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Dexamethasone reduces vascular density and plasminogen activator activity in 9L rat brain tumors

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Angiogenesis, a process dependent upon perivascular proteolysis, is required for solid tumor growth and is inhibited by certain steroids including glucocorticoids. We examined the relationship between tumor growth and vessel density in experimental rat brain 9L glial tumors following chronic treatment with the glucocorticoid dexamethasone. Tumor growth was inhibited by intraperitoneal administration of 3 mg/kg/day dexamethasone. Maximal cross-sectional areas of post-implantation day 9 tumors were 4.6 ± 1.0 mm^2 in dexamethasone-treated animals and 17.0 ± 3.4 mm^2 in controls ($P < 0.01$). Microvessel density assessed by laminin immunohistochemistry was 59% lower in dexamethasone-treated tumors ($P < 0.01$). Plasminogen activator (PA) activity, a proteolytic enzyme related to endothelial migration and vessel growth, was 4.2 ± 0.9 IU/μg protein in dexamethasone-treated tumors and 9.0 ± 1.0 IU/μg protein in control tumors ($P < 0.01$). Exposure of cultured 9L and central nervous system microvessel endothelial cells to dexamethasone concentrations comparable to those achieved in vivo had no effect on cell growth, but reduced the PA activity of culture supernatant fractions by 78% and 99%, respectively. These findings suggest that inhibition of proteolytic steps involved in vessel growth may underlie, in part, the mechanism by which glucocorticoids decrease brain tumor growth.

INTRODUCTION

Chronic treatment with glucocorticoids inhibits the growth of experimental brain tumors[16,34,41]. Angiogenesis, a requirement for solid tumor growth, also may be inhibited under certain conditions by steroids[6,12]. Whether glucocorticoids directly inhibit the proliferation of brain tumor cells in vivo, or suppress tumor growth indirectly by inhibiting angiogenesis is not known. Elucidating the underlying mechanisms of these glucocorticoid effects could aid the development of more effective agents that suppress brain tumor growth.

Glucocorticoids alter tumor cell proliferation in vitro but there is considerable variation in the response of different brain tumor cell lines[9]. Growth responses also vary depending upon specific culture conditions and steroid concentrations. Proliferation of some brain tumor cells is increased by dexamethasone concentrations below 10 μM while concentrations above 100 μM can inhibit growth[15,26,29]. Glucocorticoids are also antiangiogenic[6,7]. The mechanism of this effect appears related to basement membrane turnover[6], which is determined, in part, by proteolytic activity associated with proliferating vessels.

Plasminogen activators, serine proteases that convert plasminogen to plasmin, increase the proteolytic activity associated with capillary endothelial cells and thereby regulate endothelial invasiveness and capillary morphogenesis[28,30,33]. Plasminogen activators (PA) are also produced by glioma cells[35] and are associated with the microvasculature within brain tumors[8]. Agents that stimulate endothelial plasminogen activators induce angiogenesis while plasminogen activator inhibitors are antiangiogenic in vitro[8,28,37]. Dexamethasone inhibits plasminogen activator activity in many cell lines but this effect varies depending upon cell types and culture conditions[3,11,23,33]. The relationship between dexamethasone and plasminogen activation has not been examined in brain tumors or central nervous system endothelial cells.

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We have recently demonstrated that dexamethasone reduces the growth of experimental 9L gliomas and differentiates 9L glioma-associated blood vessels based on both biochemical and functional criteria[14]. Since differentiated vessels proliferate at slower rates, we asked if dexamethasone inhibits 9L brain tumor growth via a direct effect on 9L cell proliferation or indirectly by decreasing vessel growth. This study examines the effects of dexamethasone on 9L tumor growth in vivo, 9L cell proliferation in vitro and 9L tumor vessel density, an index of vessel growth. Tumor growth and vessel densities were significantly reduced in response to dexamethasone at concentrations that did not affect 9L cell growth in vitro. Steroid treatment reduced the PA activity associated with tumors, cultured 9L cells and cultured microvascular endothelial cells derived from the central nervous system. We present the hypothesis that alterations in plasminogen activator-dependent angiogenic mechanisms contribute to glucocorticoid-mediated inhibition of glial tumor growth.

MATERIALS AND METHODS

Cells

9L rat brain tumor cells, originally derived from a nitrosurea-induced rat glioma were obtained from Marvin Baker, University of California at San Francisco Brain Tumor Research Center, maintained in Dulbecco's Modified Eagle's Medium (DMEM, Mediatech, Washington, DC) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 2 mM L-glutamine (Mediatech), 200 U/ml penicillin (Mediatech), 200 μg/ml streptomycin sulfate (Mediatech) at 37°C in 5% CO_2/95% air.

Central nervous system endothelial cells were isolated from bovine retinal microvessels and cultured as previously described[22]. Essentially 100% of these were shown to be endothelial by labeling with acetylated-low density lipoprotein[22].

Tumor implantation

Male adult Fischer 344 rats weighing 175–200 g (Harlan/Sprague–Dawley Inc., Indianapolis, IN) were anesthetized by intraperitoneal injection of 3 ml/kg of a stock solution containing 25 mg/ml ketamine hydrochloride (Parke-Davis, Morris Plains, NJ), 2.5 mg/ml xylazine (Mobay, Shawnee, KS), and 14% ethanol in 0.85% NaCl solution. After midline incision the periosteum was displaced, a 1 mm diameter burr hole drilled, and a 10 μl suspension containing 100,000 9L cells in DMEM supplemented with 10% FBS injected into the left caudate-putamen as previously described[13].

Dexamethasone treatment of animals

For each animal, 3 mg/kg of dexamethasone sodium phosphate (Elkins-Sinn, Cherry Hill, NJ) diluted in sterile 0.85% NaCl solution to a final volume of 0.5 ml/animal was injected once per day intraperitoneally beginning 48 h after surgery as previously described[14]. Control animals received the same volume of 0.85% NaCl solution.

Perfusion

Rats were anesthetized as described above. After thoracotomy the right atrium was incised and the left ventricle canalized and perfused (60 ml/min Whatson-Marlow pump 503S, Falmouth, England) sequentially with 200 ml of 120 mM NaCl, 2.7 mM KCl in 10 mM phosphate buffer pH 7.4 (PBS) and then with 200 ml of 3.7% formaldehyde solution (Sigma, St. Louis, MO: Accustain). Brains were placed in the same fixative overnight in 4°C and processed to paraffin blocks. Coronal sections were made through the maximum cross-sectional area of the tumors.

Immunohistochemistry

Serial 5 μm coronal sections were deparaffinized and rehydrated. Laminin antigen was liberated by incubating the sections for 15–20 min at 37°C with 0.2% pepsin (Calbiochem, LaJolla, CA) in 0.01 N HCl. Sections were then incubated for 15 min in 1% H_2O_2 in methanol, rinsed, and incubated overnight at 4°C with a rabbit antilaminin antiserum (Gibco, Grand Island, NY) diluted 1:1000 in PBS containing 1% normal goat serum. Antibody binding was visualized using an Elite ABC Kit (Vector Laboratories, Burlingham, CA) according to the method of Hsu et al.[18] with 3,3′-diaminobenzidine (Sigma) as chromogenic substrate. Sections were counterstained with hematoxylin. For negative controls, immune serum was substituted with nonimmune rabbit serum (Vector).

Morphometry

The cross-sectional area of the tumors was measured with the Microcomputer Imaging Device (MCID) software package (Imaging Research Inc., Brock University, Ste. Catherines, Ontario, Canada), a Sierra Scientific High Resolution CCD camera and a Compaq DeskPro 386/25 computer. Microvessel density was quantitated in laminin-stained tissue sections by counting ten 0.1 mm^2 fields/tumor with a Leitz Aristoplan microscope with 40×objective.

Dexamethasone determinations

Blood was obtained from rats (one animal per time point) before and after the intraperitoneal injection of dexamethasone on post-implantation day 9. Dexamethasone was extracted under alkaline conditions by the procedure of Cham et al.[4] and analyzed by high performance liquid chromatography by the procedure of Lamiable et al.[12]. Briefly, 50 μl of 1 M NaOH was mixed with 3 ml of plasma and washed with 7 ml of *n*-heptane. NaCl (300 mg) was added to the aquaeous phase and the dexamethasone was extracted with 10 ml of dichloromethane. The organic phase was then dried under N_2 and resuspended in 50 μl of methanol. The samples were injected onto a C18 column (Phase sep) and the dexamethasone eluted with a linear 20–40% acetonitrile gradient in 2 mM sodium acetate, pH 4.8 at flow rate of 2 ml/min for 30 min. The UV detection wavelength was 246 nm and the sensitivity scale was 0–0.01 a.u.f.s. Prednisolone was added to the plasma samples at 1 μg/ml to determine recovery.

Cell growth

Cells were plated in triplicate onto 24-well plates at 1,000 cells/well and 12 h later the medium was replaced with medium containing dexamethasone (10^{-10} to 10^{-4} μM) or solvent only as control. Media was replenished every 24 h. Cells from four wells in each treatment group were trypsinized and counted (Coulter counter, Coulter Electronics, Hialeah, FL) every 24 h.

Plasminogen activator determination

Plasminogen activator (PA) activity was extracted from tumors as described by Markus et al.[24] with minor modifications. Rats ($n = 8$) were perfused with PBS and their brains quickly removed and placed on ice. Tumors were dissected, weighed and placed in 10 vols./weight of 0.075 M NaCl, 2.5 mM sodium phosphate, 0.25% Triton X-100, pH 7.8. The tissue was then homogenized on ice using 20 strokes of a tight fitting Dounce glass-glass homogenizer and centrifuged for 5 min at 10,000× *g*. Supernatants were used for PA and protein assays.

To collect conditioned media from cell cultures, 40,000 bovine retinal endothelial cells or 100,000 9L cells were plated on 8 chamber Permanox slides with 0.7 cm^2 surface area per chamber. The medium was replaced 24 h later with 400 μl of fresh medium containing 10 μM dexamethasone or solvent for control. The conditioned media was collected 72 h later and used for PA measurements.

PA activity was determined as previously described[20,39]. All reagents were purchased from American Diagnostica (Greenwich, CT). Briefly, 10 μl of sample were added to a mixture of 3 μl of des-AA-fibrinogen (4 mg/ml), 8 μl of human Glu-type plasminogen (1 mg/ml), 10 μl of H-D-norleucyl-hexahydrotyrosyl-lysine-*p*-nitroanilide diacetate salt (Spectrozyme PL, 5 μmol/ml) and 169 μl of 50 mM Tris-HCl, pH 7.4, in 96 well plates and incubated at 37°C in a humidified chamber. Rate of change in absorbance at 410 nm during the linear portion of the reaction was determined using a MR 600. Microplate reader (Dynatech Laboratories, Chantilly, VA). Plasminogen activator activity was determined by comparison with a standard curve generated with 2-chain tissue-PA standards.

Protein concentrations were measured according to the method of Bradford[2].

RESULTS

Tumor size and vessel density

To compare size and vessel density of treated and untreated tumors, control and dexamethasone-treated animals ($n = 6$ in each group) were sacrificed by perfusion fixation 9 days after implantation. This experiment was performed twice. Treated tumors were significantly smaller than those in controls (Fig. 1). The mean maximal cross-sectional area of dexamethasone-treated tumors was 27% of control ($P = 0.006$, 2-tailed unpaired t-test). In addition, vessel densities were dramatically lower in treated tumors than in control tumors (Figs. 2 and 3). The control tumors had 202 ± 26 while treated tumors had 83 ± 14 laminin-positive microvessels per mm^2 ($P = 0.003$).

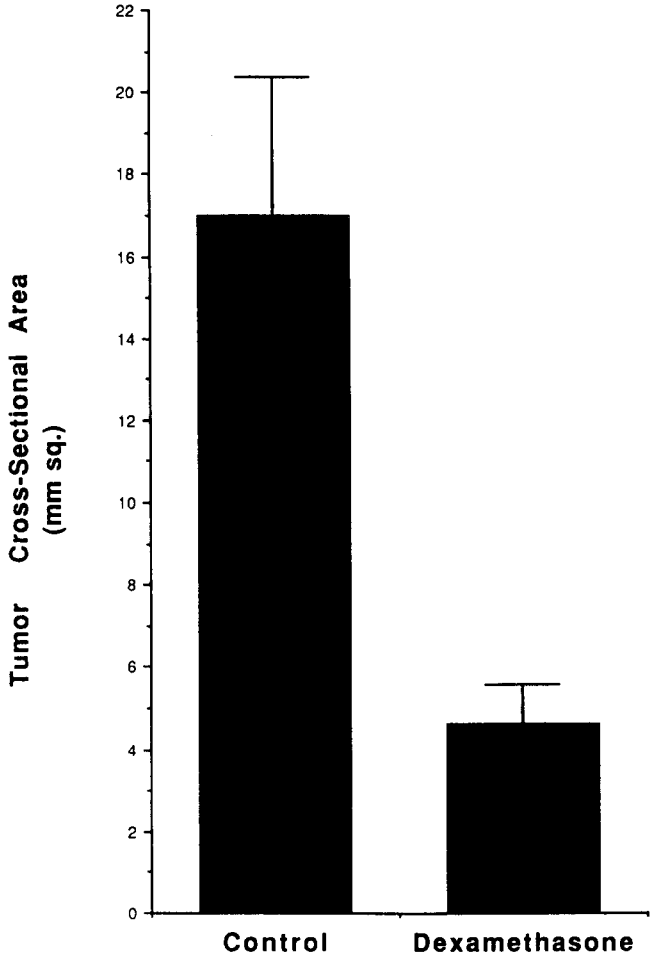

Fig. 1. Dexamethasone inhibits 9L-tumor growth in vivo. Dexamethasone-treated and control 9L tumor-bearing rats were sacrificed on post-implantation day 9. Data represents maximal cross-sectional tumor area ± standard error. $n = 6$, $P < 0.01$.

Plasminogen activator activity in tumors

At post-implantation day 9, tumor-bearing animals were perfused sequentially in approximately 0.5 h intervals from 0.5 to 5 h after the last intraperitoneal dexamethasone treatments. Tumor-associated PA activities were then determined. Mean PA activity, normalized to total protein, was reduced by 46% in dexamethasone-treated tumors ($n = 8$ in each group, $P < 0.01$, Fig. 4). In addition, there was a significant inverse relationship between the PA activity of individual tumors and the time from last dexamethasone treatment ($r^2 = 0.732$, $n = 8$, $P < 0.01$). For example, PA activity was 8 IU/μg protein 1 h after treatment and only 1. IU/μg 4 h later (Fig. 5). As expected, no significant dependence of PA activity on time of sacrifice was seen in the control tumors ($r^2 = 0.107$). When normalized to wet weight, a similar significant reduction in mean PA activity (control: 54.9 ± 5.7. IU/mg, treated 32.7 ± 5.5. IU/mg; $P = 0.02$) and a similar time dependence ($r^2 = 0.704$) was observed in the dexamethasone-treated tumors. Serum PA activity was less than 10% of the activity in tumor tissue in both control and treated animals, and likewise decreased with time after injection of dexamethasone ($r^2 = 0.778$).

Dexamethasone concentrations

We measured serum dexamethasone levels in 8 tumor-bearing animals on the 9th day after implantation to determine relevant steroid concentrations for in vitro studies. Dexamethasone was undetectable (< 0.5 μM) immediately before the last intraperitoneal injection, reached a peak of 9 μM 1 h after injection, and decreased rapidly to 1 μM 4.5 h after injection.

Cell growth

To determine whether direct inhibition of cell proliferation might be responsible for the effect of dexamethasone on 9L tumor growth in vivo, we measured the effect of dexamethasone on the doubling time of 9L cells and of central nervous system microvascular endothelial cells under conditions of exponential growth. Dexamethasone concentrations of 10^{-4} M increased 9L doubling time from 18.9 ± 0.8 h in controls to 23 ± 1.1 h and concentrations of 10^{-10} M to 10^{-5} M

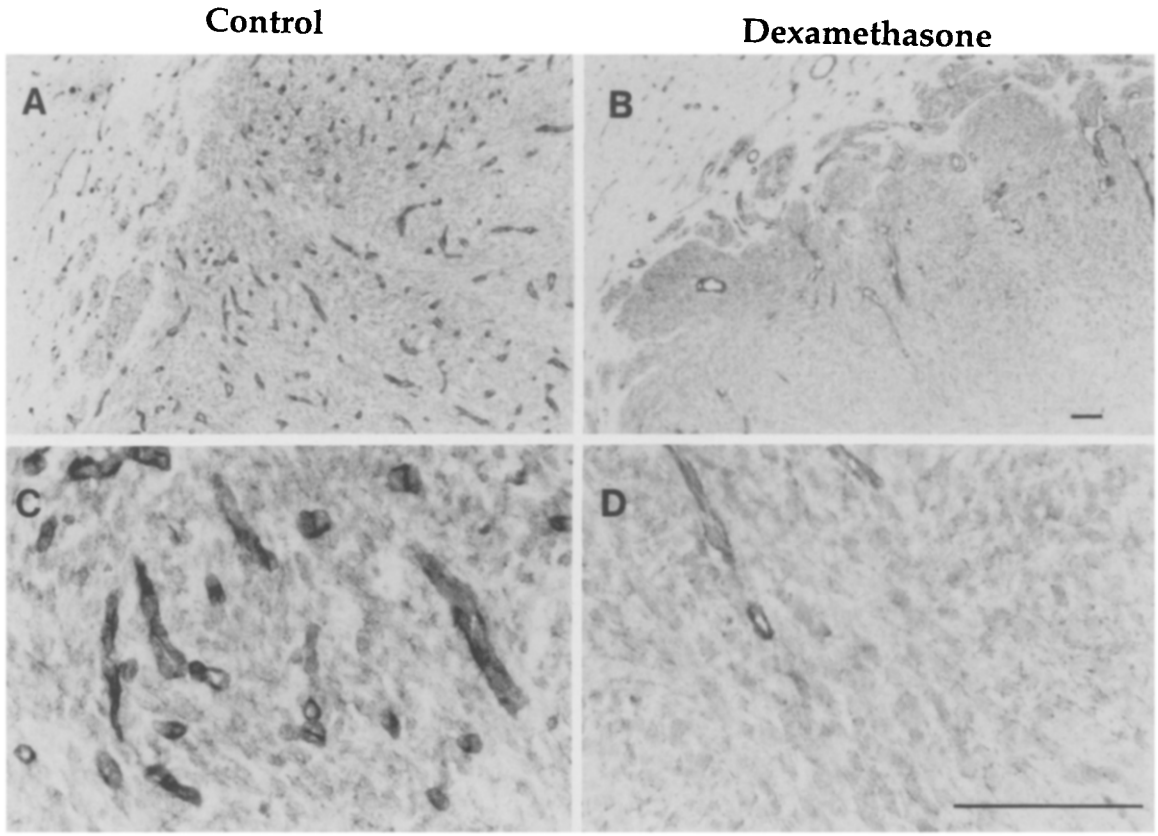

Fig. 2. Dexamethasone reduces the vascular density in 9L brain tumors. Microvessels are identified in control (A and C) and dexamethasone-treated (B and D) tumors by immunohistochemically staining for laminin. Sections are counterstained with hematoxylin. Bars = 0.1 mm.

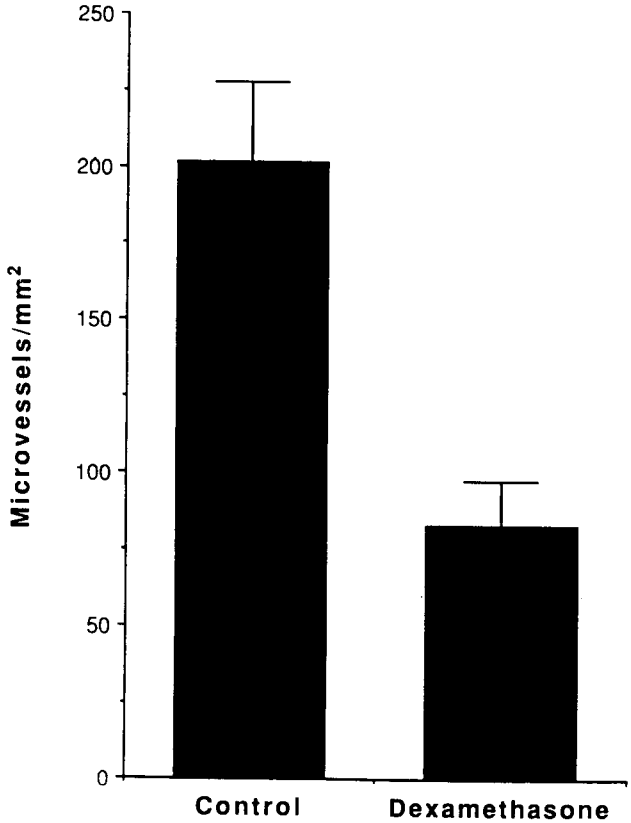

Fig. 3. Quantification of reduced microvascular density in dexamethasone-treated 9L brain tumors. Error bars indicate standard error of the mean. $n = 6$, $P < 0.01$.

were without effect. Similarly, the doubling time of endothelial cells was increased from 19.4 ± 2 h in controls to 24.5 ± 2.5 by 10^{-4} M dexamethasone and con-

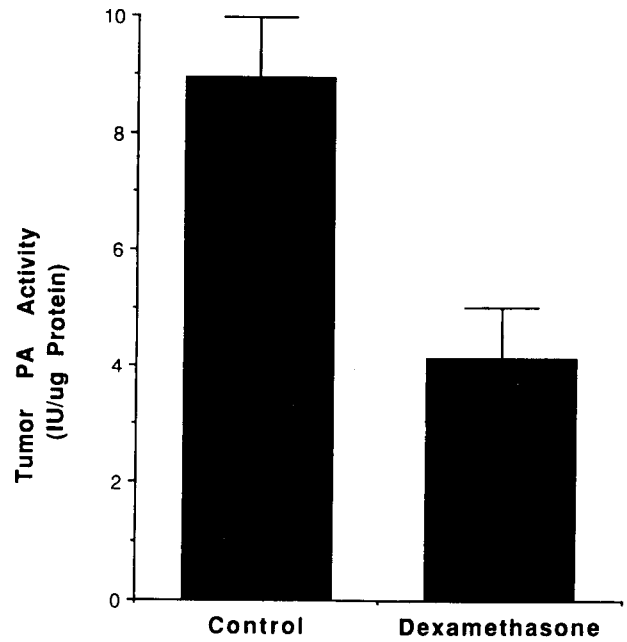

Fig. 4. Dexamethasone reduces plasminogen activator (PA) activity in 9L tumors. Dexamethasone-treated and control rats were perfused with PBS. Tumors were dissected, homogenized, and plasminogen activator activity measured as described in Materials and Methods. Data represents mean ± standard error. $n = 8$, $P < 0.01$.

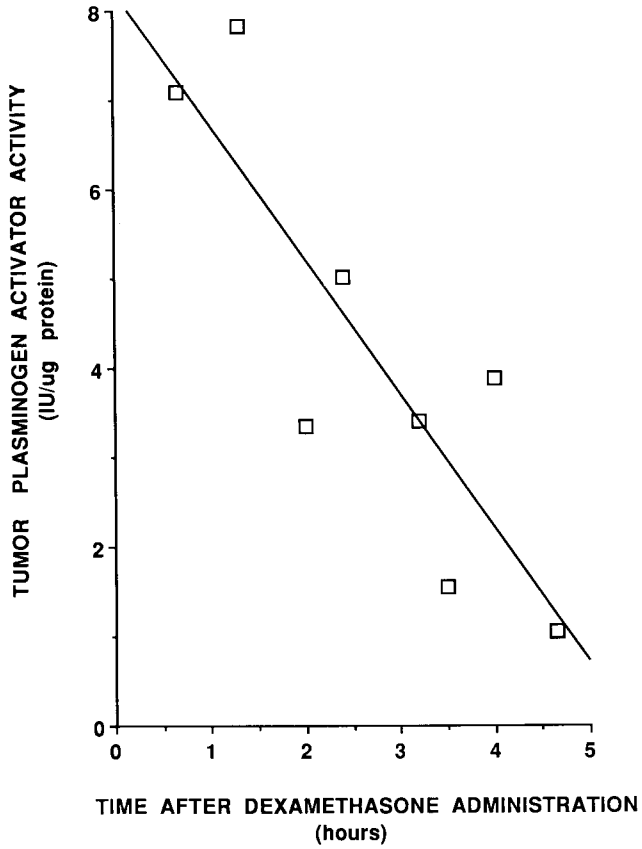

Fig. 5. Plasminogen activator activity of 9L brain tumors varies with the time after dexamethasone administration. Animals bearing 9L brain tumors were treated daily with intraperitoneal dexamethasone as described in Materials and Methods. After 7 days of dexamethasone the animals were sacrificed at the indicated times after the last steroid injection. Each point represents an individual animal ($r^2 = 0.732$, $P < 0.01$). There was no reduction in tumor PA activity in saline-treated controls.

centrations from 10^{-10} M to 10^{-5} M had no effect.

Plasminogen activator activity in cell culture

Cell cultures were used to determine if dexamethasone decreased PA activity in either 9L or central nervous system endothelial cells. Culture supernatant fractions were assayed since it has been found that the majority of PA associated with cultured cells is present in culture medium and changes in PA activity of cell extracts parallels that of culture supernatants[27]. As shown in Fig. 6, after 72 h of 10 μM dexamethasone treatment, the PA activity in endothelial and 9L culture supernatants was reduced to 1% and 12% of controls, respectively.

DISCUSSION

We demonstrate in this report that 9L brain tumor growth is inhibited by dexamethasone (Fig. 1). This confirms similar findings in other experimental rat brain tumor models[16,34,41]. The mechanism of this dexamethasone effect has not been well characterized. One potential mechanism is by direct inhibition of tumor cell proliferation. The dexamethasone treatment used in this study resulted in peak serum concentrations of 9 μM which decreased rapidly. This is similar to the study of Tamargo et al.[38] who additionally showed that the dexamethasone concentrations in 9L tumors were only 0.4% of that reached in serum. Thus, the tumor dexamethasone concentrations achieved in our study were well below 10 μM. It is widely accepted that the active concentration of drugs in vivo does not necessarily correspond to the active concentrations in vitro. However, the lowest concentration that inhibited cell growth in vitro was 100 μM, at least an order of magnitude higher than the peak conentration in vivo. Therefore, it appears unlikely that tumor growth inhibition by dexamethasone in vivo is primarily mediated by a direct effect on 9L proliferation.

The decrease in tumor vessel density found after treatment with dexamethasone (Figs. 2 and 3) suggests

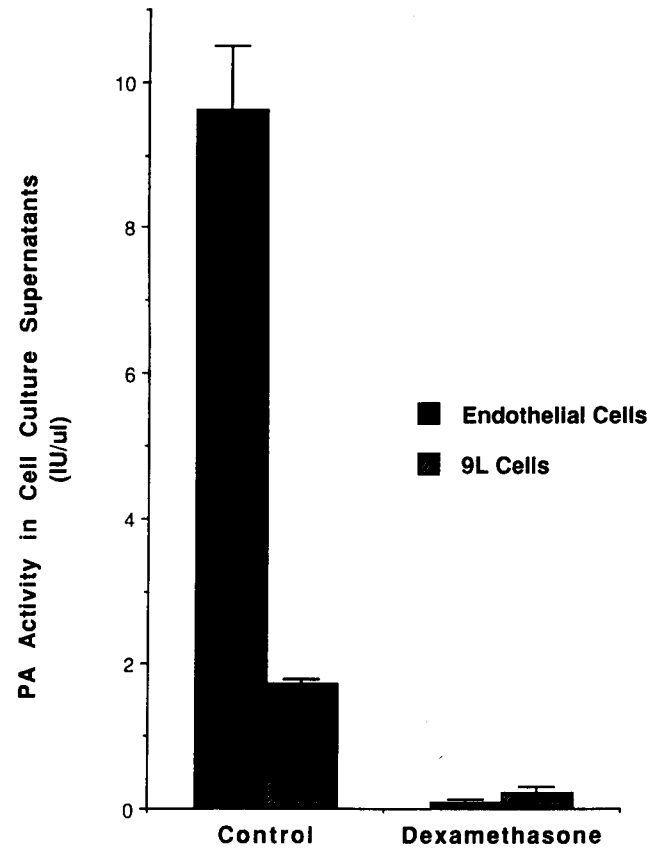

Fig. 6. Dexamethasone reduces plasminogen activator (PA) activity in cell culture supernatants of both 9L cells and endothelial cells. Endothelial cells (40,000) and 9L cells (100,000) were plated and dexamethasone added 24 h later. Conditioned medium was collected 72 h later and PA activity determined as described in Materials and Methods. Data represents mean ± standard error.

that decreased vessel growth contributed to the inhibition of tumor growth. It is well established that dexamethasone increases tumor vessel expression of blood-brain barrier as evidenced by decreased tumor-associated vasogenic edema. In addition, we have recently found that dexamethasone increases the percentage of 9L tumor vessels that express glucose transporter type-1, an endothelial blood-brain barrier marker[14]. These dexamethasone-induced blood-brain barrier effects are consistent with an increase in brain tumor vessel differentiation. The decreased vessel density reported here is also consistent with a vessel-differentiating effect since under normal conditions fully differentiated brain vessels do not proliferate[31].

The vessel density assessed in this report reflects the final product of vessel growth which involves many events potentially altered by dexamethasone. Angiogenesis is initiated by tumor-derived signals that activate endothelial cells to produce enzymes that degrade the vascular basement membrane. Endothelial cells then migrate and proliferate. We addressed the effect of dexamethasone on two of these processes by examining endothelial cell proliferation and the proteolytic activity associated with tumors and cultured cells. We found no direct effect of dexamethasone on central nervous system endothelial cell proliferation at concentrations equivalent to those achieved in vivo. In contrast, proteolytic PA activity was markedly reduced in tumors and in cell culture supernatants after dexamethasone treatment (Figs. 4 and 6). This decrease in activity may be caused by changes in the synthesis of either PA or PA inhibitor[33]. Although it is possible that retinal microvascular endothelial cells differ from brain endothelial cells in their response to dexamethasone we feel that this is unlikely since retinal endothelial cells originate from brain and express a differentiated barrier phenotype similar to brain endothelium. To our knowledge, this is the first demonstration that glucocorticoids reduce the PA activity of endothelial cells derived from the central nervous system.

Plasminogen activators may function at many levels in the complex process of angiogenesis by cleaving extracellular proteins either directly or indirectly as a result of plasmin production[33]. This alters cell–matrix interactions, liberates mitogens and angiogenic factors that are sequestered within extracellular material[32], and stimulates endothelial migration and proliferation[32,37]. Glucocorticoids inhibit endothelial cell migration in vitro[36]. Thus, inhibition of PA activity seen in this study may reduce both extracellular matrix degradation and endothelial migration and thereby block tumor angiogenesis. Consistent with this hypothesis, medroxyprogesterone, a non-glucocorticoid with anti-angiogenic properties also inhibits plasminogen activator activity in cultured endothelial cells[1].

Glucocorticoids may also alter neoplastic mechanisms not addressed in this study. They may modify tumor cell phenotype[25] altering production of tumor-derived signals that modulate endothelial proliferation or differentiation[5,40]. Altered immune cell function might also contribute to a decrease in vascular density[10,17]. In addition, inhibition of tumor-associated PA activity could decrease tumor growth independent of the effects on vessel density by reducing tumor cell invasiveness. Multiple mechanisms are likely to act synergistically to decrease angiogenesis and tumor growth.

In conclusion, we show that dexamethasone decreases PA activity, vessel density and growth of 9L rat brain tumors at concentrations that do not directly affect 9L or central nervous system endothelial cell growth in vitro. These findings suggest that inhibition of proteolytic steps involved in vessel growth is one mechanism by which glucocorticoids decrease brain tumor growth.

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