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Original Paper

Protamine Inhibits Angiogenesis and Growth of C6 Rat Glioma; a Synergistic Effect when Combined with Carmustine

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Protamine inhibits angiogenesis and blocks endothelial, fibroblast and platelet growth factors. Human and experimental gliomas spread and grow in response to both paracrine and autocrine release of these factors. Our objective was to study the effect of protamine administration on cell proliferation, angiogenesis and tumoral growth of C6 glioma. Additionally, we compared the antitumoral effect of protamine with that of another inhibitor of angiogenesis, suramin, and investigated a potential synergistic antitumoral action of low doses of protamine combined with the antineoplastic carmustine. C6 glioma cells were implanted subcutaneously in Wistar rats. A highly malignant glioma developed in 80% of animals; when the tumour reached a diameter of 1.5 cm, either protamine, suramin, carmustine or protamine plus carmustine were administered in various doses. Tumour parameters were measured and compared between groups. In a dose-dependent manner, protamine reduced tumour volume ($P < 0.001$), mitotic index ($P < 0.05$), vascular density ($P < 0.05$) and cell viability ($P < 0.005$) of C6 glioma. An ultrastructural study demonstrated membranous inclusions in the cytoplasm of 28% of tumoral and endothelial cells of tumours from animals treated with protamine. The inhibition of tumoral growth produced by moderate doses of protamine was similar to that produced by toxic doses of suramin. The combination of protamine and carmustine had a synergistic curtailing effect on tumoral growth ($P < 0.001$). Our results indicate that protamine is an effective agent against glioblastoma; in non-toxic doses it could potentiate the antineoplastic effect of nitrosoureas for the treatment of glial tumours. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

GLIOMAS ARE the most common brain tumours [1]. In humans, glioblastoma, a highly malignant glioma, acquires an intense vascularisation [2], its prognosis is poor and this has not changed greatly in the last few decades [3]. Angiogenesis is a fundamental process of tumoral growth that favours the nutrition and proliferation of neoplastic cells [4, 5]. Through complex mechanisms of autocrine and paracrine stimulation, the growth and invasiveness of glioblastoma depends on vascular neoformation [2, 6–8] promoted by, among other factors, platelet derived growth factor (PDGF) [5, 8, 9], basic fibroblast growth factor (bFGF) [10] and vascular endothelial growth factors (VEGF) [5, 11–13]. Two antagonists of PDGF binding have been reported; suramin [14] and basic proteins such as histone, polylysine and protamine [14]. The most effective is protamine [15], which curbs the effect of heparin, interacts with platelets, fibrinogen and other plasma proteins [16] and blocks the effect of PDGF [15, 17] bFGF [18, 19] and VEGF [20, 21] by attaching to their receptors.

A novel therapeutic possibility in the treatment of cancer is the inhibition of angiogenesis [5]. Suramin has already been proposed as anti-angiogenic for glioblastoma [22], renal carcinoma [23], osteosarcoma [24, 25] and prostatic cancer [26]. In this study, we tested the effect of another anti-angiogenic agent, protamine, on the tumoral growth of C6

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glioma implanted in rats. In the same model, we also compared the effect of protamine with that of suramin and studied a possible synergistic action of non-toxic doses of protamine when combined with low doses of carmustine, an antineoplastic nitrosourea.

MATERIALS AND METHODS

Glioma induction

C6 glioma cells [27], obtained from the American Tissue Culture Collection (Rockville, Maryland, U.S.A.) were cultured at 37°C under sterile conditions in Ham's F-10 medium supplemented with 2.5% fetal calf serum and 15% horse serum. To obtain a large source of C6 cells, 1×10^7 cultured C6 cells were intraperitoneally inoculated in a 12 week old Wistar rat; 20 days later, a large, multilobulated peritoneal tumour developed. The tumour was mechanically dissociated at 4°C; a suspension of 1×10^7 cells in 500 µl of saline solution was inoculated subcutaneously into the left thigh of 12 week old female Wistar rats. A subcutaneous tumour developed in 80% of the animals, it reached a diameter of 1.5 cm 19–21 days after cell implantation.

Administration of protamine, suramin, carmustine and protamine/carmustine

The rats in which a subcutaneous tumour developed were included in this study. When the tumour reached a diameter of 1.5 cm, the rats were randomly allocated to one of 10 groups: group A ($n=57$) was used as a control and injected subcutaneously with 0.5 ml saline solution every 12 h for 10 days. To study the maximum effect of protamine on glioma, animals from group B ($n=48$) were treated with the highest dose of protamine used in these experiments, 120 mg/kg by subcutaneous injection every 12 h for 10 days. To study the dose response to protamine, groups C ($n=8$), D ($n=8$), E ($n=11$), F ($n=9$), and G ($n=12$), were treated in an identical manner as rats from group B with 15, 30, 45, 60 or 90 mg/kg of protamine, respectively. To compare protamine with suramin, rats from group H ($n=16$) were treated with 60 mg/kg intraperitoneal suramin every 48 h for 10 days as described by Takano and colleagues [22]. To determine the antineoplastic effect of a low dose of carmustine, rats from group I ($n=7$) were treated with 5 mg/kg intraperitoneal carmustine as a single dose (which corresponded to 30% of the optimal antineoplastic dose of carmustine reported for this animal model, inducing a 10% mortality rate [28, 29]). To study the additive effect of protamine on the antineoplastic action of carmustine, rats from group J ($n=9$) were treated with a single intraperitoneal dose of carmustine 5 mg/kg plus protamine 60 mg/kg subcutaneaously every 12 h for 10 days.

Evaluation of antitumoral effect of treatments

After 10 days of treatment, animals from all groups were anaesthetised and perfused by intracardiac route, with either a 10% formalin solution in saline solution for the histological study or a 2.5% glutaraldehyde solution plus 0.5% osmium tetraoxide for the ultrastructural study. The tumours were dissected and their volume determined (in cm^3) by the $6/\pi\times L\times W\times H$ formula described by Tamayko and Reynolds [30]. To study the outcome of tumours in animals treated with protamine, 34 controls and 24 animals from group B were followed for an additional 10 days after the end of treatment; the tumours were measured and compared with those of animals from their same group at the end of treatment.

Histological and ultrastructural analysis

The tumours were embedded in paraffin, 10 µm sections were stained with haematoxylin and eosin for microscopic study. For electron microscopy analysis, the tumours were placed in epoxy resin and 1 µm sections were stained with toluidine blue, representative areas were selected and 80 nm sections were stained with uranyl acetate and lead citrate for ultrastructural study.

Cell proliferation study

Tumours of animals from groups A and B were studied by immunohistochemistry with monoclonal antibodies against the fluorescein-conjugated proliferation nucleocellular antigen [31, 32] (Sigma, St Louis, Missouri, U.S.A.). The incubation of monoclonal antibodies in a 1:50 dilution lasted 30 min; observations were made with a Zeiss epi-fluorescence microscope at 40× magnification. The cell proliferation index was obtained by the mean number of positive cells in 10 different microscopic fields. Observations were made without knowledge of the group source of the specimen.

Vascular density

Tumours of animals from groups A and B were studied by immunohistochemistry with polyclonal antibodies from rabbit antiserum to factor VIII R [5] (Sigma) as a marker for the cytoplasm of vascular endothelial cells. Tissue slices of 5 µm were treated with 5% trypsin in saline solution, washed and incubated with the antiserum to factor VIII R for 1 h at 37°C, followed by incubation with goat antirabbit antibodies conjugated with fluorescein. Sections of rat tongue were also stained and used as positive controls for the antiserum. Observations were made at 40× magnification; vascular density was determined by the number of capillary vessels in three microscopic fields. Observations were made without previous knowledge of the source group of the specimens.

Tumour viability

Tumour viability was determined by capture of Evans blue as follows: six rats from group A and six rats from group B were injected intravenously with 1 ml/kg of 2% Evans blue in saline solution. One hour later, the rat was anaesthetised and sacrificed, the tumour was homogenised and the dye was extracted with formamide (Sigma) for 48 h. Evans blue was measured in the supernatants by spectrophotometer at 620 nm and expressed as mg of Evans blue per mg of tumour.

Side-effects

Before perfusion, the animals were bled by intracardiac puncture in order to study haematological and chemical blood parameters.

Statistical analysis

Comparisons between groups were made by Student's t test and the Chi-square test for independent values and expressed as mean ± standard error (SE); significance was set at the 5% level.

RESULTS

Antitumoral effect of the therapeutic schemes

When the highest dose of protamine was administered (120 mg/kg) the mean tumour volume after treatment was $5.1\pm0.6\,cm^3$, the tumour volume in controls was $17.7\pm2.2\,cm^3$ ($P<0.01$) (Figure 1). With other doses of

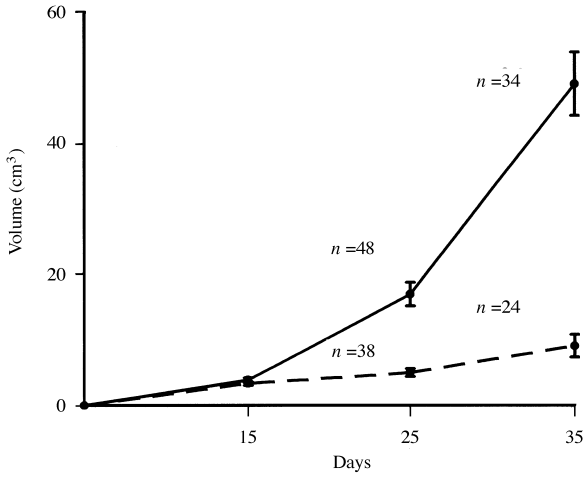

Figure 1. Comparison of the mean volume of tumours from rats treated with protamine (120 mg/kg) from day 15 to day 25 (broken line) with that from controls (solid line). The reduction in tumour size after protamine treatment was significant ($P < 0.01$ when compared with controls); these differences increased 10 days after the end of treatment ($P < 0.001$).

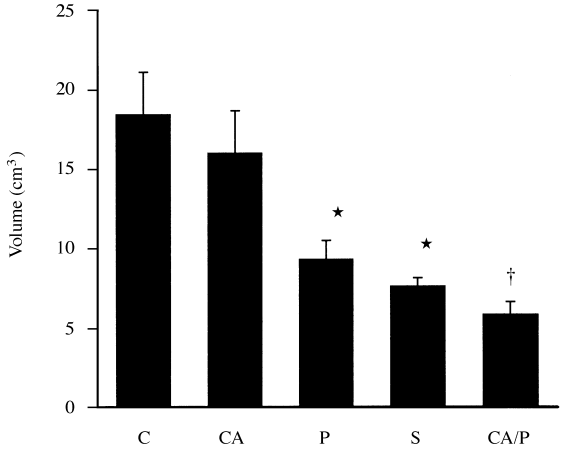

Figure 3. Comparison of mean tumour size after 10 days with various therapeutic regimens for C6 rat glioblastoma. C, controls; CA, carmustine 5 mg/kg as a single dose at day 1; P, protamine 60 mg/kg every 12 h; S, suramin 60 mg/kg every 48 h. The combination (CA/P) of a low dose of carmustine with protamine produced an intense antitumoral effect. (*$P < 0.05$ when compared with controls and †$P < 0.05$ when compared with the other regimens.)

protamine, 15, 30, 45, 60 or 90 mg/kg, the mean tumour volume was 13.6 ± 1.1, 12.6 ± 1.2 ($P < 0.05$ when compared with controls), 10 ± 1.7 ($P < 0.05$), 9.2 ± 1.2 ($P < 0.01$) and 8.3 ± 1.2 ($P < 0.01$), respectively (Figure 2). The mean volume of tumours from rats treated with suramin was 7.6 ± 0.5 cm^3 ($P < 0.01$). The mean volume of tumours from rats treated with a low dose of carmustine was 16 ± 2.6 cm^3 (not significantly different from that of controls). The mean volume of tumours in rats treated with the carmustine/protamine combination was 5.8 ± 0.8 cm^3 ($P < 0.01$ when compared with controls and $P < 0.05$ when compared with animals treated with 60 or 90 mg/kg of protamine or with animals treated with suramin). The results of treatment with protamine (60 mg/kg), suramin, carmustine and carmustine/protamine are shown in Figure 3.

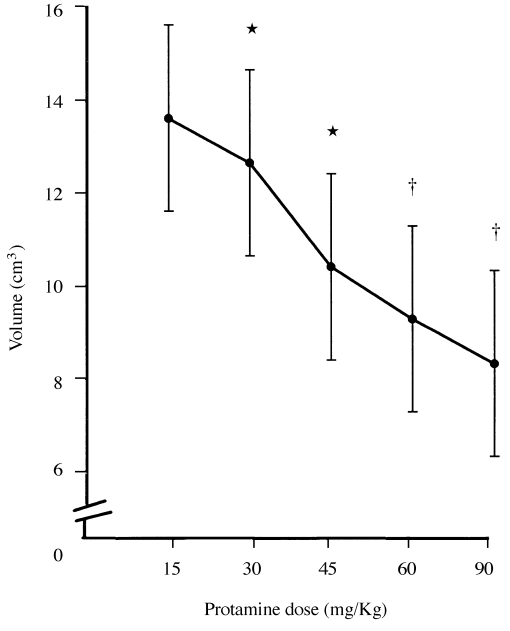

Figure 2. Effect of increasing dosage of protamine on the mean size of glioma C6 after 10 days of treatment. Tumours from controls had a mean volume of 17.7 ± 10 cm^3 (*$P < 0.05$; †$P < 0.01$ when compared with controls).

Histological and ultrastructural studies

Microscopical analysis disclosed ample areas of tumour necrosis in all animals. However, these areas were more conspicuous in animals treated with protamine; the amount of necrosis seemed to be dose dependent. The mitotic index in viable areas of tumour in animals treated with the highest dose of protamine (120 mg/kg) was 25 ± 2.5; in controls it was 32.5 ± 3.9 ($P < 0.05$). The ultrastructural study showed membranous cytoplasm inclusions and vacuoles in 28% (14/50) of tumoral and endothelial cells in animals treated with 120 mg/kg protamine (Figure 4) in contrast with 6% (3/50) in controls ($P < 0.01$).

Cell proliferation, vascular density and tumour viability

The cell proliferation index was 1.1 ± 0.5 in tumours from animals treated with 120 mg/kg protamine; in controls it was

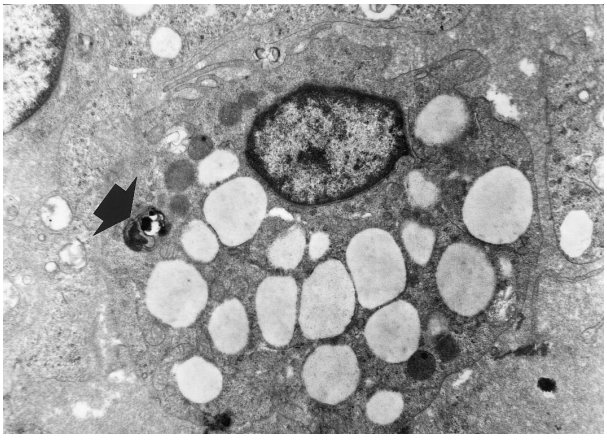

Figure 4. Ultrastructural abnormalities frequently observed in glioma cells from animals treated with protamine (×14 208). Membranous inclusions (arrow) and multiple vacuoles.

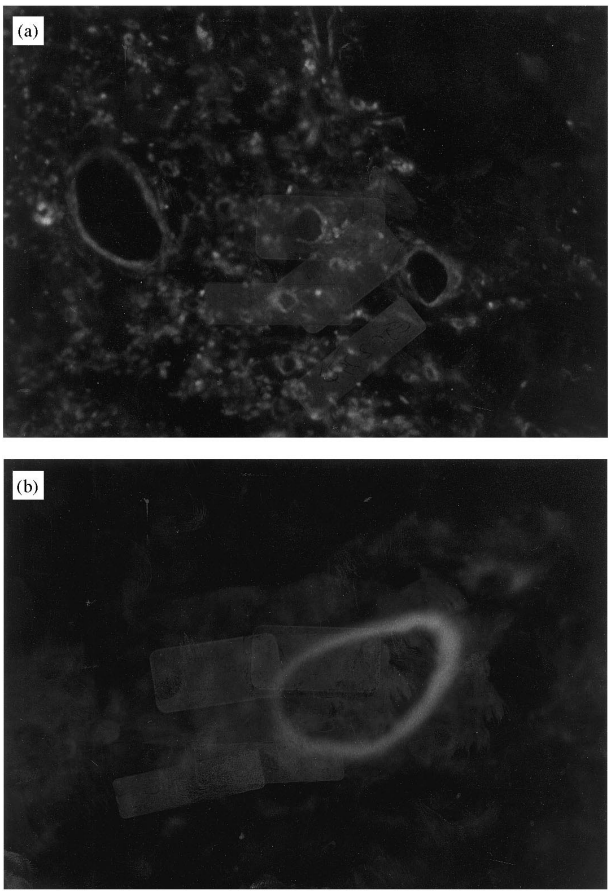

Figure 5. Immunofluorescent staining with antibodies to factor VIII-related antigen showing intense vascular proliferation in a control C6 tumour (a). A similar area in the tumour of a rat treated with protamine (120 mg/kg) shows a marked decrease in vascular proliferation (b).

1.4 ± 0.3 (non-significant). The mean number of capillary vessels in tumours from animals treated with 120 mg/kg protamine was 5.7 ± 0.9 per microscopic field; in controls it was 8.5 ± 0.8 ($P < 0.05$) (Figure 5). The Evans blue concentration was 2.1 ± 0.5 μg/mg in tumours from animals treated with 120 mg/kg protamine; in controls it was 4.5 ± 0.2 μg/mg ($P < 0.005$).

Side-effects and mortality

Chemical parameters in peripheral blood at the end of protamine treatment (120 mg/kg) showed an increase of uric acid 3.5 ± 0.3 mg/dl ($P < 0.005$) and cholesterol 80.4 ± 5 mg/dl ($P < 0.05$) above values obtained in controls. Haematological parameters showed an increase of leucocytes to $20.3 \pm 2 \times 10^3$ ($P < 0.05$) and a decrease of the haematocrit to 36 ± 2 ($P < 0.01$). Clinical abnormalities observed in animals treated with protamine 90 and 120 mg/kg included rubefaction in ears and legs and somnolence; one rat had a seizure. Mortality during treatment in animals treated with 90 and 120 mg/kg of protamine was 33% (4/12) and 25% (12/48), respectively, in contrast with 4% in controls (2/57). With all other doses of protamine, 30, 45 and 60 mg/kg, as well as with 60 mg/kg protamine combined with carmustine, no mortality was observed. Mortality was 30% in animals treated with suramin.

DISCUSSION

Protamine induced a dose-dependent significant decrease in volume, angiogenesis, mitotic index and viable tumoral areas in rats with C6 glioma. The anti-angiogenic activity of protamine has been demonstrated in chronic myocardial ischaemia [33–35], chorioamniotic membrane [36], inflammation [37], skin grafts and in tumoral cells trapped in pearls [38]. Additional effects of protamine include inhibition of collagen-induced tube formation [39], prevention of mitogenicity and inhibition of the capillary endothelial cell migration induced by FGF, as well as suppression of plasminogen stimulant factor with inhibition of DNA synthesis in endothelial cells from bovine aorta [18]. Because of its antagonistic action on FGF, protamine has been used *in vitro* as an antiproliferative agent in Kaposi cells which bear a large number of FGF receptors [40]. *In vitro* protamine inhibits the proliferation and movement of human teratoma cells stimulated by bFGF [19].

Protamine produced a clear anti-antigenic effect in C6 glioma, as measured by the vascular density and necrotic areas. Because of its rapid growth, this model of malignant glioma shows large necrotic areas, particularly in the central areas, whereas its main proliferative activity is found in the periphery of the neoplasm. When the dye Evans blue is intravenously injected, it is trapped exclusively in viable areas of the tumour; this characteristic was used in this experiment to discriminate active tumoral zones from necrotic areas, which are a consequence of inadequate vascularisation. In animals treated with protamine, necrotic areas increased, possibly due to an increase in infarcted areas secondary to inhibition of tumour angiogenesis.

Ultrastructural findings in tumoral cells from animals treated with protamine showed a high incidence of membranous inclusions, similar to those produced by suramin in the same tumour. These inclusions seem to be secondary to the accumulation of glycosaminoglycans within lysosomes [22, 41, 42] or organelle-free blebs budding from the cell membrane and may represent a direct cytotoxic effect of protamine on both the tumoral cells and the endothelial cells from nutrient vessels. Animals treated with high doses of protamine (120 mg/kg) had increased uric acid and cholesterol in serum, which could be due to either drug toxicity or increased tissular necrosis and blockage of the low density lipoprotein receptors [43]. However, with lower doses of protamine (60 mg/kg) optimal tolerance was obtained.

In humans, protamine is commonly used to antagonise heparin to prevent haemorrhages. Secondary reactions include rash, pressure increase in the pulmonary artery, vasoconstriction, systemic hypotension, anticoagulation, ventricular dysfunction and occasional death [16, 44, 45]. Similar features were observed in this experiment in rats treated with equivalent doses. However, they were minor and without mortality at a 60 mg/kg dose, which still had an important antitumoral effect and strong synergistic action with a low dose of carmustine (30% of that used as effective treatment for rat glioma) [28, 29]. The synergism of carmustine and protamine seems to be the sum of anti-angiogenic and antiproliferative effects which could be explained by an increased accessibility of the chemotherapeutic drug into the tumour secondary to decreased interstitial pressure, with the subsequent rise of vascular permeability within the tumour due to the anti-angiogenic effect [4].

In our experiments, protamine was similarly effective but less toxic than suramin, which has been the only anti-angiogenic drug tested for glioma treatment [22]. Other studies have demonstrated that the combination of steroids and protamine substantially reduce the adverse reactions to protamine [45]; as the use of steroids is common in most therapeutic schemes for malignant glioma it could entail an additional advantage for the potential use of protamine. Further studies on protamine as an anti-angiogenic agent combined with antineoplastic drugs for the treatment of glioblastoma and other malignant tumours will be necessary.

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