Karnovsky [35] that heparin inhibits the proliferation of vascular smooth muscle. Another is the finding by Sy *et al.* [36] that heparin suppresses delayed hypersensitivity.

Because three new functions of heparin have been identified only in the last 7 years, how many more functions await discovery? In summary, a central question for experimental pharmacology is to determine what additional information is stored in the fine structure of the heparin molecule.

*Clinical applications.* If specific components of heparin can be determined to be positive or negative regulators of angiogenesis, a variety of new diagnostic and therapeutic agents may be feasible. We have begun to realize that abnormal angiogenic phenomena dominate some dieseases in almost every branch of medicine [37]. For example, the surgeon depends on angiogenesis. Every incision initiates capillary growth. However, if angiogenesis is delayed, as in irradiated tissue, skin grafts die, wounds fail to heal, and anastomoses leak.

At other times, the surgeon and physician fear

new capillary growth that is out of control. New capillaries that persist or grow in the wrong place lead to blindness in retrolental fibroplasia, diabetes, retinal vein occlusion, and neovascular glaucoma to mention only a few. In fact, ocular neovascularization is one of the most common causes of blindness. Persistent neovascularization in the joint of an arthritic patient can destroy cartilage. Hemangiomas that grow swiftly on the face of young children bring about severe cosmetic, psychologic and ophthalmologic complications. The dermatologist deals with psoriasis, the otolaryngologist manages angiofibromas which may obstruct the airway or hemorrhage, and the hematologist is concerned with abnormal capillary proliferation in hemophiliac joints. The internist would like to prevent capillaries from disappearing in scleroderma. The cardiologist wishes to stimulate their growth in the infarcted heart. The oncologist is beginning to recognize that most solid tumors seem to be angiogenesis dependent and that their progressive growth requires the continuous induction of new capillaries which converge upon the tumor. These are but a few of the many diseases in which angiogenesis itself contributes to the progression of pathology.

In the face of many of these abnormal angiogenic phenomena, clinicians have until recently been passive observers, about as helpless as we were when confronted with infection in the nineteenth century and hemorrhagic disease in the twentieth century. The terms "angiogenesis inhibitor" and "angiogenesis stimulator" did not appear in the literature until about 6 years ago. Now many laboratories are beginning to work in this young field.

Will it be possible some day to employ a specific angiogenesis inhibitor to reverse neovascularization in diabetic retinopathy, or in an arthritic joint? Will angiogenesis inhibitors be useful in cancer chemotherapy or as an adjunct to radiotherapy? Will it ever be feasible to administer an angiogenesis stimulator to increase neovascularization in the infarcted heart, or to speed the appearance of new capillaries in a healing wound? The recognition that heparin can regulate angiogenesis has at least provided a basis from which to approach some of these questions.

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