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Laboratory Investigation

Growth inhibition of the 9L glioma using polymers to release heparin and cortisone acetate

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Summary

Malignant gliomas are difficult to treat systemically because of exclusion of many chemotherapeutic agents by the blood brain barrier. Furthermore, as opposed to other neoplasms, malignant gliomas recur locally, at the site of original presentation. These tumors are remarkably vascular and hence may be more dependent on angiogenesis for continued growth than other tumors.

The inhibition of tumor angiogenesis can control tumor growth by preventing the exponential vascular growth phase. We report the inhibition of the growth of the 9L glioma by the localized, controlled release of known angiogenesis inhibitors administered in a biodegradable polyanhydride polymer matrix. In the presence of heparin and cortisone and of cortisone alone there was a 4.5- and 2.3-fold reduction, respectively, in the growth of the 9L glioma. We compared these results to the inhibition of tumor neovascularization in the rabbit cornea by the localized delivery of the same agents. In the rabbit cornea model, the local release of heparin and cortisone and of cortisone alone resulted in a 2.5- and 2.0-fold reduction, respectively, in the angiogenesis response evoked by the VX2 carcinoma. This study introduces two new potential therapeutic modalities for the treatment of malignant gliomas: the use of the combination of heparin and cortisone as antineoplastic agents and the use of polymeric carriers for the local delivery of such agents in the central nervous system.

Introduction

Inhibition of angiogenesis has been shown to restrict the growth of a tumor by disrupting its vascular supply [1]. Recently, several investigators have attempted to induce the regression of tumors with the systemic administration of the combination of heparin and cortisone, a powerful inhibitor of angiogenesis. These studies, however, have yielded conflicting results. Folkman *et al.* [2] obtained tumor regression and increased survival in mice given heparin and cortisone systemically. Sakamoto *et al.* [3] documented inhibition of tumor growth in mice, but failed to obtain tumor regression or increased survival. Ziche *et al.* [4] reported inhibition of tumor growth in mice treated with systemic cortisone alone. The addition of heparin enhanced this effect slightly, but did not induce tumor regression. Penhaglion and Camplejohn [5] reported the transient inhibition of tumor growth in mice treated with systemic cortisone alone and did not observe potentiation of this effect when heparin was added, nor were tumor regressions obtained. Maragoudakis *et al.* [6] have reported that the ratio of heparin to cortisone is a critical variable in effectively inhibiting angiogenesis with these agents. Furthermore,

Folkman *et al.* [2] and Penhaglion and Camplejohn [5] noted considerable toxicity associated with the systemic administration of cortisone.

Although the different results of these studies may be due to the variability in the anti-angiogenic properties of heparins obtained from different sources (commercial heparin is a heterogeneous mixture of glycosaminoglycans [7] and heparins from different sources vary in their anticoagulant properties), it was of interest to investigate other methods of administering these agents directly to the tumor site. We therefore utilized controlled release polymers to deliver the heparin-cortisone so as to maximize tumor exposure.

Theoretically, malignant gliomas are ideally suited for the localized administration of angiogenesis inhibitors such as the combination of heparin and cortisone. The glioblastoma multiforme, the most common and most malignant glioma [8], is remarkably vascular and may be more dependent on angiogenesis than other tumors for its continued growth [9, 10]. It characteristically presents and recurs as a focal lesion intracranially [11].

We report here the treatment of the 9L glioma [12] by the localized, controlled administration of heparin and cortisone incorporated into a biodegradable polyanhydride polymer matrix [13–17]. We compare the inhibition of the growth of the 9L glioma in the rat flank in the presence of heparin and cortisone with the inhibition of the neovascularization of the VX2 carcinoma in the rabbit cornea (also in the presence of heparin and cortisone) under similar conditions and conclude that angiogenesis inhibition may be partly responsible for growth control of the 9L glioma.

Materials and methods

Experimental design

Fifty Fischer 344 rats were assigned to five treatment groups, 10 rats per group. Four groups underwent implantation in the flank of a 2–4 mm^3 piece of 9L gliosarcoma harvested from the flank of a carrier rat and of a biodegradable, controlled release polyanhydride polymer cylinder containing heparin and cortisone acetate, cortisone acetate alone, heparin alone, or no drug. The fifth group received a tumor implant alone. The rats were sacrificed 14 days after implantation, at which time the largest tumors started to ulcerate through the skin, and the tumors and polymers were removed. Tumor volumes were calculated for comparison of the different treatment modalities.

Sixty rabbit corneas were implanted with VX2 carcinoma and controlled release ethylene-vinyl acetate polymers containing the combination of heparin and cortisone acetate, cortisone acetate alone, or no drug. The neovascularization response to the carcinoma in the rabbit cornea was measured 21 days after the initial implantation, at which time the largest tumors entered the exponential growth phase.

The release kinetics of heparin and cortisone acetate from the polymer were determined *in vitro* by serial transfer of the cylinders into phosphate buffer.

Animals

Fischer 344 male rats weighing about 200 gm were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, Indiana). They were kept in standard animal facilities, five rats per cage, and given free access to food and water.

New Zealand White rabbits weighing about 1.5–2.5 kg were obtained from Bunnyville Farm (Littlestown, Pennsylvania), kept in standard animal facilities, one animal per cage, and given free access to food and water.

Tumor line

The 9L glioma [12] was obtained from Marvin Barker, Brain Tumor Research Center, University of California, San Francisco. The cells were grown in minimum essential medium (Eagle's) with 10% fetal bovine serum, L-glutamine 398 μg/ml, penicillin (base) 80.5 u/ml, and streptomycin 80.5 μg/

ml (all products from GIBCO Laboratories, Grand Island, New York) in a humidified atmosphere of 5% CO_2 at 37° C. After reaching confluence, the cells were detached with trypsin (0.25%) in Hank's Balanced Salt Solution without calcium and magnesium (GIBCO Laboratories, Grand Island, New York) and resuspended in medium.

A solid tumor was obtained upon injection of the cell suspension into the flanks of Fischer 344 male rats (Harlan Sprague-Dawley, Inc., Indianapolis, Indiana). The flank of the carrier was shaved and prepared with 70% ethyl alcohol and Prepodyne solution. Under sterile conditions, the tumor was excised, minced into pieces about 0.5 cm^3, and dissociated into smaller fragments by pressing it through a 40 mesh (380 μm) screen in a Cellector tissue sieve (Bellco Glass, Inc., Vineland, New Jersey) into a dish with Hank's balanced salt solution without calcium or magnesium. The flank of the recipient was similarly prepared and the cell mixture was injected into it. The tumors were passaged approximately every 2 weeks.

The VX2 rabbit carcinoma [18] was obtained from Dr. John Hilton, Department of Oncology, The Johns Hopkins University School of Medicine. The tumor was propagated by serial transplantation in the flank of New Zealand White rabbits. For the corneal implantation, the tissue immediately below the tumor capsule was cut into pieces measuring about 1.5 × 1.5 × 1.5 mm^3.

Anesthesia

For the flank tumor implantation, a stock solution was prepared containing ketamine hydrochloride 25 mg/ml, xylazine 2.5 mg/ml, and 14.25% ethyl alcohol in normal saline. The rats were anesthetized with an intraperitoneal injection of 3–5 ml/kg of the mixture.

For the corneal implantations, subsequent stereomicroscopic examinations, and for serial transplantation of the VX2 tumor in the thigh, the rabbits were anesthetized with a mixture of xylazine 15–17 mg/kg and ketamine 15–17 mg/kg injected intramuscularly.

Polymer and drug preparation

The biodegradable polyanhydride poly [bis (p-carboxyphenoxy) propane-sebacic acid anhydride] (PCPP-SA) in a 1: 1 ratio was synthesized and fabricated as previously described [13]. Heparin (No. H-3125, sodium salt, Grade I) and cortisone acetate (No. C-3130) were obtained from Sigma Chemical Company (St. Louis, Missouri) and incorporated into the polymer matrix to yield a 30% loading (w/w). The ratio of heparin : cortisone acetate was 1 : 8 (w/w). The polymer-drug mixture was compression-molded into cylinders weighing about 22 mg. The cylinders were then trimmed to a uniform weight of about 21.5 mg and sterilized by exposure to UV light for 1 hour.

The ethylene-vinyl acetate copolymer (EVAc) [19] (40% vinyl acetate by weight, Elvax 40P) was obtained from the Du Pont Company, Wilmington, Delaware. The polymer was washed extensively in absolute ethyl alcohol, with total volume changes every 24 hours, to extract the inflammatory antioxidant butylhydroxytoluene (BHT). The presence of BHT in the wash was monitored spectrophotometrically at 230 nm, and the washes continued until the absorbance fell below 0.03 unit [20]. The polymers were then dried in a vacuum desiccator over 4–5 days.

The agents tested for angiogenesis inhibition were incorporated into the EVAc matrix by a modification of the fabrication procedure of Rhine, Hsieh, and Langer [21]. For incorporation into the polymeric carrier, the heparin crystals were ground to a fine powder and sieved through a 200 mesh (74 μm) screen in a Cellector tissue sieve (Bellco Glass, Inc., Vineland, New Jersey). Cortisone acetate was dissolved in methylene chloride. The drugs were suspended or dissolved in a 10% EVAc-methylene chloride solution. The suspension/solution was poured into cylindrical glass molds cooled to − 70° C. The resulting polymer cylinders were first transferred to − 30° C and allowed to dry for 3 days and then placed in a vacuum desiccator for an additional 3 days. The cylinders were then cut into 1.5 × 1.5 × 1.0 mm^3 pieces for implantation in the rabbit cornea. Empty EVAc cylinders were fab-

ricated by the same procedure. All the polymers were exposed to ultraviolet irradiation for 1–2 hours prior to implantation. The final concentrations (w/w) of the angiogenesis inhibitors in the polymers were: cortisone acetate (Sigma Chemical Company, St. Louis, Missouri), 7.5 and 27%; heparin (Hepar Inc., Franklin, Ohio) and cortisone acetate, 15 and 30% combined loading, with a fixed heparin : cortisone ratio of 1 : 8. Preliminary studies showed no significant difference in the degree of angiogenesis inhibition between the two loading levels for each drug. The polymers containing cortisone alone and the combination of heparin and cortisone did not induce angiogenesis when implanted alone in the rabbit cornea. Polymers containing heparin alone, however, promoted an angiogenesis response in the cornea.

Release kinetics

The *in vitro* release kinetics of heparin and cortisone acetate were determined by serial transfer of the drug-loaded cylinders into a 0.1 M phosphate buffer (pH 7.4) at 37° C as previously described [13]. The concentration of cortisone acetate was determined by HPLC with a Hewlett-Packard 1090 M system. A mobile phase of 50% (v/v) acetonitrile in water was run in isocratic conditions at 1 ml/min through a Lichrosorb C18 5 μm 150 mm column maintained at 40° C. A diode array was used as the detector. Under these conditions, the cortisone acetate peak appeared at 3.6 min while the matrix degradation products were eluted in less than 2 min. An external standard method was used for quantification.

The concentration of heparin was determined by mixing equal volumes of a 0.1 mM Azure A dye solution with the sample. The reduction of UV absorbance at 635 nm was recorded on a Shimadzu spectrophotometer [22].

Flank implantation

A tumor was excised from the flank of the carrier and kept at 4° C during the implantation procedure. By a microsurgical technique, a strip of viable tumor was excised and cut into pieces measuring 2–4 mm^3. The flank of the recipient animal was shaved and prepared with 70% ethyl alcohol and Prepodyne solution. A 5 mm incision was made and a subcutaneous pocket created by blunt dissection. The polymer was placed in the pocket and a piece of tumor was placed on the polymer. The incision was closed with clips.

Rabbit cornea angiogenesis assay

The inhibition of angiogenesis was studied in the rabbit cornea angiogenesis assay [23]. Sixty corneas were implanted: 35 corneas with VX2 carcinoma and empty polymer, 16 with VX2 carcinoma and cortisone polymers, and 9 with VX2 carcinoma and heparin/cortisone polymers.

For the implantation of the VX2 carcinoma and the polymers in the cornea, the eye was washed with proparacaine hydrochloride 0.5% (Allergan Pharmaceuticals, Inc., Irvine, California) for local anesthesia. The globe was proptosed and secured with a latex dental dam (Hygenic Corp., Akron, Ohio). Under magnification, a 2 mm superficial incision, about 0.1 mm deep, was made on the cornea below the visual axis using a Bard-Parker No. 11 blade. A corneal micropocket was dissected by inserting an iris spatula through the corneal incision and advancing it within the corneal stroma toward the limbus, stopping at 2 mm from the corneo-scleral junction. A polymer piece measuring $1.5 \times 1.5 \times 1.0\,mm^3$ was introduced into the pocket, followed by a tumor piece measuring about $1.5 \times 1.5 \times 1.5\,mm^3$. The dental dam was removed, the globe repositioned within the orbit, and the cornea irrigated again with proparacaine hydrochloride 0.5%. The corneas were examined blindly 21 days after implantation with a Zeiss Slit Lamp Stereomicroscope (Carl Zeiss, Inc., Thornwood, New York).

The angiogenesis response was quantitated by measuring both vessel length and vessel number. To determine vessel length, the span of the blood vessels from the corneo-scleral junction to the leading edge of the angiogenesis front was measured

with an ocular microscale eyepiece. The number of blood vessels present was quantitated using a four-level scale as follows:

0 = No vessels
1 = 1–10 vessels
2 = >10 vessels, loosely packed so that details of the iris could be appreciated through the gaps between the vessels
3 = >10 vessels, tightly packed so that the iris could not be seen through the gaps between the vessels

An angiogenesis index was then determined by simply multiplying vessel length by vessel density:

Angiogenesis Index (AI) = Vessel Length × Vessel Density.

Histology

On the 14th day after implantation in the rats, the tumor masses and the polymer pieces were removed from the flank and placed in 10% phosphate-buffered formalin for 7–10 days. The fixed specimens were sectioned and the length and width of the tumor nodules were measured blindly with dial calipers (KWB Swiss, Type 5921, Thomas Scientific, Swedesboro, New Jersey). The tumor volumes were calculated using the formula:

$$\text{Volume} = (\text{length} \times \text{width}^2)/2$$ [24, 25].

If a sample contained more than one nodule, each was measured individually and the resulting calculated volumes were added. Selected tumor samples were embedded in paraffin, sectioned, and stained with hematoxylin and eosin to confirm the presence of neoplastic tissue.

Statistical analysis

The data were analyzed by using the Kruskal-Wallis test for nonparametric single factor analysis of variance and the Newman-Keuls multiple range nonparametric analogue for multiple comparisons [26].

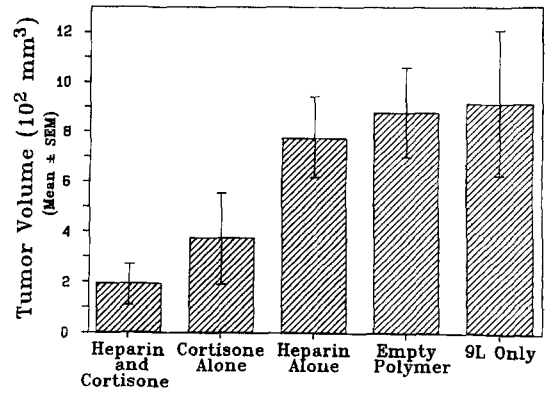

Fig. 1. Growth inhibition of the 9L gliosarcoma in the presence of heparin and cortisone acetate.

Results

The localized, controlled release of the combination of heparin and cortisone as well as of cortisone alone significantly inhibited ($p < 0.05$) the growth of the 9L gliosarcoma (Fig. 1), resulting in 78% and 57% growth inhibition, respectively. The administration of heparin alone neither inhibited nor enhanced the growth of the tumor. The polymer matrix had no effect on tumor growth. The majority of the rats receiving either heparin alone or heparin in combination with cortisone developed minor hemorrhages at the implantation site. No other adverse effects of the treatments were noted.

In the rabbit cornea, VX2 tumor angiogenesis was significantly inhibited ($p < 0.05$) by the controlled release of heparin and cortisone as well as of cortisone alone 21 days after implantation (Fig. 2), resulting in 60% and 51% angiogenesis inhibition, respectively.

The *in vitro* release studies showed that both heparin and cortisone were released from the polymer throughout the duration of the experiment (Fig. 3). Whereas the release rate of cortisone acetate was relatively constant, that of heparin was characterized by an initial burst followed by a rapidly decremental rate.

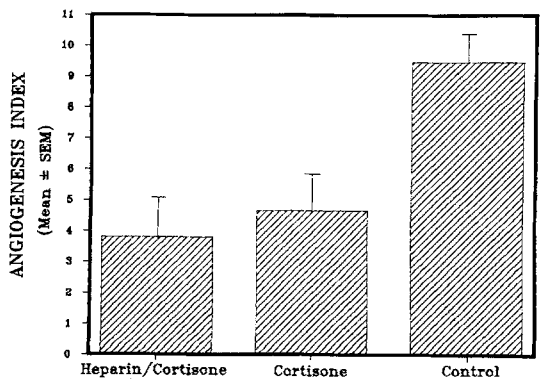

Fig. 2. Angiogenesis inhibition of the VX2 carcinoma in the presence of heparin and cortisone acetate.

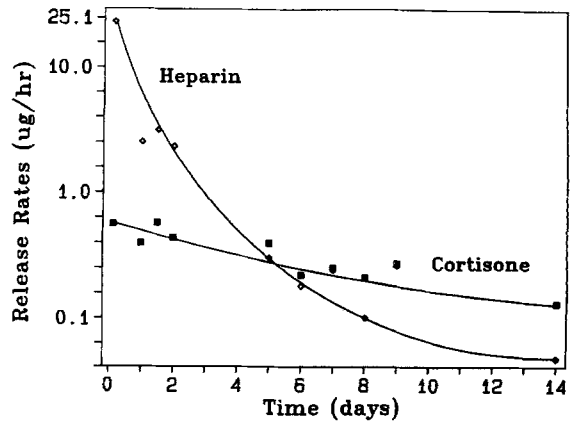

Fig. 3. Release rates of heparin and cortisone acetate from the biodegradable polyanhydride PCPP-SA (50 : 50).

Discussion

We report the inhibition of the growth of a malignant glioma, the 9L gliosarcoma, by the local, controlled release of the combination of heparin and cortisone and, to a lesser extent, of cortisone alone when administered in a biodegradable polymer matrix implanted at the site of tumor growth. The similarities between the inhibition of tumor growth in the rat flank model and inhibition of angiogenesis in the rabbit cornea model suggest that prevention of angiogenesis contributes, at least in part, to growth suppression of the 9L glioma.

Gross *et al.* [27] showed that cortisone alone, as well as other corticosteroids, can inhibit tumor-induced angiogenesis and tumor growth in the rabbit cornea. Folkman *et al.* [2] also noted this phenomenon but observed that the effect of cortisone was enhanced by the addition of heparin. In our studies, the addition of heparin to cortisone resulted in a modest, but not significant, enhancement of the cortisone-mediated tumor growth delay and angiogenesis inhibition.

The difference in the release profiles of heparin and cortisone is a result of the disparate hydrophilicity of the drugs. Since heparin is highly soluble in water, its release is predominantly controlled by diffusion due to the high chemical potential gradient. The release of cortisone acetate, which is more hydrophobic and only sparingly soluble in water, is controlled by the combination of the rate of drug dissolution, drug diffusion, and biodegradation of the polymeric matrix.

Malignant gliomas may be ideal targets for a therapeutic modality involving the localized controlled release of biological response modifiers such as angiogenesis inhibitors. The glioblastoma multiforme, the most common and most malignant glioma, remains difficult to treat [8, 28]. Arising within the central nervous system, it is sheltered by the blood-brain barrier, which limits the systemic administration of chemotherapeutic agents to those that are lipid-soluble and able to cross the barrier [28]. It is a heterogeneous lesion made up of diverse cellular populations differing in morphology, karyotypes, and chemo-sensitivities [28–30]. In contrast to other tumors, the glioblastoma multiforme usually remains a focal lesion. When it recurs, it does so locally and only rarely does it metastasize [9, 11]. It is remarkably vascular and may be more dependent on angiogenesis than other tumors for its continued growth [9, 10, 31].

The unique biological features of the glioblastoma multiforme and other malignant gliomas suggest that a novel therapeutic strategy may be required for the more effective treatment of these malignancies. An alternative to the systemic administration of a chemotherapeutic agent is the localized, controlled release of the agent using a polymeric delivery system implanted at the site of tumor growth [16, 17, 32]. The formulation of the biodegradable polyanhydride polymers utilized in

this study can be modified to shorten or lengthen the period over which the macromolecules are released, ranging from a few days to several years [13, 17]. We have shown these polymers to be biocompatible in the rat and rabbit brain [33, 34]. Theoretically, a polymeric controlled release matrix could effectively treat the local recurrence of a glioblastoma multiforme and obviate the need for lipid-soluble agents that can cross the blood-brain barrier. Indeed, many chemotherapeutic agents that have not been tested against gliomas because they are unable to cross the blood-brain barrier could be incorporated in controlled release polymers.

The use of inhibitors of angiogenesis as biological response modifiers may overcome the difficulty posed by the heterogeneity of malignant gliomas since all the cellular subpopulations within a glioma, regardless of their karyotype or morphology, ultimately depend on a homogeneous population of host capillary endothelial cells for their survival. The highly vascular glioblastoma multiforme [9, 31, 35] may be particularly vulnerable to an angiogenesis blockade.

The rat flank was utilized in this study because in the brain there is no tolerance even for small hemorrhages, which act as intracranial masses and lead to neurological deterioration. In a previously reported series [2], gliomas were unresponsive to anti-angiogenesis therapy. Since this study shows that the growth of a malignant glioma will respond to angiogenesis inhibitors, as newer anti-angiogenesis agents are developed [1, 31, 36–38] it may become possible to apply this therapeutic modality directly in the brain. Indeed, it may be possible to replace heparin with its component oligosaccharide fragments, which seem to be capable of angiogenesis inhibition when administered with cortisone but do not have the deleterious anticoagulant properties of whole heparin [2, 38].

In summary, the growth of a malignant glioma, the 9L gliosarcoma, was inhibited by the controlled release administration of heparin and cortisone from a biodegradable polyanhydride polymer matrix implanted at the site of tumor growth. This study introduces two new potential therapeutic modalities for the treatment of malignant gliomas: the use of the combination of heparin and cortisone as anti-angiogenic chemotherapeutic agents and the use of polymeric carriers for the local delivery of such agents.

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