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Effect of Heparin, Heparin Fragments, and Corticosteroids on Cerebral Endothelial Cell Growth *in vitro* and *in vivo*

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Abstract. Heparin and heparin fragments in combination with corticosteroids have been shown to markedly inhibit tumor angiogenesis. Experiments were performed to test the hypothesis that heparin, heparin fragments, and the combination of heparin and corticosteroids affect DNA synthesis and the proliferation of cerebral microvessel endothelium (ME). *In vitro,* methyl-^{3}H-thymidine incorporation in the ME cells was measured after a 24 hour pulse. Our results show that heparin, hydrocortisone, and heparin in combination with hydrocortisone had a slight inhibitory effect on DNA synthesis of ME ($p < 0.05$), and hydrocortisone in combination with heparin had a slight inhibitory effect on ME cell growth ($p < 0.05$). The hexa-, octa-, and deca-saccharide fragments of heparin stimulated DNA synthesis in ME ($p < 0.01$). *In vivo,* DNA synthesis in cerebral endothelial cells at the margin of a freeze lesion to mouse cerebral cortex was assayed by quantitation of labeling indexes from methyl-^{3}H-thymidine autoradiographs in mice treated with heparin, cortisone, or a combination of heparin and cortisone. A mean endothelial cell labeling index (LI) of 6% in the cortisone-treated animals was significantly lower than controls (32%, $p < 0.01$). The addition of heparin to cortisone did not significantly alter the endothelial cell LI compared to the cortisone-treated animals, and heparin alone did not significantly alter the LI compared to the controls. These results indicate that cortisone markedly reduces the endothelial proliferation around a cortical freeze lesion *in vivo.* This effect is independent of heparin.

Key Words: Blood-brain barrier; Cerebral endothelium; Corticosteroids; Freeze injury; Heparin.

INTRODUCTION

Heparin, in the presence of cortisone, has been shown to be a potent inhibitor of tumor angiogenesis *in vivo* (1, 2). Furthermore, several non-anticoagulant fragments of heparin, the most potent being a hexasaccharide, also demonstrate strong anti-angiogenesis activity *in vivo* in the presence of cortisone (1). Heparin and several heparin fragments alone promote tumor angiogenesis *in vivo* (1, 3), while cortisone alone fails to inhibit or induce tumor angiogenesis (1).

While heparin and corticosteroids are commonly used in the prevention and treatment of stroke, the effect of these compounds on the regeneration of cerebral microvessels surrounding an area of cerebral cortical injury such as that seen in a cerebral infarction is unknown. The purpose of the *in vitro* and *in vivo* experiments reported here was to determine the effect of heparin, heparin fragments, corticosteroids, and heparin and corticosteroids in combination on the growth of cerebral endothelium *in vitro* and the effect of these compounds on the regeneration of cerebral microvessels following a cerebral cortical injury. The results of the present study

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indicate that *in vitro*, heparin, hydrocortisone, and heparin in combination with hydrocortisone had a slight inhibitory effect on DNA synthesis and cell growth of mouse cerebral endothelial cells. In addition, several heparin fragments stimulate DNA synthesis in cerebral endothelium, the most potent being the hexasaccharide fragment.

Our *in vivo* results suggest that cortisone significantly reduces endothelial proliferation following a freeze injury to the cerebral cortex. This inhibition appears to be caused by the marked reduction in the macrophage infiltration in proximity to the necrotic vessels. The combination of heparin and cortisone did not further reduce the endothelial proliferation. Heparin alone had no effect on cerebral endothelial proliferation.

MATERIALS AND METHODS

Test Cell Cultures

All cell proliferation and methyl-^{3}H-thymidine incorporation studies were performed on cells from mouse cerebral capillary endothelial (ME) lines maintained in our laboratory. Characteristics of the cell lines, including evidence of their origin from cerebral vascular endothelium, have been extensively documented (4–6). This includes the characteristic "cobblestone" morphology of endothelial cells and immunocytochemical features of endothelium. The cells have measurable levels of angiotensin-converting enzyme, bind thrombin and maintain their characteristic endothelial morphology through 20 or more serial passages. The cells are routinely maintained by twice weekly trypsinization and splitting in T75 culture flasks at a ratio of 1:2. They are grown in modified Lewis media (LM) (7) supplemented with 20% fetal bovine serum (FBS).

Preparation of Heparin Fragments

Sodium heparin (145 U/mg) from porcine mucosa was purchased from Hepar Industries (Franklin, OH) (8). Heparinase was prepared fermentatively from *Flavobacterium heparinum* and purified from contaminating activities as previously described (9). Heparin was completely digested with heparinase (10) after which the enzyme was removed by SP-Sephadex ion-exchange chromatography (11), and the fragment mixture obtained was fractionated by low pressure gel permeation chromatography (GPC) (10). The resulting di-, tetra-, hexa-, octa-, and deca-saccharide mixtures were desalted, frozen and freeze-dried (10). Each sized mixture was then characterized for purity by high pressure liquid chromatography (HPLC) (10).

Steroid Preparation

Hydrocortisone (Sigma, St. Louis, MO), at a concentration of 1 mg/ml of 95% ethanol, was added to the culture medium immediately before use at the desired concentration. The controls contained an equivalent amount of ethanol that never exceeded 0.5%. Cortisone acetate (Merke, West Point, PA) at a concentration of 25 mg/ml was used for all *in vivo* experiments.

Methyl-^{3}H-thymidine Incorporation Studies *in vitro*

Microvessel endothelium (ME) cells (below 20th passage) were trypsinized (0.25% trypsin, 0.02% EDTA) and plated into 35 mm cluster dishes at a density of 0.5 to 1.5 $\times$ 10^5 cells per well. Cells were still subconfluent at 24 to 48 hours (h) of growth at which time they were washed once with sterile phosphate buffered saline (PBS) and fed with serum-free Lewis media (SFLM) in order to obtain baseline quiescence with regard to cell division. After 24 h the SFLM was removed and replaced with SFLM or LM supplemented with 5% FBS that had been previously stripped of endogenous steroid as described by Eckert et al (12). Heparin, heparin fragments, hydrocortisone, or the combination of heparin and hydrocortisone was added to the media at their desired concentrations. Control wells did not receive any heparin or hydrocortisone.

Methyl-^{3}H-thymidine (6.7 Ci per mM New England Nuclear) was added 24 h later (0.5 to

1 μCi per well) and left on the cells for 24 h. Medium was then aspirated from the dishes, the cells were rapidly washed twice with warm PBS and treated with cold 10% trichloroacetic acid (TCA) for ten minutes, followed by one further wash with PBS. TCA precipitable radioactivity was measured in a programmable Beckman LS7800 scintillation counter with an automatic quench control after solubilizing the cells in 2 ml of 0.3 M NaOH–2% sodium dodecyl sulfate, and mixing an aliquot of this material with AML budget solve scintillation fluid (Research Products International). In some experiments the incorporation studies were performed in 96 well tissue culture plates in order to miniaturize the system. Cells were initially seeded at 10^4 cells/well and treated in an identical fashion. These cells were harvested with a PhD Cell Harvester (Cambridge Technology, Cambridge, MA).

Cell Proliferation Assays

Cells were seeded at approximately 0.5×10^5 cells per dish in quadruplicate sets of 35 mm diameter tissue culture dishes in 2 ml of LM with 20% FBS. After 24 h each dish was washed with PBS and the media changed to SFLM. Twenty-four h later the media was removed and control or experimental media containing 5% steroid-free FBS plus heparin, hydrocortisone or heparin/hydrocortisone in various concentrations was added to each dish. At 168 h following this, cells from each dish were counted using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) that had been standardized to cell counts obtained from a hemocytometer for the same sample.

Animals

Six- to eight-week-old Swiss Webster mice (Biolabs, St. Paul, MN) were used in all *in vivo* experiments. They were fed standard laboratory chow and water *ad libitum.*

Freeze Injury to Cerebral Cortex

The animals were anesthetized with chloral hydrate, the scalp was incised, and the periosteum exposed. A focal freeze lesion was made by applying a liquid nitrogen probe to the intact skull for five seconds.

Methyl-^{3}H-Thymidine Labeling Studies

DNA synthesis of endothelial cells was assessed by studying the incorporation of methyl-^{3}H-thymidine that was injected intraperitoneally (IP) (0.025 μCi per animal) 24 h before the animals were killed by intracardiac perfusion of 3% phosphate buffered glutaraldehyde. The tissue was embedded in Spurr's and light microscopic autoradiographs prepared. The methyl-^{3}H-thymidine labeling index (LI) was quantitated by counting all the endothelial nuclei from capillaries, venules, and arterioles within one high powered field ($\times$40) of the edge of the lesion. Three sections from each lesion were quantitated by two independent investigators. Each group was comprised of four animals. Therefore, the LI from each group of animals was calculated from twelve sections. The average number of cells counted per lesion was 124 ± 9.

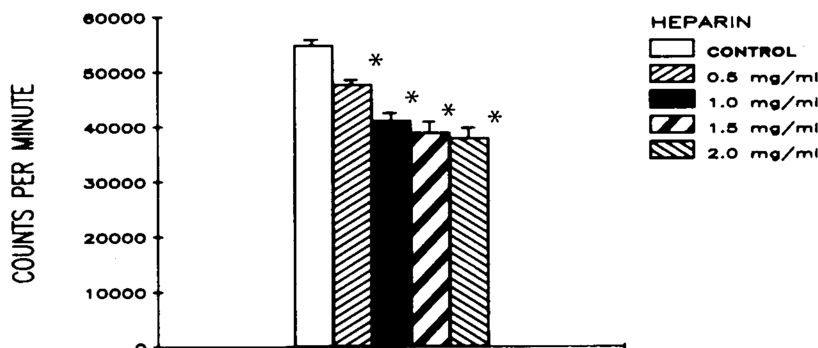
Statistical Analysis

The results of each group of experiments were evaluated using a Student's *t*-test for unpaired data.

RESULTS

Effect of Heparin, Hydrocortisone, and the Combination of Heparin and Hydrocortisone on Endothelial Proliferation and Methyl-^{3}H-Thymidine Incorporation *in vitro*

Using non-confluent cultures of cerebral endothelium, heparin (0.5 mg/ml to 2.0 mg/ml) demonstrated a modest but significant decrease in methyl-^{3}H-thymidine incorporation into cerebral endothelium compared to controls ($p < 0.05$) (Fig. 1).



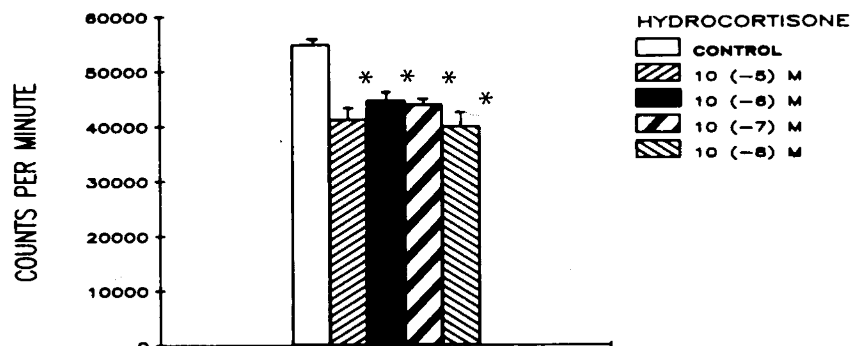


Fig. 1. Effect of heparin (top) and hydrocortisone (bottom) on methyl-^{3}H-thymidine incorporation by cerebral endothelial cells. Both heparin (0.5 mg/ml–2.0 mg/ml) and hydrocortisone (10^{-5} M–10^{-8} M) significantly reduced methyl-^{3}H-thymidine incorporation ($p < 0.05$). Mean ± standard error. * represents significant changes.

Hydrocortisone (10^{-8} M to 10^{-5} M) showed a similar significant decrease in methyl-^{3}H-thymidine incorporation into the cerebral endothelium ($p < 0.05$) (Fig. 1). Hydrocortisone (10^{-5} M to 10^{-8} M) in combination with heparin (0.5 mg/ml to 2.0 mg/ml) also resulted in a significant decrease in methyl-^{3}H-thymidine incorporation into cerebral endothelial cells ($p < 0.05$) (Fig. 2). The inhibition of methyl-^{3}H-thymidine incorporation utilizing hydrocortisone in combination with heparin was not significantly different than either heparin or hydrocortisone alone.

The effect of the di-, tetra-, hexa-, octa-, and deca-saccharide heparin fragments (50 μg/ml) on methyl-^{3}H-thymidine incorporation by cerebral endothelial cells was

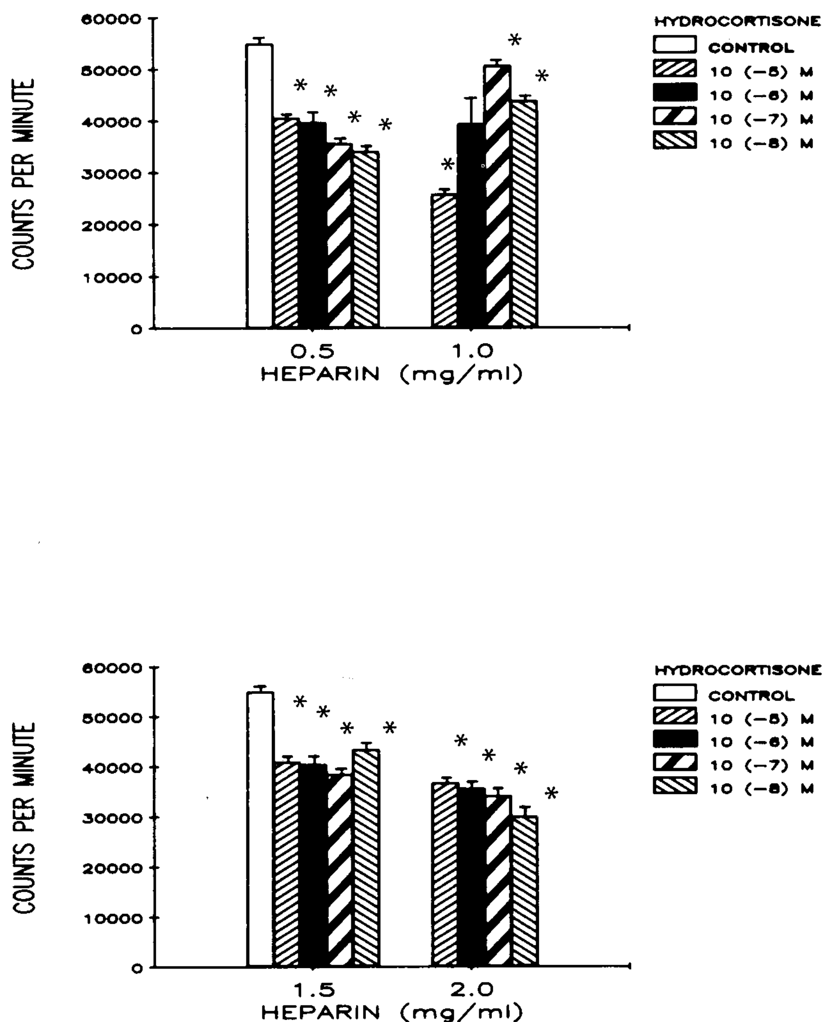


Fig. 2. Effect of heparin and hydrocortisone in combination on methyl-^{3}H-thymidine incorporation by cerebral endothelial cells. Methyl-^{3}H-thymidine incorporation was reduced at all concentrations tested. Mean ± standard error. * represents significant changes ($p < 0.05$).

also assessed. These results showed that the hexa-, octa-, and deca-saccharides all significantly increased methyl-^{3}H-thymidine incorporation by cerebral endothelial cells ($p < 0.01$). The di-, and tetra-saccharides and heparin (50 μg/ml) had no effect (Fig. 3).

Neither heparin (0.5 mg/ml to 2.0 mg/ml) nor hydrocortisone (10^{-5} M to 10^{-7} M) had a significant effect on cerebral endothelial cell proliferation at 168 h. Heparin (0.5 mg/ml to 2.0 mg/ml) in combination with hydrocortisone had a modest but significant inhibitory effect on cerebral endothelial cell proliferation ($p < 0.05$) (Fig. 4).

In none of the *in vitro* studies did the morphology of the endothelial cells change.

Fig. 3. Effect of heparin fragments on methyl-^{3}H-thymidine incorporation by cerebral endothelial cells. The deca-, octa-, and hexa-saccharide fragments all significantly increased methyl-^{3}H-thymidine incorporation ($p < 0.01$). Mean ± standard error. * represents significant changes.

Effect of Heparin, Cortisone, and Heparin in Combination with Cortisone on Cerebral Endothelium Proliferation Following a Freeze Injury to the Cerebral Cortex

Twenty mice were divided into five groups of four mice each. They all received a freeze lesion to the cerebral cortex. Group A that served as controls received normal drinking water and standard laboratory chow.They also received subcutaneous (SQ) injections of 0.25 ml saline on a daily basis. Group B mice received daily SQ injections of 250 mg/kg cortisone acetate until they were killed. Group C mice received daily SQ injections of 0.25 ml NaCl as well as 1,000 U/ml heparin in the drinking water. Group D mice received heparin (1,000 U/ml) in the drinking water as well as daily SQ injections of cortisone acetate (250 mg/kg). Group E mice received cortisone acetate (50 mg/kg) and heparin (1,000 U/ml) in the drinking water. All mice were then killed on day 4 after freeze injury. In Group A there was a marked macrophage infiltrate at the edge of the lesion and new vessel formation. The endothelial labeling index (LI) was 32% (Fig. 5). In Group B, D, and E there was a reduction in the macrophage infiltrate around the lesion, and new vessel formation was scant. The endothelial LI was significantly reduced in groups B, D and E ($p < 0.01$). In Group C there was a prominent macrophage infiltrate around the lesion, and the endothelial LI was not significantly different from controls (Fig. 5).

DISCUSSION

Heparin and non-anticoagulant fractions of heparin have been shown to be involved in the regulation of angiogenesis *in vivo* (1–3). While heparin alone fails to produce new vessel growth *in vivo,* heparin or heparin fragments in conjunction with tumor extract produce enhanced angiogenesis *in vivo* above that of tumor alone (1, 3). Heparin when bound to copper becomes angiogenic by itself *in vivo* (13) and stimulates migration of endothelial cells *in vitro* (14). In addition, heparin together with fibronectin enhances endothelial cell migration *in vitro* beyond either compound

EFFECT OF HYDROCORTISONE AND/OR HEPARIN ON CEREBRAL ENDOTHELIAL CELL PROLIFERATION IN VITRO

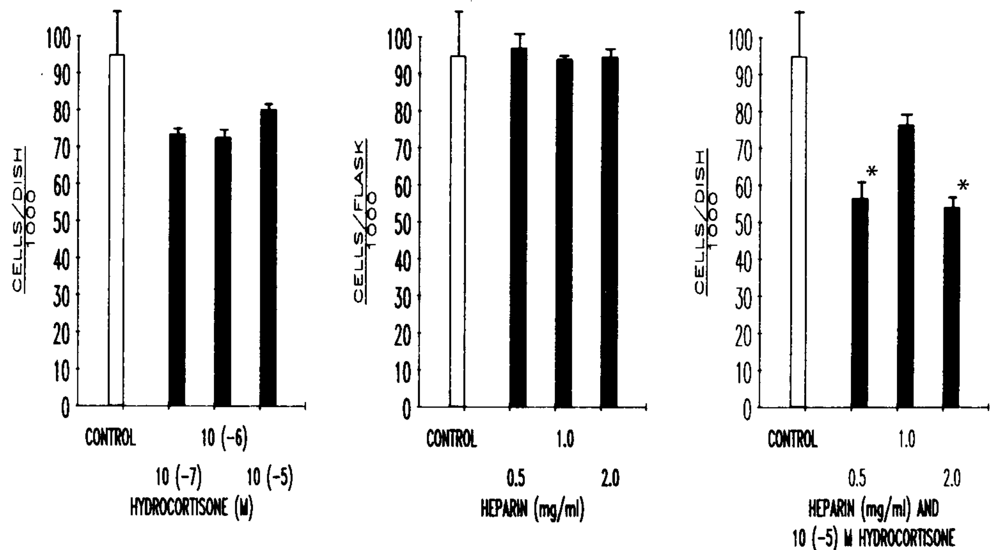


Fig. 4. Effect of hydrocortisone, heparin, and the combination of hydrocortisone and heparin on cerebral endothelial cell proliferation. Cell counts were obtained from each of quadruplicate sets of dishes at 168 h incubation. Heparin (0.5 mg/ml and 2.0 mg/ml) in combination with hydrocortisone (10^{-5} M) significantly decrease endothelial cell proliferation ($p < 0.05$). Mean ± standard error. * represents significant changes.

alone (15). Heparin has a strong affinity for a tumor-derived endothelial cell growth factor (16), as well as an endothelial cell-derived growth factor (17). Heparin may therefore serve as an intermediary in angiogenesis by potentiating growth factors or by enhancing migration of endothelial cells (18).

The experiments described in this paper were performed to answer several questions concerning heparin fragments, heparin and cortisone on the cerebral capillary endothelial cell proliferation *in vitro* and *in vivo.* While heparin and cortisone have been studied in the context of tumor angiogenesis, it is unknown whether these compounds individually or together have an effect on the neovascularization that accompanies cerebral injury or cerebral infarction. This is of great interest to the neuroscientist/clinician since these two drugs are often used together or alone in patients with cerebral cortical injury or a cerebral infarct.

Our *in vitro* results show that heparin has no effect on cerebral endothelial proliferation, and has only a mild inhibitory effect on methyl-^{3}H-thymidine incorporation, reflecting DNA synthesis in cerebral endothelial cells. Hydrocortisone, likewise, had no significant effect on the cerebral endothelial cell proliferation *in vitro.* The combination of these compounds showed a modest inhibitory effect on cerebral endothelial cell proliferation, as well as a modest decrease in methyl-^{3}H-thymidine incorporation.

We have shown that several heparin fragments produce a significant increase in methyl-^{3}H-thymidine incorporation into the cerebral endothelial cell, suggesting that these compounds directly stimulate cerebral endothelial cell growth. The hexa-sac-

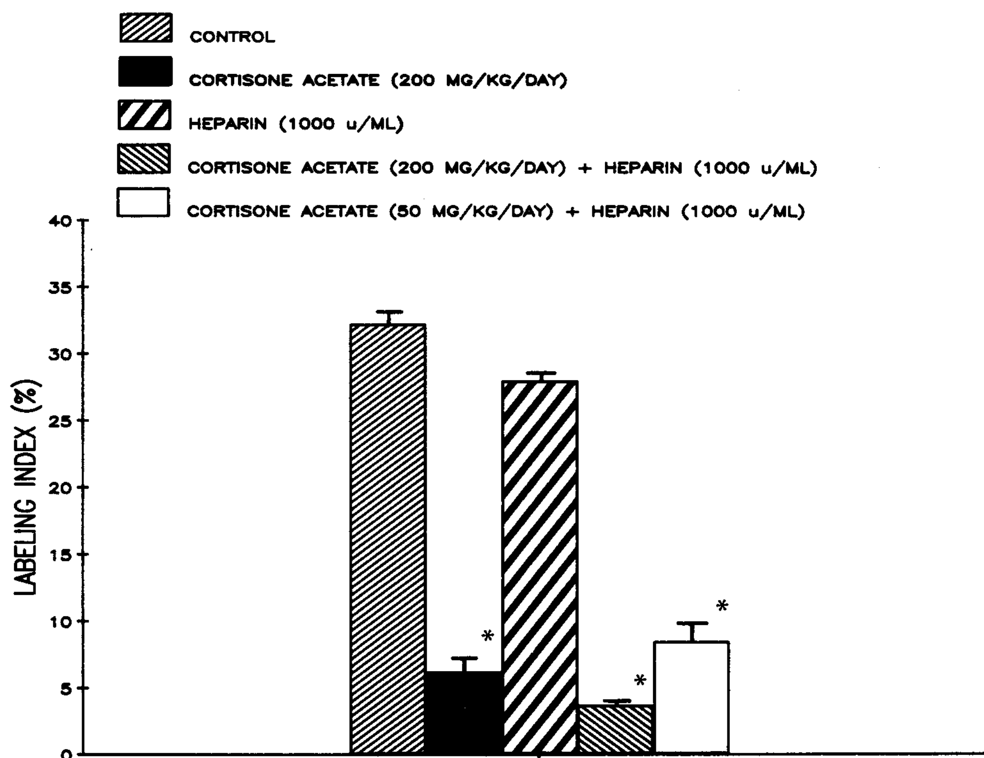


Fig. 5. Labeling indexes of methyl-^{3}H-thymidine four days after freeze injury. There is no significant difference in the labeling indexes between the control and heparin-treated group. There was a significant difference between these two groups and the cortisone plus heparin and cortisone alone groups ($p < 0.01$). Mean ± standard error. * represents significant change.

charide fragment demonstrated the most marked increase in methyl-^{3}H-thymidine incorporation. This fragment has also been shown to be the most active, both in terms of potentiating tumor angiogenesis when used alone, and inhibiting tumor angiogenesis when used in combination with hydrocortisone (1).

The effects of heparin, cortisone, and heparin and cortisone in combination on new vessel growth around a freeze injury to the cerebral cortex were quite different from that in association with angiogenesis of tumors, despite the fact that the doses used and routes of administration of the drugs were similar (1). Cortisone alone markedly reduced vascular proliferation around a freeze lesion, whereas cortisone had no effect on tumor angiogenesis (1). Cortisone resulted in a marked reduction in the macrophage infiltration around the cortical freeze lesion, and macrophage depletion by x-radiation has been shown to reduce the endothelial LI in the cortical freeze lesion model to a similar degree (19). Macrophages have been shown to induce neovascularization *in vivo* (20) and stimulate endothelial cell growth *in vitro* (21). When heparin was added to the cortisone, no further reduction of endothelial proliferation around the lesion was noted, whereas this combination markedly reduced tumor angiogenesis *in vivo* (1, 2).

Heparin alone showed no effect *in vivo* on the cerebral endothelial proliferation following a freeze lesion, whereas heparin has been shown to accelerate tumor angiogenesis (1, 2). This suggests that the endothelial mitogens released following a freeze lesion, whether they are macrophage-derived or brain-derived factors, are

different from the tumor-derived factors and endothelial cell-derived growth factors that have been shown to have a strong affinity for heparin.

Heparin has been shown to have varying effects on proliferation of mouse L-M cells *in vitro,* depending on the heparin preparation (22). The original studies showing heparin in combination with cortisone inhibit the neovascularization of several tumors were done with Panheparin (Abbott, Chicago, IL) (1), a preparation no longer available. Hepar heparin, which was used in our studies, showed a similar, but not as marked, inhibition of tumor angiogenesis in combination with cortisone (1).

Cortisone acetate and hydrocortisone have been shown to have equal effectiveness in causing tumor regression in combination with heparin *in vitro* (1). We used hydrocortisone for the *in vitro* studies because it was more soluble in alcohol than the cortisone acetate.

In conclusion, we have shown that heparin, hydrocortisone, and heparin in combination with hydrocortisone all have a mild inhibitory effect on cerebral endothelial cell proliferation *in vitro.* The hexa-saccharide fragment of heparin has a marked stimulating effect on cerebral endothelial DNA synthesis *in vitro. In vivo,* cortisone markedly decreases vascular proliferation around a freeze lesion of the cerebral cortex, an effect that is not potentiated by heparin, and is most probably due to a reduction in the macrophage infiltration around the lesion.

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