Angiogenesis Inhibition and Tumor Regression Caused by Heparin or a Heparin Fragment in the Presence of Cortisone

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We previously demonstrated that angiogenesis in vivo can be promoted by heparin and inhibited by protamine (1). The angiogenesis inhibitory property of protamine was discovered from sequential experiments in which (i) mast cells were found to accumulate at a tumor site before the ingrowth of new capillary sprouts (2); (ii) heparin released by mast cortisone are administered together, angiogenesis is abolished, regardless of the type of angiogenic stimulus. A non-anticoagulant fragment of heparin can substitute for heparin in this synergism; this fragment is a hexasaccharide. We further show that heparin administered orally is degraded into non-anticoagulant fragments which, in the presence of corti-

Abstract. *Heparin or a heparin fragment administered with cortisone inhibited angiogenesis, caused regression of large tumor masses, and prevented metastases. Oral administration of heparin resulted in the release of non-anticoagulant heparin fragments in the serum which, in the presence of cortisone, had similar anti-angiogenic and antitumor effects. Of all the heparin fragments tested, the most potent inhibition of angiogenesis in the presence of cortisone was provided by a hexasaccharide with a molecular weight of about 1600.*

cells (3) increased the migration (4) of capillary endothelial cells in vitro (5) and heparin enhanced tumor angiogenesis on the chorioallantoic membrane (CAM) of the chick embryo (1); and (iii) protamine, an antagonist of heparin, blocked the ability of mast cells or heparin to stimulate migration of capillary endothelial cells in vitro (3) and inhibited tumor angiogenesis in the chick embryo (1).

On the basis of these findings, we thought that tumor angiogenesis enhanced by heparin might be made more conspicuous in the chick embryo bioassay by adding cortisone to suppress background inflammation. The result was unexpected. While heparin alone enhanced tumor angiogenesis and cortisone alone had little or no effect, angiogenesis was inhibited by the combination of heparin and cortisone.

We now show that when heparin and

sone, bring about more potent inhibition of angiogenesis than any agent previously described, including protamine. Large tumor masses regress and metastases are prevented.

Inhibition Is Independent of Type of Angiogenic Stimulus

Tumor angiogenesis. The first evidence of the anti-angiogenic activity of heparin and cortisone came from studies of tumor angiogenesis on the CAM's of 9-day chick embryos. Plastic cover slips coated with heparin and tumor extract stimulated intense angiogenesis on the CAM. When cortisone was added with heparin, angiogenesis was abolished. Cortisone alone did not inhibit tumor angiogenesis (Table 1).

Similar inhibition was observed in the rabbit cornea. A sustained-release pellet

(6) containing heparin, cortisone, or a combination of the two was positioned between the tumor and normal vessels in the rabbit cornea (7), allowing the inhibition of new capillary growth to be measured as a function of the rate of linear capillary growth. The pellets released heparin for 14 days and cortisone for more than 30 days, whether the compounds were alone or together in the pellet. New capillaries grew toward the tumor implants at 0.44 mm per day when the pellets contained heparin, 0.22 mm per day when they contained cortisone, and 0.44 mm per day when the pellets were empty. However, no capillary growth occurred when the pellets contained heparin and cortisone together. When the heparin-cortisone pellets were depleted or removed, capillary growth resumed. Histologic sections showed that tumor cells were viable and replicating even when they were adjacent to the heparin-cortisone pellet.

Embryonic angiogenesis. New vessels appear in the yolk sac of the chick embryo at 48 hours and grow rapidly over the next 6 to 8 days. Application of methylcellulose disks (1) containing both heparin and cortisone to the 4-day yolk sac produced avascular zones within 48 hours. No avascular zones appeared with either heparin or cortisone alone.

The growing vessels of the 6-day CAM were also inhibited by heparin- and cortisone-containing disks, but not by disks containing either alone (Fig. 1). In the mature (8) CAM, where vessels were no longer growing, the heparin-cortisone combination was without effect.

Inflammatory and immune angiogenesis. Inflammatory angiogenesis induced by implantation of silica particles into the rabbit cornea, and immune angiogenesis induced by implantation of lymph node tissue from a different rabbit, were also prevented by the cortisone-heparin pellets. Cortisone alone temporarily delayed the onset of both types of angiogenesis (compared to an empty pellet), and heparin alone delayed the onset of immunologically mediated angiogenesis. Related observations have been reported for each compound alone (9, 10).

Isolation of a Non-Anticoagulant Hexasaccharide Fragment

To determine whether the whole heparin molecule was necessary for angiogenesis inhibition, we used purified bacterial heparinase to degrade commercial porcine heparin (11). The degraded heparin had no anticoagulant activity, as determined by activated partial thromboplas-

Table 1. Inhibition of tumor angiogenesis in chick embryos. Cortisone acetate (Merck) (0.9 mg) suspended in 0.9 ml of saline was flooded over the CAM of 8-day embryos through a window made previously in the shell. On day 9, tumor extract (100 μg) from hepatoma cells (4) in 5 μl of water was dried on the center of a plastic cover slip (Thermanox, 15 mm in diameter). Water (5 μl) with or without heparin (6 μg, that is, 1 unit) was

Addition to tumor extract	CAM's showing angiogenesis (%)
None	100
Heparin	100
Cortisone	80
Heparin plus cortisone	0

then added to the cover slip and dried before the cover slip was placed on the CAM. Other embryos were not treated with cortisone. The membranes were viewed on day 11 with a ×12 stereoscope. Angiogenesis was considered to be present if new capillaries converged toward the center of the cover slip. In each group there were 20 embryos.

tin time or whole blood recalcification time (11, 12). The resulting heparin fragments were purified (Fig. 2) and tested in the chick embryo and the rabbit cornea.

The various heparin fragments alone or with cortisone acetate in methylcellulose disks were applied to the 4-day yolk sac membrane of chick embryos cultured in petri dishes as described (1). In the presence of cortisone (100 μg), the hexasaccharide fragment was the most potent inhibitor of angiogenesis (Table 2) and was therefore used in subsequent experiments. In the growing 6-day CAM, disks containing hexasaccharide (12 μg) and cortisone (100 μg) produced large avascular zones up to 12.6 ± 0.1 mm in diam-

eter by 48 hours. Hexasaccharide alone did not promote tumor angiogenesis as heparin did.

In the rabbit cornea, tumor angiogenesis was also inhibited by a combination of hexasaccharide and cortisone but not by either compound alone (Fig. 3).

Oral Administration of Heparin

Subcutaneous injection of heparin together with cortisone in mice resulted in tumor regression. Increasing doses of heparin in the presence of a constant dose of cortisone led to more rapid tumor regression. However, the dose of

subcutaneous heparin was limited by its anticoagulant effects. Hexasaccharide did not act as an anticoagulant, but it was impractical to produce sufficient quantities of this fragment for systemic administration to large numbers of mice.

When administered orally, heparin does not act as an anticoagulant (13, 14). Therefore, we postulated that heparin taken orally was degraded by the gastrointestinal tract, and that non-anticoagulant heparin fragments, including hexasaccharides, might enter the bloodstream and retain their biological capacity to interact with capillary endothelium.

By using the Lewis lung carcinoma as a bioassay, we tested various combinations of orally administered heparin and subcutaneously injected cortisone acetate for their effects on tumor regression. The minimum effective dose of heparin (Panheprin; Abbott) was approximately 200 units per milliliter of drinking water. An effective regimen for cortisone acetate was an initial dose of 250 mg per kilogram of body weight injected once daily for 6 days, followed by 125 mg/kg for 1 day, 75 mg/kg for 1 day, and then a maintenance dose of 37 mg/kg per day. In another regimen we injected cortisone acetate subcutaneously at a rate of 75
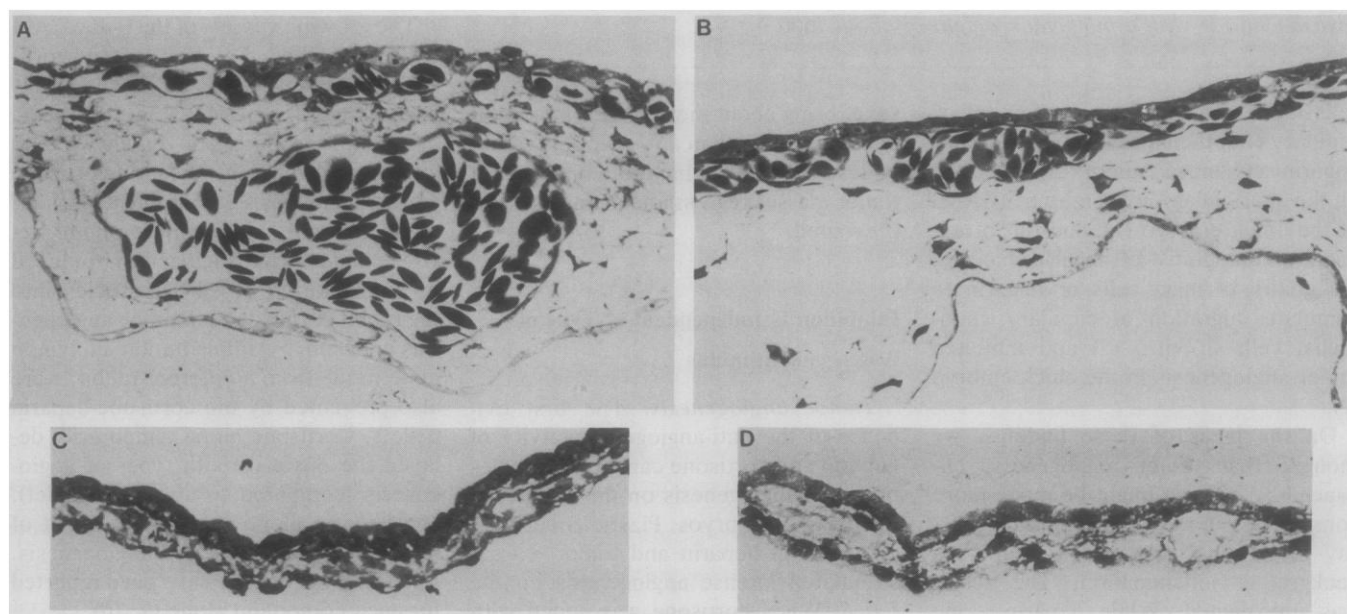

Fig. 1. Inhibition of embryonic angiogenesis. Histologic cross sections of day 8 chorioallantoic membranes. Fertilized chick embryos were removed from their shell on day 3 (or 4) and incubated in a petri dish in high humidity and 5 percent CO_2 as described (25), except that an outer dish and antibiotics were not used. On day 6, a methylcellulose (1) disk (10 μl) containing (A) cortisone acetate (Sigma, powder free of preservatives and suspending agents), (B) heparin (Sigma) (6 μg) or hexasaccharide (12 μg), (C) cortisone plus heparin, or (D) cortisone plus hexasaccharide was implanted on the chorioallantoic membrane. The embryos were examined 48 hours later, and if a clear avascular zone appeared around the methylcellulose disk, the diameter of the zone was measured with a Nikon Profile projector at ×20. Thirty embryos were used in each group. India ink was injected into the heart of some embryos just before Formalin fixation so that vessels could be followed to the edge of the avascular zone in histologic sections. Hexasaccharide plus cortisone (D) produced avascular zones in all embryos. The mean diameter of the zones was 12.6 ± 0.1 mm. Heparin plus cortisone (C) produced avascular zones of 8.9 ± 0.7 mm in diameter. There were no avascular zones in the presence of any of the compounds alone, or with methylcellulose alone. Histologic cross sections of the chorioallantoic membranes revealed that capillaries developed normally in the presence of any of the compounds alone. In contrast, capillaries were absent when either hexasaccharide plus cortisone or heparin plus cortisone had been present, although the ectodermal and endodermal cell layers remained unaffected (hematoxylin and eosin, ×500). Occasional batches of cortisone-methylcellulose did not produce zones in the presence of heparin because of cortisone acetate's low solubility, or inadequate mixing. An improvement is hydrocortisone (40 to 70 μg) with Panheprin (2 units).

mg/kg per day or added hydrocortisone to the drinking water at a concentration of 0.45 mg/ml. The optimum cortisone regimen was not determined.

Mice drinking from 200 to 1000 units of heparin per milliliter of water ingested approximately 83 to 416 times the dose of subcutaneously administered heparin necessary to completely prevent coagulation. Nevertheless, animals that drank heparin maintained normal coagulation and bleeding times, as well as normal prothrombin levels, partial thromboplastin times, and platelet counts.

To obtain additional evidence that fragments of heparin were absorbed from the gastrointestinal tract, we gave mice ^{35}S-labeled heparin (Amersham) (0.3 μC/ml or 3.0 μC/ml) in their drinking water. Serum obtained 24 hours later yielded 0.4 percent of the radioactivity that was present in the drinking water at each concentration. Gel filtration chromatography of the serum indicated that virtually all of the labeled sulfate given orally as intact heparin was recoverable as a disaccharide. Sulfated monosaccharides or inorganic sulfates were not observed.

Regression of Tumors

After determining that the heparin fragment (or fragments) essential for angiogenesis inhibition could be liberated from orally administered heparin by way of the gastrointestinal tract without affecting coagulation, we tested the effects of the heparin-cortisone combination on tumors in mice. The tumors treated were reticulum cell sarcoma (M5076), Lewis lung carcinoma, B-16 melanoma, and a bladder carcinoma (MB 49). None of these tumors undergo spontaneous regression. The first three metastasize. Tumors were implanted subcutaneously in the same (15) dorsal position in each mouse and grown to approximately 0.25 to 0.5 cm³ before treatment was initiated (Fig. 4).

All tumors eventually stopped growing or regressed when the heparin-cortisone combination was administered. In contrast, when either compound was used alone, tumor growth continued as in animals receiving only saline injections; all such control animals died with a large tumor burden.

In the majority of animals treated with heparin plus cortisone, it was possible to achieve "complete regression," that is, tumors did not recur after treatment was discontinued (Fig. 4). Thus, with orally administered heparin (200 U/ml) plus subcutaneously injected cortisone (250

mg, tapered), it was possible to obtain "complete regression" in 100 percent of reticulum cell sarcomas, 100 percent of Lewis lung carcinomas, and 80 percent of B-16 melanomas. However, when bladder carcinomas were treated with this regimen, there were no "complete regressions" until heparin was increased to 1000 U/ml, after which 80 percent of tumors regressed without recurrence. A human colon carcinoma grown in nude mice also regressed, as did V-2 carcinoma implanted in the thighs of rabbits. However, "complete regression" was not obtained with these tumors (data not shown). When both heparin and cortisone (75 mg) were administered subcutaneously, the complete regression rate

was: reticulum cell sarcoma, 80 percent; Lewis lung carcinoma, 71 percent; B-16 melanoma, 60 percent; and bladder carcinoma, 0 percent.

In early experiments, animals that received cortisone for more than 2 weeks usually died of opportunistic pulmonary infection such as *Pneumocystis carinii*. Subsequently, Bactrim and tetracycline were added to the water; cages, water, and food were autoclaved weekly; cages were fitted with filter tops (Micro-Isolator Lab Products, Inc., Rochelle Park, New Jersey); and mice were handled only by persons wearing masks and gloves. With these prophylactic measures, mortality due to infection was not a significant problem.

Table 2. Inhibition of angiogenesis by heparin fragments. The data show the percentage of embryos showing an avascular zone. Disks containing a combination of cortisone acetate (100 μg) and a heparin fragment were applied to the yolk sac membrane of 4-day chick embryos. No avascular zones developed in the presence of any heparin fragment alone, or with cortisone or methylcellulose alone. Eight embryos were used for each group. With hexasaccharide (plus cortisone), the area of the avascular zone was 17 percent of the vascular membrane at 12 μg and 15 percent at 0.1 μg. For the oligosaccharides, the maximum avascular area was 10 percent.

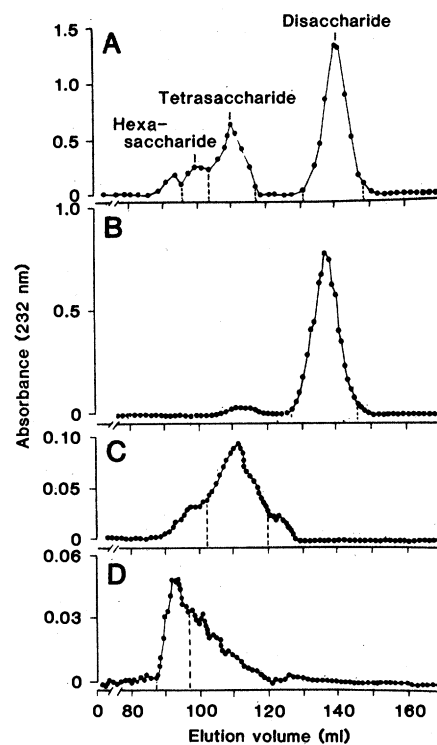| Fragment	Concentration (μg)				
	12.0	8.0	4.0	1.0	0.1
Oligosaccharides	All died	100	25	25	0
Hexasaccharide	100	100	100	50	50
Tetrasaccharide	0				
Disaccharide	0				

Fig. 2. Purification of the hexasaccharide fragment. Porcine mucosal heparin was exhaustively degraded with heparinase (11, 12), concentrated by freeze-drying, and fractionated (12, 26) on Sephadex columns equilibrated with 1M ammonium acetate. The product mixture at 250 mg/ml was eluted from a 75 by 2.5 cm G-15 column at 0.5 ml/min. This resulted in several incompletely resolved peaks corresponding to tetra-, hexa-, and higher oligosaccharides and a separate peak corresponding to disaccharide product (A). The disaccharide peak was rechromatographed on G-15 resulting in the same sharp peak (B). The mixture of tetra-, hexa-, and oligosaccharides was rechromatographed on a 50 by 1.25 cm G-50 column at 2 ml/min, resulting in an unresolved double peak corresponding to tetra- and hexasaccharide fragments and an additional peak corresponding to oligosaccharides. The tetra- and hexasaccharide fragments were combined and rechromatographed on a 50 by 2.5 cm G-25 column resulting in two peaks that were collected separately. The tetrasaccharide product was rechromatographed on G-15 and the center cut of the single peak (C) was freeze-dried. The hexasaccharide fraction was rechromatographed on a G-25 column and the center cut of the single peak (D) was freeze-dried. Fragment size was determined by dissolving a weighed amount of each fraction into 0.03M hydrochloric acid and measuring the absorbance of this solution at 232 nm. The molecular weight of each fragment was calculated (ε = 5500 per mole). The di- and tetrasaccharides were further characterized by comparing their partition coefficients on G-15 with mono-, di-, and trisaccharide standards (12). Measured molecular weights were 530, 1200, 1600, and 1870 for the di-, tetra-, hexa-, and oligosaccharide fractions, respectively. An improved method of making the hexasaccharide has recently been reported (27).

To determine whether or not hexasaccharide was active when given systemically, we injected this fragment subcutaneously (7 mg/kg, twice daily) together with cortisone acetate (250 mg, tapered) into three mice bearing reticulum cell sarcoma. Control mice received either cortisone alone or saline. Whereas the control tumors grew progressively, the tumors in mice treated with hexasaccharide plus cortisone regressed rapidly and were barely visible 4 days later (data not shown). The hexasaccharide was then discontinued because we had insufficient material to determine whether complete regression could be obtained by prolonged treatment. The tumors began to regrow 2 to 4 days later.

To directly observe avascular tumors during systemic therapy, we implanted Lewis carcinoma cells in the mouse cornea (16) and began treatment 24 hours later, that is, before the tumors were vascularized. Orally administered heparin together with subcutaneously administered cortisone significantly inhibited capillary growth (0.02 mm/day) compared to cortisone alone (0.24 mm/day), heparin alone (0.32 mm/day) or saline (0.23 mm/day). In the presence of heparin and cortisone, a thin plate of tumor remained avascular. Three-dimensional tumor growth did not occur as it did when either heparin or cortisone was administered alone.

The effect of excess heparin. In the presence of a constant dose of cortisone, increasing doses of oral heparin caused more rapid tumor regression up to a maximum, beyond which tumor growth resumed (Fig. 5).

Tumors That Did Not Regress

In the case of four tumors, neither angiogenesis nor tumor growth was suppressed by combinations of "optimal" doses of orally administered heparin and subcutaneously administered cortisone. These were sarcoma 1509a, Meth A sarcoma, glioma 26, and glioma 261B. It is interesting that these tumors were all induced by the carcinogen methylcholanthrene. Furthermore, when a nonresponding tumor (sarcoma 1509a) was implanted in the left flank of either B6AF/J mice or nude mice, and a responding tumor such as reticulum cell sarcoma was implanted in the right flank, the reticulum cell sarcoma regressed in each mouse treated with the heparin-cortisone combination, whereas the sarcoma 1509a continued to grow in the same mouse (data not shown).

Prevention of Metastases

Lung metastases were counted in all animals that died (stereoscope, ×6). In all control animals, the lungs were heavily studded with metastases from the three types of metastasizing tumors. In contrast, when any combination of heparin and cortisone was used, no metastases were found in mice bearing reticulum cell sarcoma; one metastasis was found in a mouse bearing Lewis lung carcinoma; and two avascular metastases less than 0.1 mm diameter were found in one mouse bearing B-16 melanoma. This effect can be more readily appreciated by expressing the data as the total number of lung metastases: 4553 in 73 control animals; 3 in 39 animals that received heparin plus cortisone. Furthermore, no lung metastases appeared in any surviving animals that were off treatment. We did not determine whether the antimetastatic action of heparin plus cortisone operated on vessels in the primary tumor, or on vessels in the target tissue, or by some other mechanism.

Variability of Heparin Activity

Because there is, as yet, no unit of angiogenesis inhibitory activity for heparin, units of anticoagulant activity were used throughout these experiments despite the fact that the two activities are probably unrelated. Also, units of anticoagulant activity vary with each lot of heparin and with each manufacturer (that is, from 140 U/mg to 180 U/mg). Furthermore, the angiogenesis inhibitory activity of heparin (in the presence of cortisone) varies greatly among manufacturers. We tested heparin from ten suppliers in the United States and Canada against mice bearing established reticulum cell sarcoma and Lewis lung carcinoma. Panheprin (20,000 U/ml without preservative; Abbott) was capable of bringing about complete regression in both tumors. All lots tested over a 2-year
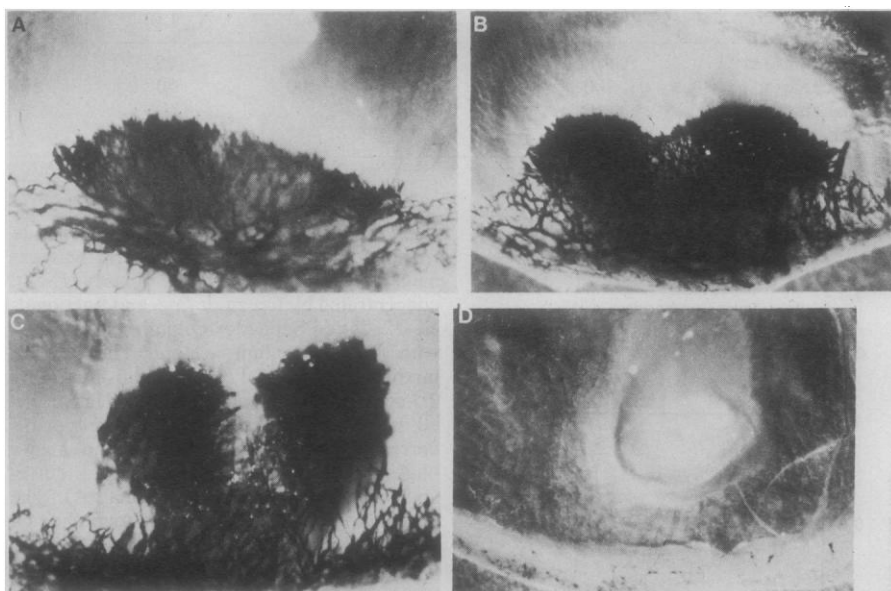

Fig. 3. Inhibition of tumor angiogenesis in the rabbit cornea at day 14 after tumor implantation. Polymer pellets of ethylene-vinyl acetate copolymer (EVA) of approximately 1 mm diameter were impregnated as described (6). (A) Cortisone acetate (1.5 mg) (Sigma), (B) heparin (180 μg) (Sigma) or hexasaccharide (300 μg), (C) neither drug, or (D) a combination of cortisone and hexasaccharide. The pellets were implanted in the cornea 1 mm from the limbus, and a 1-mm³ piece of V2 carcinoma was implanted distal to the polymer, 2 mm from the limbus. In the opposite eye of each rabbit, control pellets that were empty were similarly implanted in juxtaposition to the tumor. Release rates averaged 15 μg/day for heparin, 21 μg/day for hexasaccharide, and 5 μg/day for cortisone. When the compounds were mixed they were released at the same rates. By spectrophotometry, the pellets released heparin for 14 days, hexasaccharide for 11 days, and cortisone for more than 30 days. As capillary blood vessels grew toward the tumor implant, maximum vessel length was measured every 3 days with a stereoscopic slit lamp at ×10 (± 0.1 mm). Mean rates of capillary growth were 0.30 mm/day with hexasaccharide pellets, 0.14 mm/day with cortisone pellets, and 0.32 mm/day with empty pellets. In the presence of the hexasaccharide-cortisone combination there was no capillary growth throughout the 13-day observation period in seven rabbits, and in one rabbit a few capillaries grew at 0.07 mm/day. On day 14 the rabbits were killed, and India ink was injected into each carotid artery. The corneas were removed and photographed. In (A) and (B), new capillary blood vessels are shown growing toward the tumors and passing over the pellets containing either cortisone or hexasaccharide alone. In (D), where the pellet contained hexasaccharide and cortisone, there is only one small capillary sprout, but no other vessels in the cornea, despite the fact that the tumor continued to grow within the cornea (×10).

period were consistently active. Heparins from other suppliers were able to cause either partial or complete regression of reticulum cell sarcoma, but could not suppress the growth of Lewis lung carcinoma. The reason for this variability is unclear.

Controls

To ensure that the heparin activity was not due to a low molecular weight contaminant, we dialyzed the parent heparin exhaustively against distilled water using dialysis tubing with a 3500 molecular weight cutoff. Tumor regression was the same, whether heparin was used before or after dialysis.

To exclude the possibility that tumor regression was caused by direct cytotoxicity, we cultured four types of tumor cells in the presence of 10 percent serum
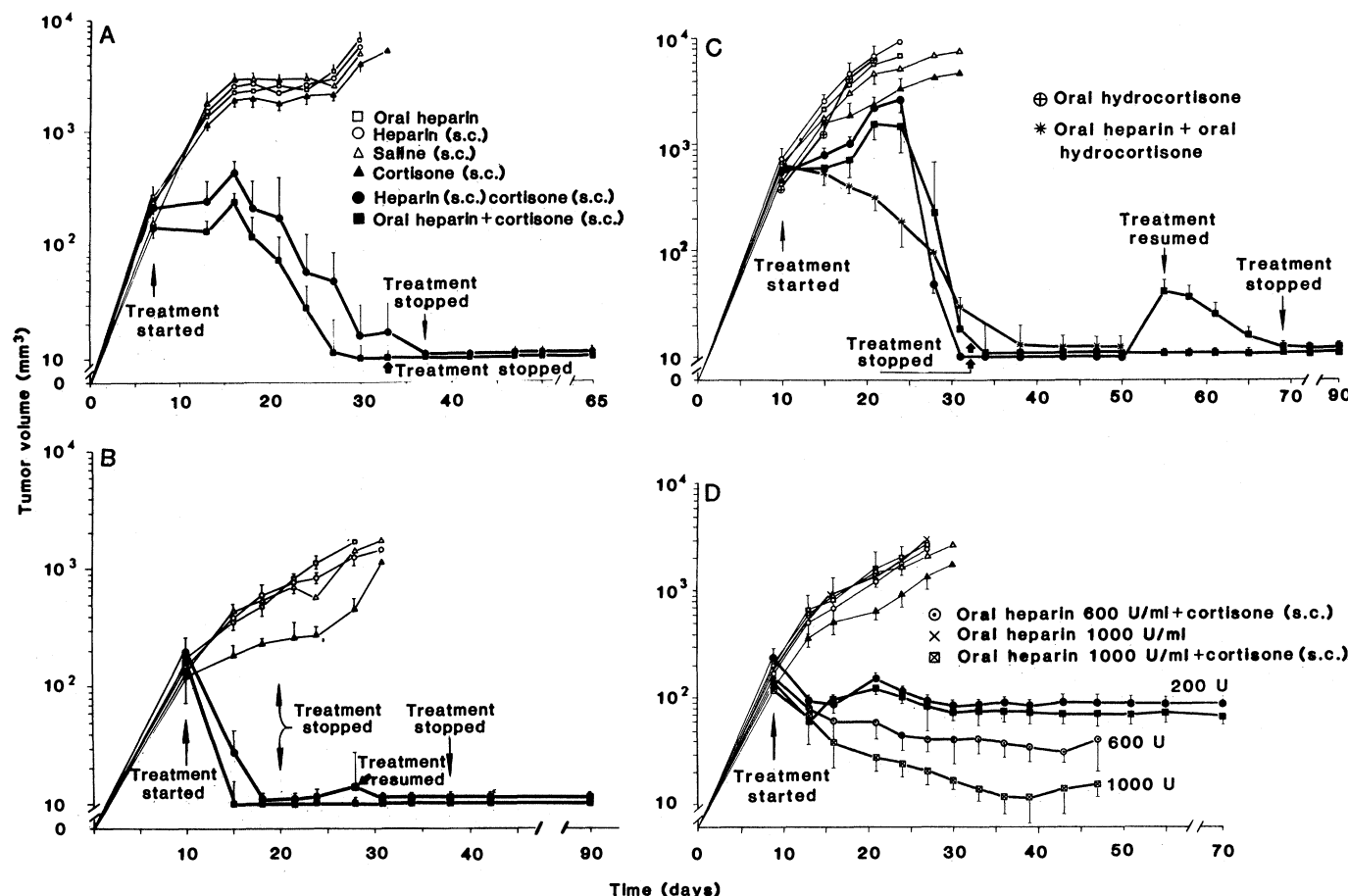

Fig. 4. Treatment of established tumors with heparin plus cortisone. Male C57B1/6 mice, 5 to 6 weeks old, were used. Tetracycline (Rochelle) and Bactrim (28) (intravenous solution; Roche) were added to drinking water until 2 weeks after the administration of heparin plus cortisone was discontinued. Tetracycline was added to autoclaved tapwater and filtered (0.45 μm Millipore) to a final concentration of 750 mg/liter. Bactrim was 2 ml per 100 ml of drinking water. Heparin (Panheprin, 20,000 U/ml, without preservative; Abbott) was then added to a final concentration of 200 U/ml or more. Heparin was not filtered. Water was changed daily because tetracycline loses activity after 24 hours. (Drinking bottles were sterilized and changed every 3 days to avoid contamination by heparin-degrading bacteria.) Tumors were measured every 3 to 5 days in two dimensions and volume was calculated by using the formula (29) vol = $(d_1 d_2 d_3 \pi)/6$, where the width of the tumor is used twice as d_1 and d_2 and the length as d_3. For subcutaneously injected cortisone the regimen was either 250 mg/kg-day with tapering to 37 mg/kg-day as described in the text, or 75 mg/kg-day. For subcutaneously injected heparin (Elkins-Sinn, Cherry Hill, New Jersey), the regimen was 627 U/kg every 12 hours. (Mice on cortisone gradually nearly doubled their water intake. It was not practical to make daily changes of the heparin concentration of cortisone-treated mice to exactly match the heparin intake of mice not treated with cortisone.) (A) Lewis lung carcinoma. Seven mice per group. All controls died by day 33 with large tumors and lung metastases. With oral heparin plus subcutaneously injected cortisone (250 mg), all mice were off treatment by day 33, and remained tumor-free. With heparin plus cortisone (75 mg), both injected subcutaneously, five mice were off treatment at day 37 and remained tumor-free. Two mice died of pneumonia on days 30 and 33, with small primary tumors and one metastasis. Other heparins (Hepar, Canada Packers, and Scientific Protein Laboratories) were ineffective up to 1000 U/ml. (B) Reticulum cell sarcoma (M5076) (30). Five mice per group. Treatment began at day 10. All controls died by day 34 with large primary tumors and lung metastases. All mice that received oral heparin plus cortisone (250 mg) became tumor-free by day 15 and remained so after treatment was discontinued. Mice treated subcutaneously with both heparin and cortisone (75 mg) had tumor recurrence after cessation of treatment, but became permanently tumor-free when treated subsequently with oral heparin plus cortisone (250 mg). One mouse died on day 31 with no gross primary tumor and no metastases. Similar regression rates were obtained with Hepar heparin at 1000 U/ml. Slower regression rates, but no "complete regressions" were obtained with heparin from Invenex, Lilly, and Scientific Protein Laboratories at 1000 U/ml. (C) B-16 Melanoma. Five mice per group, except seven per group when hydrocortisone was given orally. Symbols: ⊕, oral hydrocortisone (Sigma; 0.45 mg/ml drinking water); *, oral hydrocortisone plus oral heparin. All control animals died by day 31 with large tumors and lung metastases. In the group treated with orally administered heparin plus subcutaneously injected cortisone (250 mg), all but one mouse became tumor-free and remained so after treatment was discontinued by day 32 (31). (D) Bladder carcinoma. Seven mice per group. Heparin [Panheprin; Abbott] was administered orally (200 U/ml, 600 U/ml, or 1000 U/ml). All control animals died by day 31 with large primary tumors. In the group that received oral heparin (200 U) plus cortisone, treatment started at mean tumor volumes of 140 mm³. Tumors regressed to 70 mm³ for as long as treatment was continued (that is, 59 days). When treatment was discontinued, tumors resumed growth. When heparin was increased to 600 U/ml, tumors regressed to 45 mm³. These mice did well until day 47 when all but one died during an epidemic in the mouse colony. The survivor remains tumor-free at day 200. With 1000 U/ml, there was complete regression in six mice, and they were off treatment by day 39. One mouse with a small tumor remained on treatment until he died on day 48. The other six mice remain tumor-free through day 100.

obtained from mice receiving heparin, cortisone, heparin and cortisone, or no drug. The serum from mice treated with heparin and cortisone did not inhibit cell growth but in fact stimulated it (Table 3). Furthermore, histological sections showed no evidence of a cytotoxic effect on bone marrow or intestinal mucosa in animals receiving both heparin and cortisone.

To exclude the possibility that the heparin-cortisone combination induced tumor regression by promoting an immune reaction, we inoculated mice with fresh tumor cells at various intervals after treatment had ended. These tumors grew at the same rate as the original implants. Furthermore, if heparin-cortisone was discontinued before tumor regression was complete, the original tumor resumed its growth. Finally, tumor regression was achieved in nude mice.

To determine if other steroids could substitute for cortisone, we administered heparin with hydrocortisone, methylprednisolone, dexamethasone, or medroxyprogesterone (17). Only hydrocortisone was as effective as cortisone acetate in causing tumor regression when administered with heparin. At the highest tolerable doses, neither dexamethasone (3.2 mg/kg) nor medroxyprogesterone (112 mg/kg) caused regression of Lewis lung tumors with or without heparin. Methylprednisolone at 60 mg/kg (injected subcutaneously twice daily) caused partial tumor regression in the presence of orally administered heparin, but was ineffective if the dose was reduced to 6 mg/kg twice daily.

Because of the role proposed for prostaglandins in angiogenesis (18, 19), we determined whether a prostaglandin inhibitor could substitute for cortisone. Indomethacin was injected subcutaneously (up to 15 mg/kg, twice daily) with heparin (administered orally) to mice bearing Lewis lung carcinoma. Although tumor growth slowed slightly at the highest doses, no tumors regressed.

Discussion

These experiments demonstrate the following. (i) Heparin administered with cortisone acts as a potent inhibitor of angiogenesis. (ii) Together, these compounds inhibit the capillary proliferation of embryogenesis, inflammation, certain immune reactions, and the growth of solid tumors. (iii) The antitumor effect is more potent than other known angiogenesis inhibitors. For example, although protamine could control the growth of pulmonary metastases, it could not eradicate them nor, in most cases, could it cause regression of large subcutaneous tumors (1). (iv) The anticoagulant function of heparin is not responsible for the inhibition of angiogenesis because a nonanticoagulant hexasaccharide fragment of heparin substituted for the activity of the parent molecule. (v) The gastrointestinal tract appears to degrade heparin so that low molecular weight, non-anticoagulant fragments are absorbed into the circulation. (vi) It is possible to eradicate some established tumors by inhibition of angiogenesis alone. We had previously assumed that, at best, therapy with angiogenesis inhibitors could only confine a tumor to the avascular stage (20).

That the antitumor effect of the heparin-cortisone combination is due to angiogenesis inhibition is indicated by the following. (i) Heparin-cortisone is not cytostatic to tumor cells directly, either in vitro or in vivo. For example, histological sections of the rabbit cornea showed healthy appearing tumor cells with mitotic figures on one side of a heparin-cortisone pellet whereas capillary growth was prevented on the other side. (ii) When a heparin-cortisone or a hexasaccharide-cortisone pellet was removed from the cornea, vascular growth resumed before three-dimensional tumor regrowth. (iii) Tumors implanted in the mouse cornea failed to grow as a vascu-

Table 3. Effect of serum obtained from mice treated with heparin, hydrocortisone, or both drugs on the growth of tumor cells in vitro. The results show the fold increase in tumor cells after 72 hours of incubation. Four groups of ten mice each drank water that contained heparin (200 U/ml) [Panheprin (Abbott), 151 U/mg], hydrocortisone phosphate (0.45 mg/ml; Sigma), or heparin plus cortisone at these concentrations, or water alone. Serum was obtained by sterile cardiac puncture on day 4, and made up to 10 percent in Dulbecco's modified Eagle's medium. This mixture was not filtered to avoid the possibility of heparin binding to the filter. Cells derived from solid tumors that had grown in C57B1/6 mice were plated in Falcon 24-well plates at low density (5000 cells per well) in 10 percent calf serum. The next day a cell count was taken at hour 0, the mouse serum test media were substituted, and quadruplicate wells were counted again at 72 hours.

Addition to drinking water	Reticulum cell sarcoma	B-16 melanoma	Lewis lung carcinoma	Bladder carcinoma
Heparin	2.9	3.7	24.2	39.5
Hydrocortisone	3.0	5.9	33.2	44.8
Heparin plus hydrocortisone	3.4	7.0	37.4	57.4
Control (water only)	2.9	3.8	20.0	38.8

Fig. 5. Effect of increasing doses of oral heparin when cortisone dose is constant. Mice with established reticulum cell sarcomas received subcutaneous injections of cortisone acetate (75 mg/kg-day). The mice in each group (four mice per cage) were allowed to drink water containing heparin [Panheprin; Abbott] from 200 U/ml up to 10,000 U/ml. Water intake was measured daily for each cage. Symbols: ○, no heparin or cortisone (saline injection); □, cortisone alone (75 mg/kg); △, heparin alone (5000 U/ml); ●, heparin (200 U/ml) plus cortisone; ■, heparin (1000 U/ml) plus cortisone; ▲, heparin (2500 U/ml) plus cortisone; ⊠, heparin (5000 U/ml) plus cortisone; ×, heparin (10,000 U/ml) plus cortisone. The amount of heparin ingested by each group (in units per kilogram per day) is shown in the figure. The most rapid tumor regression occurred in mice drinking the 1000 U/ml dose of heparin, but tumors began to grow when mice drank heparin at 2500 U/ml.
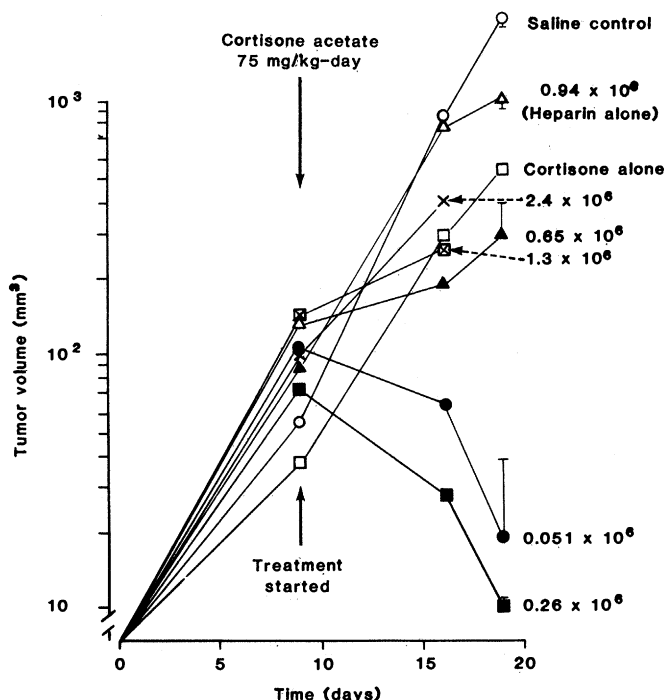
larized mass as long as the animals were maintained on systemic heparin-cortisone. (iv) Tumor regression did not seem to be immunologically mediated. (v) The inhibitory effect of heparin-cortisone was specific for growing microvessels; mature, nongrowing vessels remained unaffected.

It is not understood how angiogenesis inhibition results in complete regression of a large tumor mass. The sudden cessation of capillary proliferation within a tumor could result in necrosis and "bystander" killing of residual tumor cells (21). The data reported here support the concept (20, 22) that solid tumors are angiogenesis-dependent. They also indicate that responsive tumors do not develop "drug resistance" to anti-angiogenesis therapy.

The mechanism of angiogenesis inhibition by heparin-cortisone or hexasaccharide-cortisone is unknown. It is possible that one of the compounds facilitates the rapid uptake of the other into endothelial cells. The appearance of an optimum heparin-cortisone ratio for tumor regression supports this idea (Fig. 5). An alternative interpretation is that heparin contains both a promoter and an inhibitor of angiogenesis and that only the latter acts in the presence of cortisone.

Also unexplained is the ability of several tumors to stimulate angiogenesis and grow despite the administration of heparin and cortisone. These nonresponders may represent a certain class of tumors that can degrade heparin or in some other way interfere with the effect of heparin and cortisone on endothelium.

It has been reported that steroids can partially suppress tumor angiogenesis under certain conditions in the hamster cheek pouch (23) and the rabbit cornea (17). Although the highest doses of cortisone alone temporarily slowed tumor growth, no dose of steroid alone was able to cause complete regression in any tumor in the present study.

It is too early to say what the clinical effectiveness of the heparin-cortisone combination might be. Clinical use of the drug combination might be limited by the immunosuppressive effects of cortisone that are not ameliorated by heparin and may be enhanced by it (10). Nevertheless, these studies, when considered with the results of previous experiments (1–3, 24), suggest the importance of heparin in capillary growth control and offer a potential future role for angiogenesis inhibitors as a new class of pharmacologic agents.

Note added in proof: After this study was completed, Panheprin (Abbott) became unavailable. The next most potent heparin (Hepar Inc., Franklin, Ohio) can cause regression only of reticulum cell sarcoma.

References and Notes

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31. In the B-16 melanoma treated with subcutaneous injections of both heparin and cortisone (75 mg), one mouse died on day 18 and another on day 21; neither had lung metastases. Treatment was discontinued for the other three mice by day 32; tumors recurred 3 weeks later and were successfully retreated with orally administered heparin plus subcutaneous cortisone (250 mg); these mice have remained tumor-free. In the group that received both oral heparin plus oral hydrocortisone, five mice became tumor-free and were off treatment by day 47. Two mice died on day 55 (although tumor-free).
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