Heparin-Steroid Conjugates: New Angiogenesis Inhibitors with Antitumor Activity in Mice

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ABSTRACT

Inhibitors of angiogenesis hold potential in the treatment of cancer and other diseases where the disease is caused or maintained by the inappropriate growth of blood vessels. In the present study, a novel inhibitor of angiogenesis was synthesized by covalently linking a nonanticoagulating derivative of heparin, heparin adipic hydrazide (HAH), to an acid-labile bond to the antiangiogenic steroid, cortisol. The rationale was that the heparin derivative, which binds to sulfated polyanion receptors on endothelial cells, should concentrate the steroid on the surface of vascular endothelial cells. Endocytosis of the conjugate and decomposition of the acid-labile linkage inside lysosomes and other acidic intracellular compartments should then lead to release of the cortisol and expression of its antiproliferative activity. Analysis of the stability of HAH-cortisol showed that it was stable at pH 7.4 and broke down rapidly (t½, 15 min) at pH 4.8 at 37°C. Treatment of murine pulmonary capillary endothelial cells with HAH-cortisol at 10⁻⁷ M (with respect to cortisol) suppressed their DNA synthesis by 50% and inhibited their migration into wounded areas of confluent monolayers. HAH-cortisol at 10⁻⁴ M (with respect to cortisol) did not suppress the DNA synthesis of Lewis lung carcinoma cells. Daily i.p. injections of HAH-cortisol into mice bearing s.c. sponge implants retarded vascularization of sponges for as long as the injections were continued. Daily i.v. injections of HAH cortisol at doses causing no apparent toxicity retarded the growth of solid s.c. Lewis lung carcinomas in mice by up to 65%. In all of these assays, equivalent treatments with a mixture of the HAH plus cortisol was significantly less effective. The antiproliferative effect of HAH-cortisol on endothelial cells appeared independent of the glucocorticoid activity of the steroid since HAH conjugated to 5ß-pregnane-3a,17«,21-triol-20-one, a steroid lacking glucocorticoid or mineralocorticoid activity, was even more effective at inhibiting DNA synthesis by murine pulmonary capillary endothelial cells than was HAH-cortisol. In conclusion, HAH-cortisol represents the prototype of a new class of angiogenesis inhibitors for the treatment of cancer and other angiogenic diseases.

INTRODUCTION

Angiogenesis, the formation of new blood vessels, occurs during tumor growth (1–3) and during certain other pathological conditions such as rheumatoid arthritis, psoriasis, and diabetic retinopathy (4–7). It rarely occurs in healthy adult humans except during wound healing and during phases of the female reproductive cycle (8). Within the last decade, efforts have been made to exploit this difference by using angiogenesis inhibitors to control the growth of tumors and other angiogenic diseases. Several inhibitors have been identified which have promising antitumor activity (9–13).

A dramatic antitumor effect of an antiangiogenic therapy was reported by Folkman et al. (14) who administered heparin and cortisone, p.o. or s.c., to mice bearing established tumors of various types. The heparin, or heparin metabolites, acted together with the cortisone to inhibit angiogenesis and tumor growth. Unfortunately, the magnitude of the antitumor effect was critically dependent upon the source of the heparin and further attempts to repeat and extend these findings were thwarted by unavailability of the most effective heparin source. Later studies performed in several laboratories have thus shown modest (15, 16) or no (17, 18) antitumor effects.

The mechanism underlying the additive antiangiogenic activity of heparin and cortisone has not been positively identified, although it may be related to the fact that the mixture increases the rate of dissolution of the basement membrane beneath newly formed capillaries (19). Structure-activity studies have shown that steroids such as tetrahydro S,3 which lack glucocorticoid and mineralocorticoid activity, can act in conjunction with heparin to inhibit angiogenesis. These studies have led to the description of a new biological activity of steroids, termed the angiostatic activity (20). More recently, the synthetic heparin substitute, β-cyclodextrin tetradecasulfate, was reported to augment the antiangiogenic effects of angiotensic steroids on corneal neovascularization in rabbits when applied locally or topically (21). It was suggested that the β-cyclodextrin tetradecasulfate might act by forming a noncovalent complex with the steroid and promote its binding to the surface of endothelial cells.

In the present study, we prepared a nonanticoagulating derivative of heparin, HAH, and linked it by an acid-labile bond to cortisol (hydrocortisone). We reasoned that the HAH, like heparin itself, should be taken up preferentially by vascular endothelial cells (22–28) and by cells of the reticuloendothelial system (29–33) after i.v. administration. Although heparin receptors are present on several other cells types, including smooth muscle cells (34), hepatocytes (35), fibroblasts (32), and neurons (36), the majority of systemically administered heparin is taken up by vascular endothelial cells, presumably on account of the vast surface area of these cells which is in contact with the blood. In addition, dividing endothelial cells bind and endocytose about 10-fold more heparin than nondividing endothelial cells (37), suggesting that the dividing vascular endothelial cells which are abundant in tumors (8, 38) and other angiogenic disease sites might be especially susceptible to its effects. Endocytosis and delivery of HAH-cortisol to acidified endosomes and lysosomes, which are known to occur with heparin itself (25, 31), should then lead to release of active free cortisol within the vascular endothelial cells resulting in changes in gene transcription and inhibition of cell proliferation (39). We report here that HAH-cortisol inhibits the proliferation of vascular endothelial cells in vitro, retards the vascularization of sponges implanted into mice, and significantly inhibits the growth of solid Lewis lung carcinomas in mice. A conjugate of HAH and tetrahydro S was even more effective than was HAH-cortisol at inhibiting proliferation of vascular endothelial cells, indicating that the inhibitory effects of HAH-cortisol are independent of the glucocorticoid and mineralocorticoid activities of the steroid.

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The abbreviations and trivial names used are: tetrahydro S, 5ß-pregnane-3a,17«,21-triol-20-one; HAH, heparin adipic hydrazide; cortisol, hydrocortisone; PBS, phosphate-buffered saline; vWF, von Willebrand factor; MPCE, murine pulmonary capillary endothelial cell; PE, Lewis lung carcinoma.
HEPARIN-STEROID CONJUGATES AS ANGIOGENESIS INHIBITORS

MATERIALS AND METHODS

Materials. Heparin (sodium salt; H3125; Grade 1 from porcine intestinal mucosa, 181 USP units/mg), adipic dihydrazide, cortisol (hydrocortisone, H4001), tetrahydro S, and Russell’s viper venom (RVCL-1, freeze-dried powder reconstituted to 3 ml with saline) were from Sigma Chemical Co., St. Louis, MO. Carbazole, sodium tetraborate, EDC, and other chemicals were from Aldrich Chemical Co., Milwaukee, WI. All reagents were Analar or equivalent, or the best grade available.

[3H]Thymidine (TRK 120, 25 Ci/mmol or 25 GBq/mmol) and 133Xe [XAS 120P, 10 mCi (370 MBq)] were from Amersham International, Amersham, United Kingdom.

Polyether-polyurethan sponge (VP45) was a gift from M. Robinson, Vitafarm Ltd., Ashton-under-Lyne, United Kingdom. Disks with diameters of 0.8 cm were cut from sheets of the sponge using a cork-borer.

Dalboce’s modified Eagle’s tissue culture medium was obtained from Gibco BRL, Gaithersburg, MD. Fetal calf serum was obtained from ICN Biomedicals, Inc., Costa Mesa, CA. Insulin-transferrin-sodium selenite media were obtained from Sigma. Ninety-six-well flat-bottomed microtitre plates and 6-well tissue culture plates were obtained from Falcon (Becton Dickinson and Co., Lincoln Park, NJ).

MECA 20, a pan anti-mouse vascular endothelial cell antibody, was kindly provided by Dr. Adrian Dujvvestijn, Rijksuniversiteit Limburg, Maastricht, the Netherlands. Details of the antibody have been published (40).

Measurement of Heparin Concentration. The concentration of heparin solutions was determined by using a modification of the carbazole assay described by Bitter and Muir (41). In brief, the heparin solutions to be analyzed were diluted until their approximate concentration was in the range of 25–250 μg/ml. Two hundred-μl of the heparin solutions were added dropwise to glass tubes containing 1 ml of an ice-cold solution of sodium tetraborate (5.03 mg/ml) in concentrated sulfuric acid. The solutions were mixed and heated at 95°C for 10 min. Forty μl of carbazole (1.25 mg/ml) in absolute ethanol were added to each tube and the solutions were mixed and heated at 95°C for 15 min. The tubes were allowed to cool and the absorbance was measured at 530 nm. The heparin or HAH concentrations were calculated by comparing the absorbances of duplicate samples of the test solutions with those of standards prepared from heparin or HAH solutions with concentrations ranging from 0 to 250 μg/ml.

Preparation of HAH-Cortisol. A solution of heparin (10 mg/ml) in distilled water was concentrated in an Amicon ultrafiltration cell fitted with a YM30 membrane until about 30% of the heparin appeared in the filtrate. This was done to remove low molecular weight heparin present in the starting material. Heparin was then condensed with adipic dihydrazide in a manner similar to that described for the synthesis of heparinylglycine (42). The concentrated heparin was adjusted to 10 mg/ml and adipic dihydrazide was added to a final concentration of 0.5 μM. The pH of the solution was adjusted with 1 M HCl to 4.75. A freshly prepared solution of EDC in water was added to give a final EDC concentration of 10.7 mg/ml. The reaction was allowed to proceed at room temperature while maintaining the pH at 4.75 ± 0.02 (SD) by the periodic addition of 1 M HCl. When the pH had stabilized (2–3 h), the mixture was kept at 4°C for 48 h and then dialyzed (using tubing with a molecular weight cutoff of 3500) into 1 M HCl in water at 4°C with four changes of dialysate over 4 days. The mixture was dialyzed into 0.1 M sodium acetate buffer, pH 4.0, and the HAH solution was adjusted to 1 mg/ml by addition of 0.1 M sodium acetate buffer, pH 4.0. A solution of cortisol (11 mg/ml) in ethanol was added to the HAH solution to give a final cortisol concentration of 3.2 mg/ml and a final ethanol concentration of 30% (v/v). The mixture was allowed to react for 16 h at room temperature and was then adjusted to pH 10 with 10 M NaOH. The resultant HAH-cortisol was dialyzed extensively into water the pH of which had been adjusted to 10 with a few drops of 10 M NaOH. The dialyzed product was finally freeze-dried and stored in a desiccator at 4°C.

The molar ratio of cortisol to HAH in the product was determined by measuring the bound cortisol and HAH concentrations in a standard solution. The HAH concentration was determined using the carbazole method described above and the molar concentration was calculated assuming an average molecular weight of 15,000 (25). The cortisol concentration was measured spectrophotometrically at 274 nm, using an extinction coefficient of 21,900 M⁻¹cm⁻¹ for the acyl hydrazide derivative of cortisol. This extinction coefficient was determined by dissociating samples of HAH-cortisol at acidic pH and measuring the released cortisol concentration from the absorbance at 242 nm (using the extinction coefficient of 16,100 M⁻¹cm⁻¹ (43)) after correcting for 242 nm absorbance due to the heparin-hydrazide. The cortisol:HAH molar ratio in the product was usually between 5 and 8. Fig. 1 shows a schematic representation of the product.

Preparation of HAH-Tetrahydro S. A HAH-tetrahydro S conjugate was prepared in a manner similar to that described above for HAH-cortisol except that, for the coupling reaction, 7 volumes of HAH at 1 mg/ml in sodium acetate buffer, pH 4.0, were mixed with 3 volumes of tetrahydro S at 10.4 mg/ml in ethanol. The mixture was heated at 56°C for 14 days and then was adjusted to pH 10, diapezized, and freeze-dried as before. The molar ratio of tetrahydro S to HAH in the resultant conjugate was calculated by determining the content of HAH in the conjugate by the carbazole assay as above and the content of tetrahydro S by releasing the steroid under acidic conditions and measuring the released steroid using the blue tetrazolium assay described by Chen et al. (44). In brief, 1 ml of a 10-mg/ml solution of HAH-tetrahydro S in H₂O was diazlated twice for 2–3 days at room temperature into 10 ml of 0.01 M acetic acid solution in H₂O. The dialysates were combined and freeze dried. The dry residue was dissolved in 5 ml of ethanol. To 2 ml of the ethanol solution were added 1 ml of a solution of 0.14 mg/ml of 5 M NaOH in 25 ml of ethanol and 2 ml of a 2.5-mg/ml solution of blue tetrazolium in ethanol. After 1 h at room temperature, the absorbance of the solution was measured at 510 nm and the steroid concentration was calculated by reference to the absorbance of a set of standard steroid solutions prepared and assayed on the same occasion. The product consisted of 0.5 mol tetrahydro Smol HAH. Attempts to improve on this level of loading by increasing the temperature or the duration of the reaction or by increasing the tetrahydro S concentration in the reaction mixture were unsuccessful. The product is similar in structure to that shown in Fig. 1 except that the HAH forms an acyl hydrazide bond with C-20 of the steroid not C-3.

Acid Lability of HAH-Cortisol. A solution of HAH-cortisol (0.75 ml, 200 μg/ml) in distilled water was added to a 1.5-ml spectrophotometer cell in a thermostatically controlled holder maintained at 20°C or 37°C. 0.75 ml of 0.1 M sodium acetate, pH 4.8, was added and the contents of the cell were rapidly mixed. The UV spectrum from 400 nm to 200 nm was scanned immediately and every 5 min thereafter until the decomposition of the HAH-cortisol was complete (Fig. 2). The half-life of the decomposition was taken as the time for the absorbance at 274 nm to decay to one-half its initial value.

Anticoagulant Activity. The anticoagulant activity of heparin, HAH, and HAH-cortisol was measured by their ability to inhibit coagulation of human plasma by Russell’s viper venom. Twenty μl of solutions of heparin at a range of concentrations from 0 to 100 μg/ml or HAH or HAH-cortisol at a range of concentrations from 1 to 1000 μg/ml in PBS were added to glass tubes which had been prewarmed to 37°C. Fresh citrated human plasma (150 μl) at 37°C was added to each tube. The contents were immediately mixed and the tubes were incubated at 37°C for 1 min. One hundred μl of Russell’s viper venom (Sigma No. RVCL-1 reconstituted to 3 ml) were added and the contents were mixed. After 15 s, 100 μl of a prewarmed solution of CaCl₂ (0.2 M) in PBS

![Fig. 1. Predominant structure of HAH-cortisol conjugate. Heparin is a heterogeneous sulfated polysaccharide with a varying number of repeating uronic acid and glucosamine residues. The polymer is modified in a random manner by (a) O-, N-sulfation, (b) O-acetylation, and (c) N-acetylation of glucuronic acid to iduronic acid.](image-url)
were added. The contents of the tubes were immediately mixed and the tubes were incubated at 37°C. The time for the plasma to clot was recorded. The clotting times for plasma treated with HAH or HAH-cortisol were compared to those treated with unmodified heparin and the differences in anticoagulant activities were calculated.

**DNA Synthesis by MPCE Murine Vascular Endothelial Cells in Vitro.** MPCE cells were a kind gift from Professor Adam S. G. Curtis, University of Glasgow, Glasgow, United Kingdom. The cells are a pulmonary capillary endothelial cell line isolated from BIO.D2 mice. They are vWF positive and Gryphimia lectin positive, and have endothelial morphology. The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal calf serum, 0.7% insulin-transferrin-sodium selenite media supplement, 2 mM L-glutamine, 2 units/ml penicillin G, and 2 µg/ml streptomycin. Cultures were maintained at 37°C in an atmosphere of 10% CO₂.

Experiments designed to determine inhibition of DNA synthesis were performed as follows. MPCE cells were removed from tissue culture flasks with trypsin (0.625% w/v) and EDTA (0.2% w/v). A cell suspension was prepared at 5 x 10⁴ cells/ml in culture medium containing 10% serum and supplements as above. The cell suspension was dispersed in 100-µl aliquots into the wells of 96-well flat bottomed microtiter plates. The cells were incubated for 24 h at 37°C during which time they adhered to and spread onto the surface of the wells. One hundred µl of solutions of HAH-cortisol or various control substances, such as cortisol, HAH, or a mixture of HAH and cortisol, dissolved in tissue culture medium were then added to designated wells and the cells were incubated for 24 h at 37°C. All cultures were set up in quadruplicate. Each culture was then pulsed with 1 µCi [³H]thymidine for 24 hours. The cells were washed with PBS, detached with trypsin-EDTA, and harvested onto glass fiber disks using an automated cell harvester. Radioactivity in treated cultures was expressed as a percentage of that in cultures treated with medium alone to obtain the percentage of inhibition of DNA synthesis.

**DNA Synthesis by 3LL Lewis Lung Carcinoma Cells in Vitro.** Experiments designed to measure inhibition of DNA synthesis were performed in a manner similar to that described for MPCE cells. One hundred µl of a suspension of 3LL cells at 2.5 x 10⁴ cells/ml in complete medium [Eagle’s minimum essential medium supplemented with 10% (v/v) fetal calf serum, 2.4 mM L-glutamine, 2 units/ml penicillin, and 1 µg/ml streptomycin] were distributed into 96-well microtiter plates and incubated for 24 h at 37°C. One hundred µl of solutions of HAH-cortisol or various control substances were then added and cultures were incubated for 24 h at 37°C. 1 µCi/culture of [³H]thymidine was added and the quantity of label incorporated by the cells was determined.

**Repair of Wounded MPCE Monolayers.** MPCE cells were cultured in 6-well tissue culture plates and allowed to grow to confluence. The confluent monolayers were wounded with a plastic pipet tip (Oxford Universal large tip). The wounds were approximately 3 cm long and 0.15 cm wide. The wounded cultures were either treated with HAH-cortisol conjugate at a final concentration of 2 x 10⁻⁴ (with respect to cortisol) or with an unconjugated mixture of HAH (at a final concentration of 2.4 x 10⁻⁵ M) plus cortisol (at a final concentration of 2 x 10⁻⁴ M). The cultures were incubated at 37°C for 5 days and photographed.

**Vascularization of Implanted Sponges.** Measurements of the extent of vascularization of sponge implants in mice were made using the method of Andrade et al. (45) with slight modifications. Sterile polyethylene-putrethene sponge disks (0.8 cm diameter x 0.4 cm thick) were prepared which contained a 1.2 cm length of polypropylene tubing (1.4 mm external diameter) protruding from the center of one circular face of the sponge and located 0.2 cm deep into the sponge (i.e., midway through its thickness). The cannula was sewn into the sponge with silk sutures and its open end was sealed with a removable plastic plug. The sponges were not treated with angiogenic stimuli before implantation. Male BALB/c mice 8–12 weeks old were anesthetized and the sponges were aseptically implanted into a s.c. pouch which had been fashioned with curved artery forceps through a 1-cm-long dorsal midline incision. The cannula was exteriorized through a small incision in the s.c. pouch.

Vascularization of the sponge was assessed twice weekly after implantation by anesthetizing the mice and injecting 10 µl of a sterile solution of ¹³³Xe in saline (5 x 10⁵ dpm/ml) into the sponge via the cannula. The cannula was immediately plugged to prevent escape of the gas. A collimated gamma scintillation detector was then situated 1 cm above the sponge and the radioactivity was measured for 5 s each min for 9 min. The radioactivity-versus-time data were fitted to an exponential decline curve using a least-squares regression algorithm, and the half-life of clearance was computed. In freshly implanted avascular sponges the ¹³³Xe was cleared slowly with a half-life of about 25 min whereas in a fully vascularized sponge the ¹³³Xe was cleared rapidly with a half-life of about 7 min. Normally, full vascularization of an implanted sponge occurred in about 16 days.

Two types of experiments were performed in which the effect of HAH-cortisol on vascularization of sponges was determined. In the first, HAH-cortisol (0.078 mg) in 25 µl saline was injected into the sponge via the cannula every day for 10 days starting the third day after implantation. Other groups of mice received equivalent quantities of HAH (0.65 mg), cortisol (0.13 mg), an unconjugated mixture of HAH (0.65 mg) and cortisol (0.13 mg) or an unconjugated mixture of native heparin (0.65 mg) and cortisol (0.13 mg). In the second series of experiments, mice received i.p. injections of HAH-cortisol (2.35 mg) or HAH (1.95 mg) plus cortisol (0.39 mg) in 0.25 ml saline every day for 5 days starting on the third day after implantation, followed by one-half of these doses each day for the next 7 days. These doses and schedules had been found in pilot experiments to inhibit vascularization. Each treatment group contained 10 mice. Effects on vascularization were judged from changes in the ¹³³Xe clearance rate. Differences in clearance rates were tested for statistical significance using the Mann-Whitney-Wilcoxon test for two independent samples (46).

**Enumeration of Blood Vessels and Endothelial Cells in Implanted Sponges.** An experiment was performed in which HAH-cortisol (1 mg) was injected daily into implanted sponges via the cannula for 10 days starting 3 days after implantation. Other mice received equivalent doses of HAH (0.83 mg), cortisol (0.17 mg), or an unconjugated mixture of HAH (0.83 mg) plus cortisol (0.17 mg). A further group received an unconjugated mixture of native heparin (0.83 mg) plus cortisol (0.17 mg). Sponges were dissected out and transverse frozen sections were cut from halfway through the sponge’s thickness. Endothelial cells and blood vessels were identified by indirect immunoperoxidase staining with goat anti-human vWF antibody which cross-reacts with mouse vWF and with a rat monoclonal antibody, MECA 20, which reacts with mouse vascular endothelial cells (40). The developing antibodies were horseradish peroxidase-conjugated rabbit anti-goat immunoglobulin and rabbit anti-rat immunoglobulin, respectively, and were used according to the manufacturer’s recommended procedure. Blood vessels were visible as vWF-positive structures with discernible lumina. About one-half of the vessels contained erythrocytes. Endothelial cells were visible as vWF-positive cells with the morphology of endothelial cells but lacking an adjacent lumen. The vessels were counted using a light microscope.
and endothelial cells present in 15 microscope fields (in a diametric line across the section) were counted.

**Antitumor Experiments.** Male C57BL/6 mice ages 8–12 weeks were given s.c. injections of $3 \times 10^6$ 3LL cells which had been maintained in tissue culture. The tumor is an anaplastic epidermoid carcinoma which originated as a spontaneous lung tumor in a C57BL mouse in 1951 (47). When the tumors had grown to measurable dimensions (about 0.3 cm in diameter), the mice were given i.v. injections of 0.1 ml of saline containing 1 mg of HAH-cortisol every day for 9 days. Other mice received equivalent quantities of HAH (0.83 mg), cortisol (0.17 mg), or a mixture of both. On the tenth day, the mice were sacrificed and their tumors were removed and weighed. All treatment groups contained 8–10 mice. Differences in tumor weights were tested for statistical significance using the Mann-Whitney-Wilcoxon nonparametric test for two independent samples (46).

### RESULTS

**Acid Lability of HAH-Cortisol.** HAH-cortisol broke down to give free cortisol and HAH with a half-life of 35 min at pH 4.8 at 20°C (Fig. 2). At 37°C the breakdown was faster, with a half-life of 15 min. At pH 7.4, the conjugate was relatively stable; only 8% breakdown had occurred after 24 h at 37°C. The conjugate was as stable when incubated at 37°C in complete tissue culture medium which had been conditioned by MPCE cells as it was in PBS (results not shown).

**Anticoagulant Activity.** HAH-cortisol and HAH were virtually devoid of anticoagulant activity. They had <1% of the anticoagulant activity of native heparin, i.e., < 2 USP units/mg (Table 1).

**Inhibition of DNA Synthesis by MPCE Cells by HAH-Cortisol.** At low concentrations, HAH-cortisol inhibited DNA synthesis by MPCE cells to a significantly greater extent ($P < 0.001$) than did free cortisol, HAH, or an unconjugated mixture of HAH and cortisol (Fig. 3a). The conjugate inhibited DNA synthesis by 50% at a concentration of $5 \times 10^{-6}$ m with respect to cortisol ($6.25 \times 10^{-7}$ m with respect to HAH). A consistent finding was that HAH-cortisol failed to inhibit DNA synthesis completely at high concentrations (i.e., $3 \times 10^{-4}$ m or greater with respect to cortisol). As shown in Fig. 3a, the maximum inhibition of DNA synthesis induced by HAH-cortisol was 75%. Cortisol itself was less inhibitory. It produced a flat dose-response curve with a maximal inhibition ranging between 40 and 60% at $10^{-4}$ m. Mixing the cortisol with unconjugated HAH did not affect the inhibitory activity of the cortisol. Similar results to those in Fig. 3a were obtained in 4 separate experiments.

**Lack of Inhibition of DNA Synthesis by 3LL Lung Carcinoma Cells by HAH-Cortisol.** 3LL cells were at least 20-fold less sensitive to HAH-cortisol than were MPCE cells ($P < 0.001$) (Fig. 3b). Treatment of 3LL cells with HAH-cortisol at $10^{-4}$ m with respect to cortisol did not reduce their DNA synthesis.

**Inhibition of Repair of Wounded MPCE Monolayers by HAH-Cortisol.** HAH-cortisol retarded the healing of wounded MPCE murine vascular endothelial cell monolayers. In wounded monolayers treated with an unconjugated mixture of HAH (2.4 $\times 10^{-5}$ m) and cortisol (2 $\times 10^{-4}$ m), migration of MPCE cells into the wounded area began within 24 h and was complete by 72 h. In contrast, the wound was still evident on day 5 in monolayers treated with HAH-cortisol at an equivalent concentration (Fig. 4). This indicates that HAH-cortisol inhibits the migration of MPCE cells. Importantly, the cells in the unwounded areas of the culture remained morphologically healthy showing that HAH-cortisol inhibited migration and proliferation but was not toxic to quiescent cells.

**Toxicity of HAH-Cortisol to Mice.** Neither HAH nor HAH-cortisol caused any loss of body weight or other outward signs of toxicity when administered i.v. to mice at doses of 10 mg/day for 14 days. By comparison, i.v. administration of cortisol itself at 0.4 mg/day killed three of three mice within 7 days. Unmodified heparin was also toxic: two of three mice that were given i.v. injections of 0.5 mg heparin/day died within 3 days and the third died after 6 days.

**Inhibition of Vascularization of Implanted Sponges by HAH-Cortisol.** Daily injections of HAH-cortisol (0.78 mg) into implanted sponges in mice abolished vascularization of the sponge. This was evident from the fact that (a) the $^{133}$Xe clearance rate of the HAH-cortisol-treated sponges remained the same as, or greater than, that of freshly implanted (avascular) sponges for as long as the injections were continued (Fig. 5a) and (b) frozen sections of sponges from mice that had received daily injections of HAH-cortisol over a 10-day period were completely devoid of endothelial cells or blood vessels, as identified by anti-vWF and MECA 20 staining (Table 2).

In a further experiment, mice were given daily i.p. injections of high doses of HAH-cortisol (2.35 mg) for 5 days and then one-half of this dose (1.17 mg) for 7 days (Fig. 5b). Measurements of $^{133}$Xe clearance rates showed that vascularization was significantly ($P < 0.05$) retarded only while the larger doses were being given. Mice treated with equivalent amounts of a mixture of unconjugated HAH and cortisol showed $^{133}$Xe clearance rates similar to those in recipients of saline alone.

The $^{133}$Xe clearance rate of sponges treated by injection of HAH-cortisol was actually slower than that of freshly implanted (avascular) sponges (Fig. 5a). The same was true of sponges in mice receiving HAH-cortisol i.p. at the 7- and 10-day post-implantation time points.

### Table 1 Reduced anticoagulant activity of HAH-cortisol

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<th>USP (units/mg)</th>
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Fig. 3. Inhibition of DNA synthesis in MPCE cells by HAH-cortisol conjugate and lack of effect on 3LL cells. MPCE cells or 3LL cells were grown in 96-well plates for 24 h and were then treated for 24 h at 37°C with HAH-cortisol conjugate (O), with an equivalent mixture of unconjugated HAH plus cortisol (●) or with cortisol (□) alone. Bottom abscissa, cortisol concentration in each treatment; top abscissa, HAH concentration. The ability of the cells to incorporate [3H]thymidine into DNA was determined 24 h later. [3H]thymidine incorporation is expressed as a percentage of that in untreated control cultures (84,000 dpm for MPCE cells, 54,300 dpm for 3LL cells). Bars, SD.
HEPARIN-STEROID CONJUGATES AS ANGIogenesis INHIBITORS

Fig. 4. Inhibition of repair of wounded MPCE cell monolayers by HAH-cortisol conjugate. Confluent MPCE cell cultures were wounded with a plastic pipet tip. The wounds were 3 cm long and 0.15 cm wide. a. A culture immediately after wounding. The wounded monolayers were then incubated with HAH-cortisol conjugate (2 × 10⁻⁴ M with respect to cortisol) (b) or with an equivalent mixture of unconjugated HAH and cortisol (c). b and c, appearance of the wounds after 5 days. The HAH-cortisol conjugate inhibited the migration of cells into the wounded area, whereas the unconjugated mixture did not. The untreated culture healed at a rate similar to that treated with the unconjugated mixture. Bar, 100 μm.

HAH-cortisol, but not its unconjugated constituents, retarded the ¹³³Xe clearance rates. In control groups which received equivalent quantities of HAH alone, cortisol alone, or a mixture of the two, ¹³³Xe clearance rates were the same as in mice which received saline alone (Fig. 5). However, the number of blood vessels and endothelial cells present in sections of sponges which had received HAH injections, either alone or mixed with cortisol, were reduced by about 45 and 65%, respectively, as compared with sponges receiving injections of saline alone (Table 2). These differences were statistically significant (P ≤ 0.05).

In other systems, native heparin and glucocorticosteroids have synergistic antiangiogenic activity (20, 48). We therefore determined the effect of injecting a mixture of native heparin and cortisol daily into implanted sponges on the vascularization of the sponge. The treatment neither retarded the ¹³³Xe clearance rate nor reduced the number of blood vessels in the sponge (Fig. 5a; Table 2); however, it did deplete the number of endothelial cells somewhat, relative to those in recipients of cortisol alone (640 cells/cross-section of sponge as compared with 1128, respectively).

Antitumor Effects of HAH-Cortisol. In four separate experiments, daily i.v. injections of HAH-cortisol (1 mg) into mice bearing established s.c. Lewis lung carcinomas reduced the average weight of the tumors to between 38 and 58% of that in mice treated with saline alone (Table 3). In all these experiments, the retardation of tumor growth was highly statistically significant (P < 0.001). The antitumor effect of the conjugate was consistently superior to that of an equivalent dose of an unconjugated mixture of HAH and cortisol which reduced tumor weights to between 58 and 82% of that in mice treated with saline alone. In two of these experiments, the superiority of the conjugate over the mixture was statistically significant (P < 0.05) and in the third experiment it bordered on being significant (P = 0.08). In mice receiving cortisol alone (0.17 mg/day), the average tumor weights were the same as in the group receiving both HAH and cortisol. No reduction in tumor weight was observed in mice receiving HAH alone (0.83 mg/day).

Fig. 5. Delayed vascularization of implanted sponges in mice by administration of HAH-cortisol conjugate. Polyurethane sponges were implanted s.c. into mice. The extent of vascularization of the sponges was assessed at various times thereafter by injecting ¹³³Xe into the sponges and measuring the rate of disappearance of radioactivity. As vascularization proceeds, the half-life of ¹³³Xe clearance falls from about 25 min to about 7 min. In a, starting 3 days after implantation, HAH-cortisol (0.78 mg) was injected daily for 10 days directly into the sponge (C). Other mice received equivalent quantities of a mixture of unconjugated HAH (0.65 mg) plus cortisol (0.13 mg) (○), cortisol (0.13 mg) alone (●), unmodified heparin (0.65 mg) plus cortisol (0.13 mg) (△), or diluent (▲). In b, starting 3 days after implantation, mice were given i.p. injections of 2.25 mg HAH-cortisol for 5 days and then with 1.17 mg HAH-cortisol for a further 7 days (□). Other mice received equivalent quantities of unconjugated HAH plus cortisol (●) or diluent (▲). Each treatment group consisted of 10 mice. Points, arithmetic mean of the results in each group; bars, SD.
of sponges taken from 4 to 7 mice ± SD. In the case of native heparin plus cortisol, only
and endothelial cells present in an entire cross-section of sponge was calculated. The
edges to the center of each sponge. From these measurements, the number of blood vessels
plus 0.17 mg cortisol, or 0.83 mg native heparin plus 0.17 mg cortisol. Frozen sections of sponges were cut. The blood vessels and endothelial cells present
anti-vWF antibody and MECA 20. Vessels were identified as vWF-positive structures with
2 sponges were analyzed; numbers in parentheses, total number of vessels and endothelial
numbers given are the average number of vessels and endothelial cells in cross-sec tion s
lumina usually containing erythrocytes.

cortisol <1 mg) or equivalent amounts of unconjugated HAH (0.83 mg). cortisol (0.17 mg),
Crooked S. Tetrahydro S, a steroid which lacks glucocorticoid or miner-
recipients of HAH plus cortisol. 'T/C, treated versus saline control.

Inhibition of DNA Synthesis of MPCE Cells by HAH-Tetrahy-
dro S. Tetrahydro S, a steroid which lacks glucocorticoid or min-
eralocorticoid activity, yielded a HAH conjugate which was as potent as
HAH-cortisol at inhibiting DNA synthesis by MPCE cells in vitro.

In a further experiment, the treatment with HAH-cortisol was started 1 day after the 3LL cells were injected and was continued for 10 days. The conjugate again reduced the tumor growth rate by about 60% whereas the unconjugated mixture reduced it by 30% (Fig. 6). Hence, earlier treatment did not improve the antitumor effects of the HAH-cortisol.

**DISCUSSION**

The major findings to emerge in the present study are that HAH-cortisol inhibits DNA synthesis and migration of vascular endothelial cells in vitro, retards vascularization of sponges implanted into mice and significantly inhibits the growth of solid Lewis lung carcinomas in mice. In each assay, the activity of the HAH-cortisol conjugate exceeded that of a mixture of its components showing that chemical linkage of the components improves the biological activity of the conjugate.

The HAH-cortisol was constructed by first condensing the carboxyl groups of accessible glucuronic acid and iduronic acid residues in heparin with adipic dihydrazide. This resulted in the introduction of hydrazide groups into heparin which, on mixing with cortisol, condensed with the ketone group on C-3 of the steroid to form a conjugate in which the heparin and the steroid were joined by an acyl hydrazine

| Material injected | No. present in cross-section of sponge | | |
|-------------------|----------------------------------------|-----------------|-----------------|-----------------|
|                   | Blood vessels | Endothelial cells | |
| Blood vessels (with lumina) | Endothelial cells (without lumina) |
| Saline | 930 ± 274 | 1460 ± 335 | |
| HAH-cortisol conjugate | 50 ± 274 | 447 ± 213 | |
| HAH | 716 ± 183 | 1128 ± 152 | |
| HAH + cortisol mixture | 503 ± 198 | 533 ± 91 | |
| Native heparin + cortisol | 793 (625-960) | 640 (533-731) | |
| a Doses were 1.0 mg HAH-cortisol, 0.83 mg HAH, 0.17 mg cortisol, 0.83 mg HAH plus 0.17 mg cortisol, or 0.83 mg native heparin plus 0.17 mg cortisol.
| b Frozen sections of sponges were cut. The blood vessels and endothelial cells present in an entire cross-section of sponge was calculated. The numbers given are the average number of vessels and endothelial cells in cross-sections of sponges taken from 4 to 7 mice ± SD. In the case of native heparin plus cortisol, only 2 sponges were analyzed; numbers in parentheses, total number of vessels and endothelial cells of the 15 fields of the 2 sponges.
| c Endothelial cells were identified by indirect immunoperoxidase staining with rabbit anti-vWF antibody and MECA 20. Vessels were identified as vWF-positive structures with lumina usually containing erythrocytes.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Treatment</th>
<th>Tumor wt (g)</th>
<th>T/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HAH-cortisol</td>
<td>0.78 ± 0.47</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td>saline</td>
<td>1.66 ± 0.82</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>HAH-cortisol</td>
<td>0.64 ± 0.39</td>
<td>0.38</td>
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<tr>
<td></td>
<td>HAH + cortisol</td>
<td>0.97 ± 0.43</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>saline</td>
<td>1.68 ± 0.61</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>HAH-cortisol</td>
<td>0.46 ± 0.07</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>HAH + cortisol</td>
<td>0.63 ± 0.30</td>
<td>0.61</td>
</tr>
<tr>
<td></td>
<td>saline</td>
<td>1.04 ± 0.30</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>HAH-cortisol</td>
<td>1.11 ± 0.24</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>HAH + cortisol</td>
<td>1.56 ± 0.03</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>HAH</td>
<td>2.21 ± 0.47</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>cortisol</td>
<td>1.62 ± 0.25</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>saline</td>
<td>1.90 ± 0.54</td>
<td></td>
</tr>
</tbody>
</table>

| a Mice bearing 0.2-0.3-cm s.c. 3LL tumors were given daily i.v. injections of HAH-cortisol (1 mg) or equivalent amounts of unconjugated HA (0.83 mg), cortisol (0.17 mg), or a mixture of both.
| b Tumor weight ±SD 9 days after onset of treatment.
| c Tumor weight significantly (P < 0.05) less than in saline controls.
| d Tumor weight in HAH-cortisol recipients significantly (P < 0.05) less than in recipients of HAH plus cortisol.
| e T/C, treated versus saline control.

Fig. 6. Antitumor effects of HAH-cortisol conjugate. Mice were given s.c. injections of 3LL cells. One day later and daily thereafter, the mice were given daily i.v. injections of 1 mg HAH-cortisol () or equivalent quantities of unconjugated HAH plus cortisol (O) or diluent alone (A). Each treatment group contained 10 mice. Tumors were measured daily. * time points at which the tumor volumes in the recipients of unconjugated HAH plus cortisol were significantly smaller than those in the diluent recipients; ** time points at which the tumor volumes in the recipients of HAH-cortisol were significantly smaller than those in recipients of unconjugated HAH plus cortisol. At all time points the tumors in the HAH-cortisol recipients were significantly smaller than in the diluent recipients. Statistical significance was judged using the Mann-Whitney-Wilcoxon non-parametric test for two independent samples (46).

Fig. 7. Inhibition of DNA synthesis of MPCE cells by HAH-tetrahydro S. MPCE cells were treated for 24 h at 37°C with HAH-tetrahydro S (D), with an equivalent mixture of unconjugated HAH plus tetrahydro S (3), or with tetrahydro S (O) or HAH (X) alone. Bottom abscissa, HAH concentration. The ability of the cells to incorporate [3H]thymidine into DNA was determined 24 h later and is expressed as a percentage of that in untreated control cultures (179,500 dpm).
bond. This bond was stable at pH 7.4 but rapidly dissociated at pH 4.8 with a half-life of 15 min at 37°C. Thus, uptake of HAH-cortisol by endothelial cells and delivery of the conjugate to acidified endosomes and lysosomes would be expected to lead to rapid release of the steroid since these intracellular compartments have a pH of approximately 4.8.

There are two ketones in cortisol which could potentially react with the hydrazide, one at C-3 on the A-ring and the other at C-20 on the side chain. However, significant reaction appears to occur with the C-3 ketone only under the conditions used in the present study to give the product depicted in Fig. 1. This is evident from the fact that the chromophoric A-ring of cortisol, which has a UV spectrum with a λmax at 242 nm, changes on reaction with hydrazide to a product with λmax at 274 nm; a change in the absorption properties of the A-ring would not be expected if the linkage were with the C-20 ketone. Studies with another steroid, tetrahydrocortisol, which has the same C-20 ketone as in cortisol but which lacks a C-3 ketone in the A-ring revealed that conjugation through the C-20 ketone occurs at a rate more than 100-fold slower than through the C-3 ketone of cortisol (results not shown).

Derivatization of heparin to obtain HAH destroyed its anticoagulant activity, in accordance with the findings of Danishefsky and Siskovic (42) for heparinylglycine and heparinylglycine methyl ester. Presumably, modification of either or both of the two iduronic acid residues in the pentasaccharide sequence near to the nonreducing terminus (49) distorts its anti-thrombin-binding activity. The absence of any anticoagulant activity of the product is advantageous because hemorrhage is unlikely to be a problem when HAH-cortisol is administered to animals. The HAH-cortisol should, however, retain its ability to bind to endothelial cells because the sulfate groups which are important for tight binding (26) are unaltered.

HAH-cortisol inhibited DNA synthesis in cultured proliferating MPCE vascular endothelial cells at lower concentrations than did a mixture of its components. In addition, it delayed the repair of wounded MPCE monolayers, suggesting that, in addition to inhibiting cell proliferation, it inhibited endothelial cell migration. These two effects may explain why total abolition of vascularization was observed in sponges receiving injections of HAH-cortisol. Vascularization is a complex process requiring both the migration of vascular endothelial cells into the sponge and then proliferation to enable the capillary sprout to elongate. Administration of unconjugated mixtures of heparin and cortisol, or HAH and cortisol, did not affect vascularization of sponges, in contrast to results in other angiogenesis assays (20, 48).

The antitumor effects induced by injecting HAH-cortisol into mice bearing Lewis lung carcinomas were modest but statistically significant. HAH-cortisol-treated mice showed a reduction in tumor mass of 40–60% relative to that in untreated controls. In three of four experiments, the reduction of tumor growth in mice treated with HAH-cortisol significantly exceeded that in recipients of unconjugated HAH and cortisol. In addition, in the sponge experiments, systemically administered HAH-cortisol was less effective at preventing vascularization than it was when injected directly into the sponge. It is possible that the inhibitory effects of systemically administered HAH-cortisol on tumor growth and sponge vascularization might be improved by a different dose regimen designed to increase and sustain blood levels. Since the effects of steroids on cell activation/proliferation rapidly reverse when the steroid is removed (14), it is possible that a wave of endothelial cell proliferation occurs towards the end of each 24-h interval between injections. If so, the problem should be solved by a continuous infusion of the conjugate. Another possible explanation for the relatively modest antitumor effects relates to the finding (as in Fig. 3e) that the maximum inhibition of DNA synthesis of MPCE cells that could be achieved with high concentrations of HAH-cortisol was only 75%. Thus, if vascular endothelial cells in the mouse tumor have the same sensitivity to HAH-cortisol as do MPCE cells, it would be unlikely that complete inhibition of tumor angiogenesis and, consequently, of tumor growth could be attained. Finally, despite compelling evidence to the contrary (50), it is possible that solid tumors are not completely angiogenesis-dependent and that tumor growth can continue, even in the presence of an angiogenesis inhibitor, by infiltration of tumor cells along existing vascular tracts. This appears to be true of lymphomas (51) and it is possible that the Lewis lung carcinoma used in the present study resembles lymphomas in this regard. Measurements of the rate of endothelial cell division in tumors in mice on HAH-cortisol therapy are needed to resolve this point.

In an effort to determine whether it was the glucocorticoid activity of cortisol which was responsible for the antiproliferative effects of HAH-cortisol on endothelial cells, we conjugated tetrahydro S to HAH. Tetrahydro S had been shown by Crum et al. (20) and by others (52, 53) to lack glucocorticoid and mineralocorticoid activity yet be capable of suppressing angiogenesis and tumor growth when applied in a mixture with heparin and similar compounds. In accordance with these earlier studies, HAH-tetrahydro S potently inhibited DNA synthesis by MPCE cells in vitro, indicating that the angiogenic effects of the present conjugates is independent of the glucocorticoid activity of the steroid.

Tetrahydro S and related steroids offer a number of advantages over cortisol for use as HAH conjugates in vivo. Being metabolically inert in all but angiostasis assays, they should not cause the toxic side effects (e.g., osteoporosis) associated with long term corticosteroid therapy. Long term treatment with HAH conjugates is likely to be necessary in tumor therapy to suppress the outgrowth of dormant tumor cells. In addition, HAH-tetrahydro S inhibited DNA synthesis more completely than did HAH-cortisol, suggesting that it should be a better antitumor agent in vivo. Unfortunately, tetrahydro S and related steroids lack a C-3 ketone by which they can be conjugated efficiently to HAH. Conjugation can be achieved through the C-20 ketone but the coupling efficiency (0.5 mol steroid/mol HAH) is poor compared with cortisol (5–8 mol steroid/mol HAH). We are currently developing new methods for forming HAH-tetrahydro S and will report these at a later date.

In conclusion, the HAH-cortisol conjugate described herein is the prototype of a new class of angiogenesis inhibitors with antitumor activity. The angiogenic activity of heparin and cortisol, which has previously been shown for unconjugated mixtures of these agents (20), appears to be improved by their combination into a single molecule. By refining the choice of steroid, the coupling chemistry for developing new methods for forming HAH-tetrahydro S and will report these at a later date.

In conclusion, the HAH-cortisol conjugate described herein is the prototype of a new class of angiogenesis inhibitors with antitumor activity. The angiogenic activity of heparin and cortisol, which has previously been shown for unconjugated mixtures of these agents (20), appears to be improved by their combination into a single molecule. By refining the choice of steroid, the coupling chemistry for forming the conjugates, and the dose regimen for administration, we hope to improve activity, yield, and safety to the extent that these drugs become clinically useful.

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HEPARIN-STEROID CONJUGATES AS ANGIogenesis INHIBITORS

Heparin-Steroid Conjugates: New Angiogenesis Inhibitors with Antitumor Activity in Mice

Philip E. Thorpe, Elaine J. Derbyshire, Silvia P. Andrade, et al.