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Inhibition of Angiogenesis Through Modulation of Collagen Metabolism

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Combinations of angiostatic steroids and heparin induce basement membrane breakdown, capillary retraction, and endothelial cell rounding as part of their antiangiogenic action (Ingber DE, Madri JA, Folkman J: Endocrinology 119:1768, 1986). Specific modulators of matrix metabolism were examined for their effects and on capillary development in the growing chick chorioallantoic membrane to explore the possibility that structural alterations of extracellular matrix (ECM) could be causally involved in the antiangiogenic mechanism. Regression of growing capillaries was induced by proline analogs (L-azetidine-2-carboxylic acid, *cis*-hydroxyproline, D,L-3,4-dehydroproline, thioproline) and an inhibitor of prolyl hydroxylase (α,α-dipyridyl) which interfere with triple helix formation and prevent collagen deposition. β-aminopropionitrile, an inhibitor of collagen cross-linking, was also antiangiogenic although an inhibitor of glycosaminoglycan deposition (β-methyl D-xyloside) was not. Combination of suboptimal doses of active collagen modulators with either angiostatic steroids or heparin resulted in great potentiation of their antiangiogenic effects. Coadministration of proline analogs, angiostatic steroids, and heparin resulted in complete inhibition of angiogenesis (avascular zones in 100% of chorioallantoic membranes) and larger and more extensive avascular zones than previously observed with steroid-heparin combinations. The antiangiogenic effects of these compounds (alone and in combination) were found to be directly related to their ability to inhibit collagen accumulation. Screening of additional compounds that are known to alter ECM turnover resulted in identification of a new angiogenesis inhibitor, all trans-retinoic acid. These findings suggest that growing capillaries may be "switched" into a regressive mode through alterations of collagen metabolism that result in loss of ECM structural integrity. Identification of ECM turnover as a major control point in angiogenesis may also provide a more rational approach for development of new antiangiogenic regimens.

Additional key words: Heparin, Steroid, Retinoic acid, Extracellular matrix, Basement membrane, Cell shape, Tension.

We have previously identified a class of natural steroid metabolites, known as angiostatic steroids, that inhibit angiogenesis when coadministered with heparin or nonanticoagulant heparin fragments (9, 16, 23). The mechanism by which these steroids produce regression of growing vessels remains unknown. However, angiostatic steroids appear to induce basement membrane dissolution as part of their antiangiogenic action (23). Basement membrane breakdown correlates with capillary retraction, endothelial cell rounding, cessation of growth, and vascular involution. Extracellular matrix (ECM) configurations that prohibit cell spreading and induce rounding similarly inhibit capillary endothelial cell growth *in vitro* (24). Basement membrane dissolution is also observed during involution of other epithelial tissues (21, 49). Thus, it is possible that steroid-induced changes of capillary ECM could act in a causal fashion to promote endothelial cell retraction and to inhibit angiogenesis.

We now show that regression of growing capillaries can be induced through specific inhibition of deposition or cross-linking of collagen. When administered in suboptimal doses, collagen modulators also strongly potentiate the antiangiogenic actions of angiostatic steroids and can obviate the requirement for heparin. Screening of additional compounds that are known to alter ECM turnover resulted in identification of a new antiangiogenic agent that can also potentiate angiostatic steroids.

EXPERIMENTAL DESIGN

EXPERIMENTAL SYSTEM

In the present study, previously characterized modulators of ECM metabolism were tested for their ability to induce capillary regression in the chick chorioallantoic membrane (CAM). These compounds were administered alone or in combination with angiostatic steroids and heparin. Methods for adaptation of the CAM system for study of angiogenesis have been previously described in

detail (1, 9, 23). In brief, methylcellulose sustained drug-release polymers were used for application of test substances on the exposed ectodermal surface of 6-day CAMs. Regression of capillaries within the subectodermal vascular bed resulted in formation of avascular zones (>4 mm diameter) within 48 hours of culture (8-day equivalent). Avascular zones were scored using a binocular dissecting scope at 10 × magnification (+, 4–6 mm diameter; ++, 6–8 mm; +++, >8 mm).

Materials

Cis-hydroxyproline (CHP), L-azetidine-2-carboxylic acid (LACA), and D,L-3,4-dehydroproline (DHP) were obtained from Calbiochem (La Jolla, California). Thioproline (TP) was acquired from Aldrich (Milwaukee, Wisconsin). α,α-dipyridyl (DPY), β-aminopropionitrile fumarate (BAPN), β-methyl-D-xyloside, and all trans-retinoic acid were purchased from Sigma Chemical Company (St. Louis, Missouri). Heparin (Hepar Inc., Franklin, Ohio) and angiostatic steroid (6α-fluoro-17,21-dihydroxy-16β-methyl-pregna-4,9-(11)-diene-3,20-dione; kindly supplied by Upjohn Co., Kalamazoo, Michigan) were applied to CAMs at 50 and 70 μg, respectively when used in these studies.

Light Microscopy

To better visualize regions of capillary regression, india ink was first mixed at a ratio of 1:7 with a lipid emulsion (Liposyn 10%, Abbot Laboratories, North Chicago, Illinois) and then injected directly into the vascular system of living embryos using a 33 gauge needle (Hamilton, Reno, Nevada). Injected CAMs were fixed in formalin, whole-mounted, and photographed from above using a Zeiss dissecting microscope and AGFA 25 film. Collages were reconstructed from overlapping printed images representing adjacent regions directly beneath and surrounding the methylcellulose release polymer.

Effects on Collagen Accumulation

Regions of the CAM present directly beneath methylcellulose release polymers were dissected away from 8-day CAMs. Normal controls consisted of regions of CAM that were exposed to polymer that was free of drug. Tissue samples (approximately 4 × 4 mm squares) were hydrolyzed in 6 N HCl and their total amino acid composition was determined by the Microchemistry Facility, Harvard University. Hydrolyzed amino acids were derivatized to phenylisothiocyanate and resolved on a Hewlett-Packard 1084B high performance liquid chromatography apparatus using an Altex-ODS 3-μm column.

Effects on collagen accumulation were estimated based upon the observation that hydroxyproline is primarily found in collagens, whereas most other amino acids are found almost equally distributed in all proteins. In this study, we measured changes in the molar ratio of hydroxyproline:alanine as an estimate of effects on collagen content relative to general protein accumulation (standard error was consistently less than 15% of the mean for all ratios measured in this study). Alanine was chosen as a reference amino acid because the ratio of hydroxyproline to alanine varied least from experiment to experiment within control 8 day CAMs. Maximal inhibition of the hydroxyproline/alanine ratio produced in this study was a 40% reduction below control values. This method only serves as an estimate of the relative amount of collagen and does not distinguish between effects on hydroxylation versus accumulation of collagen.

RESULTS AND DISCUSSION

Inhibitors of Collagen Deposition

Analogs of proline (*e.g.*, CHP, LACA, DHP, and TP) are incorporated into collagen in place of proline during collagen synthesis. The presence of these analogs within the collagen peptide interferes with protein folding (25). Aberrant triple helix formation results in impaired secretion of interstitial collagens and acceleration of intracellular collagen degradation (5). While incorrectly folded basement membrane collagen appears to be able to be secreted by cells (33), it cannot be deposited within organized extracellular matrices *in vitro* (32, 33, 48) or *in vivo* (49). The net result of treatment of cells with proline analogs is therefore inhibition of total collagen deposition into ECM although general protein accumulation is usually not significantly affected (45).

Application of the proline analog, CHP, to the 8-day chick CAM resulted in dose-dependent inhibition of angiogenesis as measured by the percentage of CAMs exhibiting avascular zones on day 8 (Fig. 1). CHP induced avascular zones in 100% CAMs at doses of 600 μg and above. The size (4 to 6 mm diameter) and appearance of these zones were similar to those induced by combinations of angiostatic steroid and heparin (see Fig. 1 in Ref. 23). Other proline analogs (LACA, DHP, and TP) were tested on the CAM and were found to display similar antiangiogenic activity. It should be noted that the proline analog, DHP, can also alter collagen deposition by inhibiting prolyl hydroxylase activity (27, 35). Avascular zones were also produced using DPY to interfere with collagen deposition exclusively via inhibition of the enzymes prolyl and lysyl hydroxylase. The relative efficacy of these different inhibitors of collagen deposition is shown in Figure 2.

When used in suboptimal doses, proline analogs greatly potentiated the antiangiogenic effect of angiostatic steroids and heparin (Fig. 3). As previously described (9), combination of angiostatic steroid and hep-

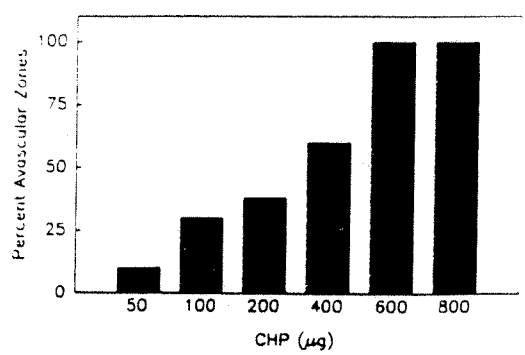

FIG. 1. Dose-dependent induction of avascular zones in the chick CAM by the proline analog, *cis*-hydroxyproline (*CHP*).

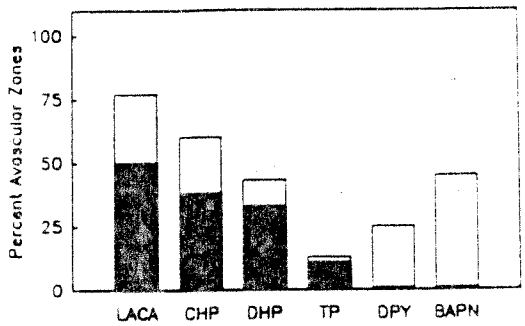

FIG. 2. Comparison of the antiangiogenic effects of different collagen modulators at 200 μg (*closed bars*) and 400 μg (*open bars*). *LACA*, L-azetidine-2-carboxylic acid; *CHP*, *cis*-hydroxyproline; *DHP*, D,L-3,4-dehydroproline; *TP*, thioproline; *DPY*, α,α dipyridyl; *BAPN*, β-aminopropionitrile.

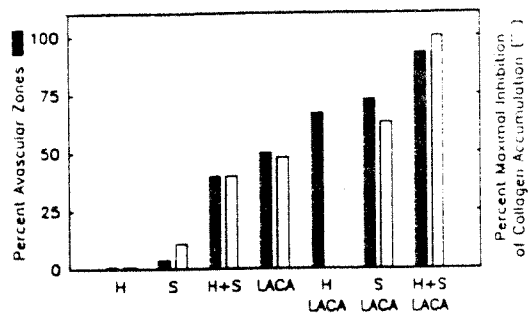

FIG. 3. Comparison of effects of heparin, steroid, and LACA on angiogenesis (*closed bars*) and collagen accumulation (*open bars*). Method for determination of collagen accumulation is described in the "Experimental Design" section. Addition of LACA to heparin, steroid, or heparin-steroid combinations resulted in significant potentiation of antiangiogenic activity ($p < 0.001$ as determined using a nonparametric test for significance between different proportions). *H*, heparin (50 μg); *S*, angiostatic steroid (70 μg); *LACA*, L-azetidine-2-carboxylic acid (200 μg).

arin produced avascular zones (4 to 6 mm in diameter) in approximately 40% of CAMs even though each compound has little effect when administered alone. Interestingly, coadministration of a half-maximal concentration of LACA (200 μg) with either steroid or heparin resulted in potentiation of antiangiogenic activity. Addition of LACA (200 μg) to the steroid-heparin combination produced avascular zones in over 90% of CAMs. Zones were also larger in diameter (>6 mm) than those observed with only the steroid-heparin combination and regions outside the zone even exhibited a drastic decrease in capillary density (Fig. 4). Combination of heparin and steroid with higher concentrations of either LACA or CHP (>200 μg) induced avascular zones in 100% of CAMs (not shown).

The antiangiogenic effects of these drug combinations were found to correlate directly with inhibition of collagen accumulation within 8-day CAMs as estimated by amino acid analysis (Fig. 3). In this series of experiments, maximal inhibition of collagen accumulation was observed with combinations of LACA (200 μg), heparin (50 μg), and steroid (70 μg). The same dose of LACA was also able to inhibit collagen deposition when administered alone or in combination with steroid. Angiostatic steroid alone produced a small decrease in collagen accumulation. These steroids have little antiangiogenic activity in the absence of heparin. Heparin did not alter collagen accumulation or produce avascular zones. Importantly, the antiangiogenic heparin-steroid combination produced a large depletion of collagen relative to total protein content in the CAM (40% of maximal inhibition exhibited by the LACA-heparin-steroid combination).

L-proline was coadministered with proline analogs to confirm that their antiangiogenic effects resulted from specific perturbation of proline metabolism. The antiangiogenic effects of CHP (400 μg) were almost entirely abrogated by simultaneous application of equimolar amounts of L-proline (Fig. 5). Similarly, the potentiation of the heparin-steroid combination of LACA (increase from 40% to 93% avascular zones) was completely inhibited by coadministration of L-proline (Fig. 5). Further evidence that the antiangiogenic effects of proline analogs did not result from inhibition of general protein synthesis came from studies using cycloheximide. This potent inhibitor of protein synthesis never produced avascular zones in the CAM. Cycloheximide was inactive at lower doses (<0.05 μg) and resulted in death of the embryo at higher concentrations. In contrast, application of collagen modulators never resulted in systemic toxicity (*i.e.*, even at doses as high as 800 μg; not shown).

INHIBITION OF COLLAGEN CROSS-LINKING

BAPN was tested in the CAM system to directly determine whether alterations of ECM structural integrity could be involved in the antiangiogenic mechanism. BAPN inhibits collagen cross-linking by preventing aldehyde formation and by inhibiting the enzyme lysyl oxidase. An increase in noncross-linked collagen results in decreased tensile strength of collagenous matrices. In the present study, BAPN inhibited angiogenesis when administered alone and was as potent as many of the proline analogs (Fig. 2). Furthermore, the antiangiogenic effects of both angiostatic steroids and steroid-heparin combinations were strongly potentiated by low concentrations of BAPN (200 μg) that were inactive when administered alone (Fig. 6).

SCREENING OF OTHER MATRIX MODULATORS

The present findings in conjunction with those of a previous study (23) strongly suggest that changes in ECM may be central to the antiangiogenic mechanism. If so, then screening of compounds that are known to alter ECM turnover may serve to facilitate identification of new inhibitors of capillary development.

We first tested β-methyl-D-xyloside, an inhibitor of glycosaminoglycan deposition, but found it to be inactive in the CAM system. Xylosides substitute as initiation sites for glycosaminoglycan polymerization. The absence of linkage to proteoglycan core protein apparently inhibits deposition of xyloside-containing glycosaminoglycans into ECM. In this manner, they stimulate synthesis and secretion of glycosaminoglycans in a soluble form (18). It should also be noted that β-methyl-D-xyloside affects

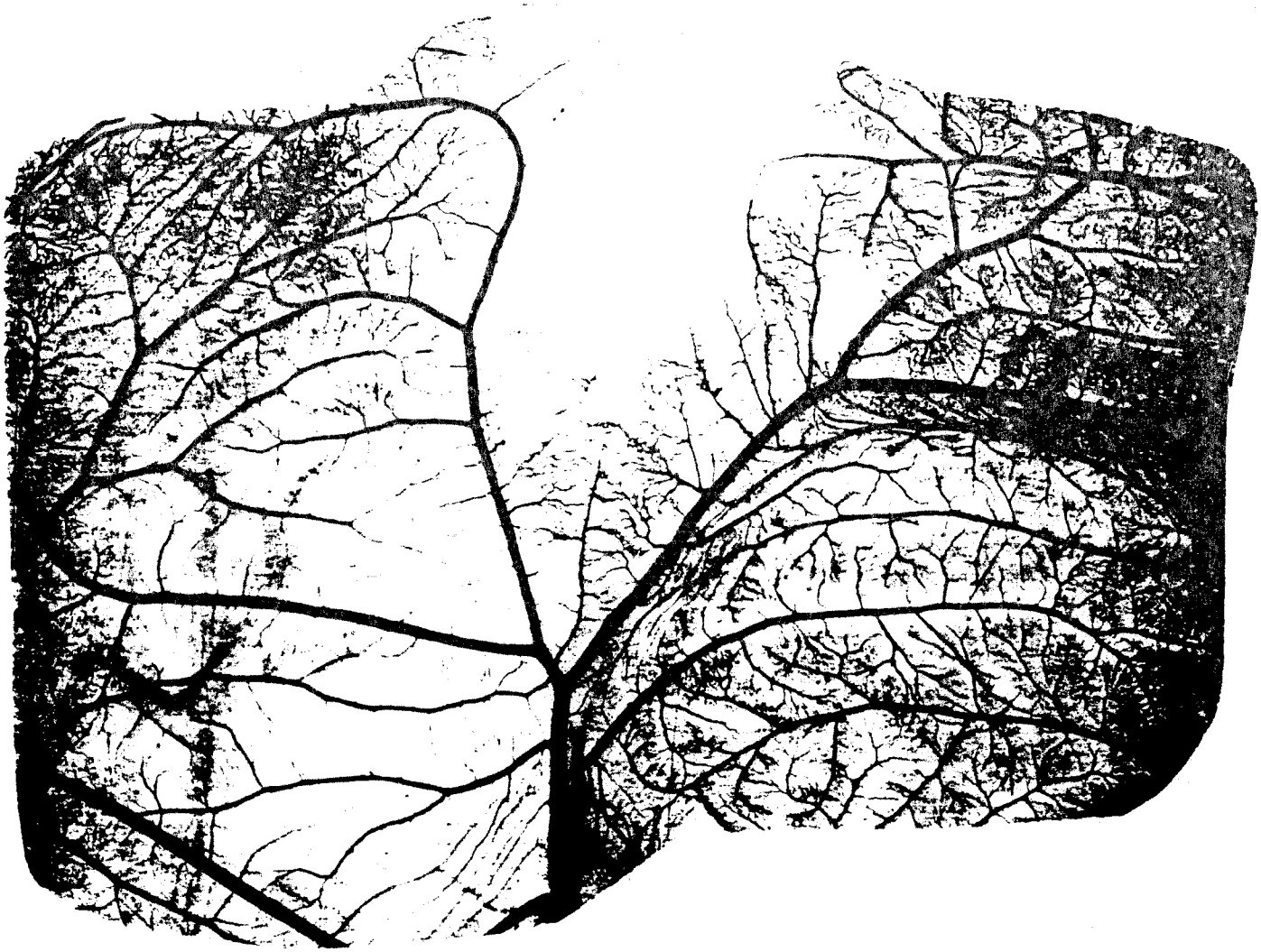

FIG. 4. Composite light micrograph collage of an india ink-injected CAM displaying regression of the vascular network in response to 48-hour exposure to a combination of LACA (200 μg), heparin (50 μg), and angiostatic steroid (70 μg). Bar = 1 cm.

production of chondroitin sulfate more than heparan sulfate (18) which would be of much greater interest in context of angiogenesis. The lack of antiangiogenic activity of this xyloside therefore does not rule out an important role for proteoglycans during angiogenesis.

Retinoic acid has also been reported to alter the metabolism of a variety of ECM components including glycosaminoglycans (41), laminin (7, 39, 47), fibronectin (39), and collagens (36, 39, 47). When all trans-retinoic acid was tested alone, it was found to inhibit angiogenesis in 100% of CAMs at 1 μg. However, it was toxic and resulted in death of embryos at higher doses. Once again, suboptimal doses of retinoic acid potentiated angiostatic steroid and heparin as well as combinations containing proline analogs.

The most effective antiangiogenic regimens observed in these studies were either (a) angiostatic steroid (70 μg), heparin (50 μg), and LACA (400 μg), or (b) angiostatic steroid (70 μg), LACA (400 μg), and all trans-retinoic acid (1 μg). The latter combination did not require heparin. Application of methycellulose polymers containing these antiangiogenic combinations on 6-day CAM resulted in initiation of zone formation within 24 hours. Of these CAMs, 100% exhibited avascular zones that extended to cover regions over 1 cm in diameter (Fig. 4). Avascularity was maintained for an additional 72 hours. This inhibition was reversible since avascular regions always revascularized if maintained in culture for extended periods without readministering drug. Furthermore, it is most important to note that no antiangiogenic regimen could produce capillary regression when applied to 11-day CAM. Vessels within the subectodermal capillary bed of the CAM cease growing at day 10 to 11 of development (2). Thus, these potent inhibitors of angiogenesis only produce involution of capillaries that are actively growing.

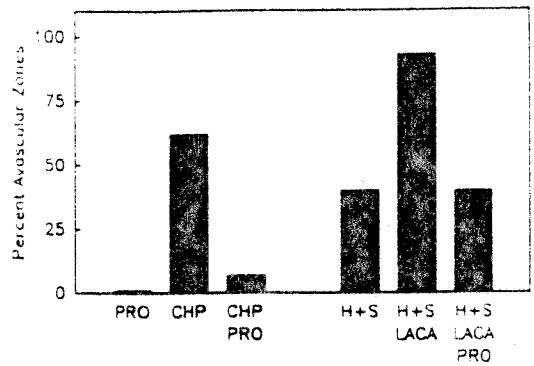

FIG. 5. Abrogation of the effects of proline analogs using exogenous L-proline. Coadministration of L-proline with angiogenic regimens containing proline analogs resulted in a significant decrease in the percentage of avascular zones ($p < 0.001$). *PRO*, (400 μg and 200 μg in the CHP and LACA experiments, respectively); *CHP*, 400 μg; *H+S*, heparin (50 μg) + angiostatic steroid (70 μg); *LACA*, L-azetidine-2-carboxylic acid (200 μg).

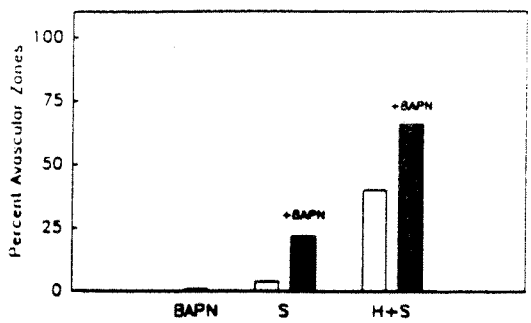

FIG. 6. Potentiation of the antiangiogenic effects of angiostatic steroid and heparin-steroid combinations by an inactive dose of BAPN. Coadministration of an inactive dose of BAPN with the heparin-steroid combination resulted in a significant increase in antiangiogenic activity ($p < 0.05$). *BAPN*, β-aminopropionitrile (200 μg); *S*, angiostatic steroid (70 μg); *H+S*, heparin (50 μg) + angiostatic steroid (70 μg). *Solid bars*, in the presence of 200 μg of BAPN.

DISCUSSION

In our past study on the mechanism of capillary involution, we demonstrated a direct correlation between local basement membrane dissolution, vessel retraction, endothelial cell rounding, and capillary regression (23). However, it was unclear whether these ECM alterations were the cause or result of capillary involution. In the present study, we set out to examine whether specific modulators of matrix metabolism could actively inhibit angiogenesis. We focused on inhibitors of collagen synthesis and maturation because capillary ECM contains various collagen subtypes. Types IV and V collagens are present within vascular basement membrane and type III collagen is found within the interstitial matrix that surrounds and supports the entire vasculature (31). Endothelial cells accumulate collagens as they grow *in vivo* (17, 40) and their migration can be inhibited by using proline analogs to interfere with collagen deposition *in vitro* (32). Endothelial cell growth and migration also appear to require ongoing collagen degradation (26). Thus, we previously suggested that controlled alterations of ECM metabolism could serve as an important control point in capillary development (16, 23, 24).

We now show that inhibition of collagen accumulation is sufficient to induce capillary regression in the growing chick CAM. Specific inhibitors of collagen deposition such as the proline analogs, CHP and LACA, were highly antiangiogenic when administered alone. In suboptimal doses they were also strong potentiators of angiostatic steroids. Furthermore, antiangiogenic potency of all drug combinations studied correlated directly with their ability to inhibit collagen accumulation. Capillary involution was similarly produced by DPY which interferes with collagen synthesis by inhibiting prolyl and lysyl hydroxylases. Proline analogs and DPY also interfere with morphogenesis of other tissues including cornea (8), cartilage (3), thyroid gland (20), lung (43), salivary gland (43), and mammary gland (49).

For the first time it is also clear that angiostatic steroids have a direct effect on ECM turnover as they produce a net decrease in collagen accumulation when administered in combination with heparin. This biochemical finding supports our previous immunofluorescence data obtained with antibodies against basement membrane glycoproteins (23) and collagens (D. E. Ingber, J. A. Madri, and J. Folkman, unpublished observation). Yet, it is still unknown whether steroid-heparin combinations inhibit collagen deposition, increase its degradation, or interfere with the activity of endogenous collagenase inhibitors. Interestingly, angiostatic steroids appear to have little effect on either angiogenesis or collagen accumulation when administered on their own.

Heparin has no effect on collagen accumulation when administered alone. Yet, it clearly potentiates the anti-collagen and antiangiogenic effects of both steroids and proline analogs. The mechanism of this potentiation is unknown. Heparin could alter steroid metabolism locally within mesenchymal cells (11) or endothelium. For example, heparin could potentiate steroid action by reducing angiostatic steroid deactivation or by facilitating production of a more potent metabolite. Heparin probably does not act by altering steroid binding or uptake by endothelial cells since heparin appears to act in a similar fashion to potentiate the anti-angiogenic effects of the other nonsteroidal matrix modulators.

An alternative explanation for the effects of heparin on angiogenesis relates to the observation that addition of heparin in the absence of steroids normally accelerates capillary growth *in vivo* (42) as well as endothelial cell migration *in vitro* (4). Endothelial cell migration involves both collagen deposition (32) and breakdown (26). Heparin-dependent acceleration of capillary elongation may therefore be mediated by coordinated increases in both ECM synthesis and degradation. If additional inhibitors of collagen deposition were administered in this context (*e.g.*, proline analogs, angiostatic steroids), then collagen-containing substrates might rapidly break down since ongoing degradation would no longer be matched by new synthesis. Heparin could also modulate capillary development via changes in collagen fibrillogenesis (30) or alterations of the biophysical characteristics of collagenous matrices (34). In this manner, heparin may serve to potentiate antiangiogenic compounds by altering the local connective tissue environment surrounding the growing endothelium.

BAPN also produced capillary regression. The inhibitory activity of BAPN most likely resulted from its ability to inhibit collagen cross-linking. Collagen that contains fewer cross-links may also be more labile metabolically (38). However, in the present study, even low doses of BAPN that had no effect on angiogenesis were found to strongly potentiate the antiangiogenic effects of steroid and heparin. As previously shown, BAPN has no effect on the morphogenesis of certain tissues (43) although it can severely retard the development of others (37, 44).

The antiangiogenic effects of collagen modulators must be understood in terms of effects on capillary growth and form. Collagens are present both in a fibrillar form in the interstitium and within a network arrangement in capillary basement membrane. Inhibitors of hydroxyproline formation (*e.g.*, proline analogs, DPY) interfere with deposition of collagen into both types of ECM. In addition, in some systems, basement membrane production is in turn dependent upon accumulation of adjacent interstitial collagen fibrils (10, 12). Inhibitors of collagen deposition may therefore induce basement membrane alterations by both direct and indirect means.

Inhibitors of collagen cross-linking would be expected to have little direct effects on basement membrane integrity since lysine cross-links do not play a major role in basement membrane assembly. However, BAPN could alter the structural integrity of capillary basement membrane indirectly by decreasing the loadbearing strength of neighboring interstitial collagen fibrils. Fibrillar collagen may provide much of the structural integrity that is responsible for maintenance of branching tissue morphology as it usually accumulates within clefts at sites of epithelial folding (6, 43).

Capillaries normally undergo extensive branching morphogenesis and can rapidly extend to reach over large distances within developing and healing tissues (*e.g.* approximately 0.2 mm/day in response to implantation of endothelial mitogens within rabbit cornea; 42). The extensive cell elongation that is observed during angiogenesis *in vivo* (14) and within mitogen-stimulated endothelial cells *in vitro* (24) requires a sufficient number of attachment points. Cell spreading also requires an underlying anchoring foundation that can physically resist cell-generated tensile forces (12, 19, 22). Any increase in malleability or loss of structural integrity of the endothelial cell attachment foundation (*e.g.*, collagen breakdown, decreased tensile strength of fibrillar collagens, gradual basement membrane dissolution) would result in cell retraction and rounding.

Capillary endothelial cell DNA synthesis decreases in an exponential fashion in direct response to linear decreases in cell extension (*i.e.*, increases in cell rounding) (24). In fact, endothelial cells rapidly lose their viability when maintained in a round or unattached state (13). Thus, any combination of drugs that results in loss of structural integrity of basement membrane (the *in vivo* substratum for cell anchorage) would negate the growth-promoting effects of angiogenic stimuli and eventually result in endothelial cell death. As previously suggested (22, 23), controlled involution of growing epithelium or endothelium may therefore be the result of natural coordination between matrix resorption and spontaneous cell retraction. It is interesting that none of our inhibitors induce capillary regression in older CAMs that contain nongrowing capillaries. This may relate to the observation that these vessels contain thicker and more complete basement membranes that most likely exhibit greater structural integrity and lower baseline rates of matrix turnover.

The concept that alterations of ECM metabolism and structure play a central regulatory role in angiogenesis also provides a more effective means for identification and development of new inhibitors of capillary growth. For example, our initial screening using this rationale resulted in identification of a highly antiangiogenic compound, all trans-retinoic acid. On the other hand, 3-methyl-D-xyloside, an inhibitor of glycosaminoglycan deposition, was found to be inactive demonstrating that "matrix modulation" is not a guarantee of antiangiogenic activity.

Angiogenesis inhibitors could be of potential clinical importance because it is becoming clear that many ostensibly disparate diseases may be "angiogenesis-dependent" (15, 16). Proline analogs, on their own, would most likely make poor chemotherapeutic agents since they can have generalized nonspecific effects (*e.g.*, secondary to general protein synthesis inhibition) when administered in high concentrations *in vivo*. Similarly, retinoic acid is limited by its systemic toxicity at high doses. The importance of the angiostatic steroids is: (a) they have little if any systemic toxicity (b) they are potentiated by suboptimal (nontoxic) doses of other matrix modulators, and (c) they apparently can focus the action of general ECM modulators so that their effects are limited only to growing capillaries.

Our long-term objective is the treatment of diseases (*e.g.*, solid tumors) that depend upon continued neovascularization. We envision that angiostatic steroids may be combined with suboptimal doses of the matrix modulators to selectively inhibit the pathologic growth of capillaries. Past investigators have shown that the growth of some tumors can be inhibited by proline analogs (28, 46, 50). Whereas much of this inhibitor effect appeared to be due to effects on the tumor cells themselves (46), it is possible that some of this activity related to inhibition of collagen deposition by associated capillary endothelial cells within growing tumor vessels. Coadministration of angiostatic steroids with these inhibitors of collagen deposition could serve to amplify and focus these inhibitory effects on the tumor vasculature. Steroid-matrix modulator combinations that are free of heparin could also be applied topically or in conjunction with sustained drug-release polymer systems (29).

In conclusion, these data indicate that capillary regression can be induced through inhibition of collagen deposition or via interference with collagen cross-linking in the growing chick CAM. Similar drug combinations have no effect on older, nongrowing CAM vessels. Capillary growth and elongation during early stages of angiogenesis may therefore normally require synthesis of an extracellular collagen-containing substrate that can support capillary endothelial cell spreading and growth *in vivo*. Capillary regression in turn appears to result from loss of

ECM tensile strength or through compromise of its structural integrity. Thus, as previously proposed (22), the growth of large populations of endothelial cells seem to be controlled and coordinated in space through directed structural alterations of a common matrix attachment scaffolding. In this manner, matrix-dependent alterations of cell shape may serve as a "solid state" switching control during angiogenesis as well as a site for specific pharmacological perturbation.

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REFERENCES

1. Auerbach R, Kubai L, Knighton D, Folkman J: A simple procedure for the long-term cultivation of chicken embryos. Dev Biol 41:391, 1974
2. Ausprunk DH, Knighton DR, Folkman J: Differentiation of vascular endothelium in the chick chorioallantois: a structural and autoradiographic study. Dev Biol 38:237, 1974
3. Aydelotte MB, Kochmar DM: Development of mouse limb buds in organ culture: chondrogenesis in the presence of a proline analog, L-azetidine-2-carboxylic acid. Dev Biol 28:191, 1972
4. Azizkhan RG, Azizkhan JC, Zetter BR, Folkman J: Mast cell heparin stimulates migration of capillary endothelial cells in vitro. J Exp Med 152:931, 1980
5. Berg RA, Schwartz ML, Crystal RG: Regulation of the production of secretory proteins: intracellular degradation of newly synthesized "defective" collagen. Proc Natl Acad Sci USA 77:4746, 1980
6. Bernfield MR, Banerjee SD: The basal lamina in epithelial-mesenchymal interactions. In Biology and Chemistry of Basement Membranes, edited by Kefalides N, p 137. New York, Academic Press, 1978
7. Carlin BE, Durkin M, Bender B, Jaffee R, Chung AE: Synthesis of laminin and entactin by F9 cells induced with retinoic acid and dibutyryl cyclic AMP. J Biol Chem 258:7729, 1983
8. Coulombre AJ, Coulombre JL: IV. Interruption of collagen excretion into the primary stroma of the cornea with L-azetidine-2-carboxylic acid. Dev Biol 28:183, 1972
9. Crum R, Szabo S, Folkman J: A new class of steroids inhibits angiogenesis in the presence of heparin or a heparin fragment. Science 230:1375, 1985
10. David G, Bernfield MR: Collagen reduces glycosaminoglycan degradation by cultured mammary epithelial cells: possible mechanism for basal lamina formation. Proc Natl Acad Sci USA 76:786, 1979
11. Dougherty TF, Berliner DL: The effects of hormones on connective tissue cells. In Treatise on Collagen, edited by Gould BS, Vol 2, p 367. New York, Academic Press, 1968
12. Emerman JT, Pitelka DR: Maintenance and induction of morphological differentiation in dissociated mammary epithelium on floating collagen membranes. In Vitro 13:316, 1977
13 Folkman J, Moscona A: Role of cell shape in growth control. Nature 273:345, 1978
14. Folkman J: Angiogenesis: initiation and control. Ann NY Acad Sci 401:212, 1982
15. Folkman J, Klagsbrun M: Angiogenic factors. Science 235:442, 1987
16. Folkman J, Ingber DE: Angiostatic steroids: method of discovery and mechanism of action. Ann Surg 206:374, 1987
17. Form D, Pratt BM, Madri JA: Endothelial cell proliferation during angiogenesis. *In vitro* modulation by basement membrane components. Lab Invest 55:521, 1986
18. Galligan L, Horwood J, Schwartz NB, Dorfman A: Stimulation of synthesis of free chondroitin sulfate chains by β-D-xylosides in cultured cells. J Biol Chem 256:5400, 1985
19. Harris AK, Wild P, Stopak D: Silicone rubber substrata: a new wrinkle in the study of cell locomotion. Science 208:177, 1980
20. Hilfer SR, Pakstis GL: Interference with thyroid histogenesis by inhibitors of collagen synthesis. J Cell Biol 75:446, 1977
21. Ikawa H, Treistad RL, Hutson JM, Manganaro TF, Donahoe PK: Changing patterns of fibronectin, laminin, type IV collagen, and a basement membrane proteoglycan during rat Mullerian duct regression. Dev Biol 102:260, 1984
22. Ingber DE, Jamieson JD: Cells as tensegrity structures: architectural regulation of histodifferentiation by physical forces transduced over basement membrane. In Gene Expression During Normal and Malignant Differentiation, edited by Andersson LC, Gahmberg CG, Ekblom P, p 13. Orlando, Academic Press, 1985
23. Ingber DE, Madri JA, Folkman J: A possible mechanism for inhibition of angiogenesis by angiostatic steroids: induction of capillary basement membrane dissolution. Endocrinology 119:1768, 1986
24. Ingber DE, Madri JA, Folkman J: Endothelial growth factors and extracellular matrix regulate DNA synthesis through modulation of cell and nuclear expansion. In Vitro Cell Dev Biol 23:387, 1987
25. Jimenez SA, Yankowski R: Role of molecular conformation on secretion of chick tendon procollagen. J Biol Chem 253:1420, 1978
26. Kalebic T, Garbisa S, Glaser B, Liotta L: Basement membrane collagen:degradation by migrating endothelial cells. Science 221:281, 1983
27. Kerwar SS, Felix AM, Marcel RJ, Tsai I, Salvador RA: Effect of L-3,4-dehydroproline on collagen synthesis and prolyl hydroxylase activity in mammalian cell cultures. J Biol Chem 251:503, 1978
28. Kidwell WR, Taylor S, Bano M, Grantham F: Growth arrest of mammary tumors by proline analogs. In Progress in Cancer Research and Therapy, edited by Bresciani F, King RJB, Lippman ME, Namer M, Raynaud J-P, Vol 31, p 129. New York, Raven Press, 1984
29. Langer R, Folkman J: Polymers for the sustained release of proteins and other molecules. Nature 263:797, 1976
30. Lindahl U, Hook M: Glycosaminoglycans and their binding to biological macromolecules. Ann Rev Biochem 47:385, 1978
31. Madri JA, Furthmayr H: The collagenous components of the subendothelium: correlation of structure and function. Lab Invest 43:303, 1980
32. Madri JA, Stenn KS: Aortic endothelial cell migration. I. Matrix requirements and composition. Am J Pathol 106:180, 1982
33. Maragoudakis ME, Kalinsky HJ, Wasvary J: Basement membrane biosynthesis: secretion without deposition of underhydroxylated basement membrane collagen by parietal yolk sacs. Biochim Biophys Acta 538:139, 1978
34. Newman S, Frenz DA, Tomasek JJ, Rabuzzi DD: Matrix-driven translocation of cells and nonliving particles. Science 228:885, 1985
35. Nolan JC, Ridge S, Oronsky AL, Kerwar SS: Studies on the mechanism of reduction of prolyl hydroxylase activity by D,L-3,4 dehydroproline. Arch Biochem Biophys 189:448, 1978
36. Oikarinen H, Oikarinen AI, Tan EML, Abergel RP, Meeker CA, Chu M-L, Prockop DJ, Uitto J: Modulation of procollagen gene expression by retinoids: inhibition of collagen production by retinoic acid accompanied by reduced type I procollagen messenger ribonucleic acid levels in human skin fibroblast cultures. J Clin Invest 75:1545, 1985
37. Pratt RM Jr, King CTG: Inhibition of collagen cross-linking associated with β-aminopropionitrile-induced cleft palate in the rat. Dev Biol 27:322, 1972
38. Prockop DJ, Kivirikko KI, Tuderman L, Guzman NA: The biosynthesis of collagen and its disorders. New Engl J Med 301:77, 1979
39. Salomon DS, Liotta LA, Rennard SI, Foidart J-M, Terranova V, Yaar M: Stimulation by retinoic acid of synthesis and turnover of basement membrane in mouse embryonal carcinoma-derived endodermal cells. Coll Relat Res 2:93, 1982
40. Sariola H, Timpl R, Von Der Mark K, Mayne R, Fitch JM, Linsenmayer TF, Ekblom P: Dual origin of glomerular basement membrane. Dev Biol 101:86, 1984
41. Shapiro SS, Poon JP: Effect of retinyl acetate on sulfate glycosa-

minoglycan biosynthesis in dermal and epidermal cells in vitro. Connect Tissue Res 6:101, 1978
42. Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M: Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science 223:1296, 1984
43. Spooner BS, Faubion JM: Collagen involvement in branching morphogenesis of embryonic lung and salivary gland. Dev Biol 77:84, 1980
44. Tanzer ML: Experimental lathryism. Int Rev Connect Tissue Res 3:891, 1967
45. Uitto J, Hoffmann H-P, Prockop DJ: Synthesis of elastin and procollagen by cells from embryonic aorta: Differences in the role of hydroxyproline and the effects of proline analogs on the secretion of the two proteins. Arch Biochem Biophys 173:187, 1976
46. Vembu D, Liotta LA, Paranjpe M, Boone CW: Correlation of tumorigenicity with resistance to growth inhibition by *cis*-hydroxyproline. Exp Cell Res 124:247, 1979
47. Wang S, Gudas LJ: Isolation of cDNA clones specific for collagen IV and laminin from mouse teratocarcinoma cells. Proc Natl Acad Sci USA 80:5880, 1983
48. Wicha MS, Liotta LA, Garbisa S, Kidwell WR: Basement membrane collagen requirements for attachment and growth of mammary epithelium. Exp Cell Res 124:181, 1979
49. Wicha MS, Liotta LA, Vonderhaar BK, Kidwell WR: Effects of inhibition of basement membrane collagen deposition on rat mammary gland development. Dev Biol 80:253, 1980
50. Wicha MS, Liotta LA, Lewko WM, Kidwell WR: Blocking basement membrane collagen deposition inhibits the growth of 7,12-dimethylbenzanthracene-induced rat mammary tumors. Cancer Letters 12:9, 1981