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Low-dose Chemotherapy Combined with an Antiangiogenic Drug Reduces Human Glioma Growth *in Vivo*[1]

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ABSTRACT

This study evaluates the efficacy of the combination of an antiangiogenic drug and conventional chemotherapeutics for the treatment of experimental human gliomas. As an antiangiogenic, we used recombinant human PEX, a fragment of matrix metalloproteinase-2 that we have previously shown to have a significant antimitotic, anti-invasive, and antiangiogenic properties against human glioblastoma *in vitro* and *in vivo*. We used carboplatin and etoposide as the two chemotherapeutic drugs routinely used in our institution (Ospedale Maggiore de Milano) for the treatment of malignant gliomas. Conventional chemotherapeutic drugs were administered at high dose or at a low and semicontinuous regimen. Combined treatment of high-dose chemotherapy and PEX did not produce an improvement of survival in comparison with chemotherapy alone, but it was associated with a decrease in tumor volume, vascularity, and proliferative index and an increased apoptosis. All of these animals experienced severe side effects. The longest survival was documented in animals submitted to low and semicontinuous chemotherapy and antiangiogenic treatment. This regimen was associated with no side effects, marked decrease in tumor volume, vascularity, and proliferative index, and an increased apoptosis. Our data suggest that low-dose chemotherapy in combination with PEX can be successfully used against human malignant glioma *in vivo*.

INTRODUCTION

Malignant gliomas are highly vascularized and invasive neoplasms characterized by a high incidence of recurrence and a poor prognosis (1). The standard treatment of this disease consists of surgery plus radiotherapy eventually followed by chemotherapy (1–3). Chemotherapy is usually reserved to recurrent tumors already treated with surgery and radiotherapy or to tumors in which surgery was partial or not feasible and the effect of radiotherapy was limited (1). Various chemotherapy schemes are used, most of them consist of the association of separate drugs, usually administered at high dose. Toxicity, drug resistance, overcome in the blood brain barrier, and limited and transient effect in patients are the main problems associated with standard chemotherapy regimens (1, 2, 4–6).

The growth of malignant gliomas depends on the balance between stimulating and inhibiting factors. Many of these factors are directly secreted by the tumor itself, and some of them have been isolated (7–9). Some of these factors have been also used for the treatment of malignant gliomas, achieving a significant growth suppression *in vivo* (7, 10). Antiangiogenesis seems to be a promising approach for the treatment of gliomas (7, 10, 11).

A strategy combining continuous low-dose chemotherapy used in combination with antiangiogenesis inhibitors has been developed recently (12–18). These studies have pointed out that this combination suppressed the growth of experimental tumors more effectively than conventional therapy alone and also when the tumors were made drug resistant before therapy. The effect of this combination in gliomas remains to be evaluated.

Preliminary studies[3] from our laboratories have identified a 210 amino-acid fragment of human metalloproteases-2, called PEX, that has significant antimitotic, anti-invasive, and antiangiogenic properties against human glioblastoma cells *in vitro* and *in vivo*.

In the present study, we evaluated the efficacy of the combination of an antiangiogenic treatment (PEX) and the conventional chemotherapy regimen that is in use in our institutions (Ospedale Maggiore di Milano) for the treatment of patients with glioblastomas and anaplastic gliomas (11). Conventional chemotherapeutic drugs were administered at high dose or at low and semicontinuous regimens. The study was focused on occurrence of growth suppression and of chemo-related side effects and on the analysis of histopathological findings on tumors removed from the treated animals. Our data showed that the association between low dosage and semicontinuous chemotherapy combined with an antiangiogenic agent can be successfully used against human malignant gliomas *in vivo*.

MATERIALS AND METHODS

Production of Recombinant PEX

PEX RNA was extracted from U87 glioblastoma cells using two primers: 5′-CCGCTCGAGCCTGTCACTCCTGAG-3′ (sense) and 5′-CGGAATCTCAGCAGCCTAGCCAG-3′ (antisense). The extracted RNA was amplified by PCR. The amplified fragment was then purified, cloned into a PGEM-T easy vector, and amplified in DH-5 α cells. Plasmid DNA was cut with *Xho*I and *Eco*RI and purified on an agarose gel. The fragment was cloned into the pRSET vector (Invitrogen, Carlsbad, CA) and transformed in BL21 bacteria. Transformed BL21 bacteria were grown in LB media, followed by induction with 1 mM isopropylthiolgalactoside. Recombinant PEX was maximally expressed 4 h after isopropylthiolgalactoside induction. PEX was purified under denaturing conditions because the majority of the protein was found in inclusion bodies. To isolate recombinant 6× His-tagged protein, cells were washed and lysed, and inclusion bodies were dissolved in 8 M urea, 100 mM NaH_2PO_4, and 10 mM Tris-HCl buffer (pH 8). PEX was then purified by Ni-charged chelating agarose. Elution of bound PEX is performed with the same buffer at pH 5.9 (monomer). Recombinant protein was refolded in 0.1 M $Na_2B_4O_7$ buffer (pH 10). Fractions containing PEX are then dialyzed against water and the protein concentration determined by BCA reagent (Pierce). Purity of the preparation was checked by running the purified protein on a 12% SDS-PAGE gel. Approximately 2 mg of protein were extracted from bacteria grown in 1

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liter of LB media. The biological activity of the recombinant protein was tested *in vitro* using an angiogenic assay and *in vivo* in glioma nude mice models.

In Vitro Angiogenic Assay

In vitro angiogenic assays were performed to test the biological activity of the protein. The assay was performed as described previously (19) with slight modifications. Porcine aortic endothelial cells stably transfected with KDR (PAE/KDR) were used (20). The cells were cultured in Ham's F-12 media supplemented with 10% nonheat-inactivated FCS and 1000 units/ml penicillin/streptomycin. PAE/KDR cells were seeded in 96-well plates coated with 0.5-mm thick type I collagen gel ($4 \times 10{,}000$ cells/cm^2) and allowed to attach and spread for 3 h. The seeded cells were subjected to three different conditions: condition 1, cells grown in Ham's F-12, 10% FCS; condition 2, cells incubated with U87-conditioned media; condition 3, cells incubated with U87-conditioned media plus PEX at different concentrations.

Design of Animal Experiments

The study consists of two parts. In the first part, we tested the effect of a combination of high-dose chemotherapy and antiangiogenic treatment. In the second part, we investigated whether the combination of semicontinuous low-dose chemotherapy associated with antiangiogenic therapy caused sustained regression of tumors and reduced the systemic toxicity usually encountered when conventional chemotherapy regimens are used.

Chemotherapy is based on the scheme in use in our institution (Ospedale Maggiore di Milano) for the treatment of glioblastomas and anaplastic astrocytomas (2). This scheme comprises of the association between carboplatin (300 mg/m^2) and etoposide (100 mg/m^2) on day 1 followed by etoposide (100 mg/m^2) on day 2 to day 5. Courses are repeated every 4 weeks. To be closer to the human scenario, chemotherapeutic drugs were used at RD[4] and not at MTD. The RD doses used in mice for this study were calculated considering for each drug the relationship between human MTD and the human RD used in the conventional scheme and those reported for mice (2, 3, 21–23).

First Part. Four groups of 10 five-week-old Swiss male nude mice (Charles River) were implanted with 5×10^4 U87 human glioblastoma cells (from the American Type Culture Collection) intracranially using an open window technique. Tumors were grown for 14 days. At this stage, four animals from each group were sacrificed, and the whole brain was removed, fixed in OCT, and stored at −70°C. Five-μm sections were cut, and the tumor volume was measured. Afterward, the animals were randomly divided into four groups according to the treatment regimen.

For the first group, these animals received cycles of conventional chemotherapy plus antiangiogenic therapy as follows: day 1, 40 mg/kg of carboplatin i.p. plus 20 mg/kg of etoposide i.p.; day 2 to day 5, 20 mg/kg etoposide i.p.; and day 6 to day 13, PEX at a dose of 2 mg/kg i.p. Treatment was stopped for 1 week followed by a new cycle of therapy.

For the second group, these animals received cycles of chemotherapy as scheduled in the previous section without receiving any antiangiogenic treatment (chemotherapy-alone control group). Treatment was conducted as described above for the first group.

For the third group, these animals received for 1 week at each cycle 2 mg/kg of PEX (PEX control group).

For the fourth group, these animals were used as a control and treated with vehicle alone (sterile saline) according to the regimen reported for the first group.

Second Part. Four groups of 10 five-week-old Swiss male nude mice (Charles River) were implanted with 5×10^4 U87 human glioblastoma cells intracranially using a open window technique. Tumors were grown for 14 days. At this stage, four animals from each group were sacrificed, and the whole brain was removed, fixed in OCT, and stored at −70°C. Five-μm sections were cut, and the tumor volume was measured. Subsequently, the animals were randomly divided into four groups according to the treatment regimen as follows.

For the first group, animals received low regimen of chemotherapy plus antiangiogenic treatment as follows: day 1, bolus with 6 mg/kg of carboplatin i.p. and 4 mg/kg of etoposide i.p.; every 3 days, a cycle composed of 2 mg/kg of carboplatin plus 2 mg/kg of etoposide and 2 mg/kg of PEX i.p. for 2 days. Treatment was continued for more than 120 days.

For the second group, animals received low regimen of chemotherapy alone without receiving any antiangiogenic treatment (chemotherapy control group).

For the third group, animals received 2 mg/kg/day of PEX according to the previous scheme (PEX control group).

For the fourth group, animals received vehicle alone (sterile saline) and were used as a control (control group).

The body weight and the general clinical status of the animals were recorded every 2–3 days. At the end of the experiment, animals were sacrificed, and the whole brain was removed, embedded in OCT, and stored at −70°C. Sections of the brain were obtained, and part of them was submitted to routine histological examinations with hematoxylin and the tumor volume was measured and expressed as a mean ± SE. The remaining slides were stained with anti-CD31, anti-αSM actine, and anti-Ki67 and with a TUNEL assay as described below in the following sections.

Each animal experiment was repeated two times. Tumor volume was estimated using the formula for ellipsoid, (width^2 × length)/2. Growth curves were statistically analyzed using repeated measures ANOVA.

Immunohistochemistry

Tumor specimens were embedded in OCT, frozen in dry ice, and stored at −70°C. Five-μm sections of each specimen were stained using H&E for morphology. Immunohistochemistry was carried out using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA). Primary antibodies included anti-CD31 (R&D Systems, Minneapolis, MN), anti-αSM actine (Serotec, Cambridge, United Kingdom), and anti-Ki67 (Dako, Carpinteria, CA). Detection was carried out using 3,3′-diaminobenzidine chromogen, which resulted in a positive brown staining. Sections were counterstained with hematoxylin, dehydrated in ethanol, and mounted with glass coverslips. Negative control slides were obtained omitting the primary antibody. Ki-67 staining was quantified by counting the number of positively stained cells of 100 nuclei in 20 randomly chosen fields.

Apoptosis Studies

Sections of tissue were fixed with 1% paraformaldehyde in PBS, and apoptotic cells were detected with TUNEL staining with ApopTagTM plus Kit (Intergen, Gaithersburg, MD) with 1% methyl green as a counterstaining. Apoptosis was quantified by determining the percentage of positively stained cells for all of the nuclei in 20 randomly chosen fields/section at 200× magnification.

Slides of the immunohistochemical studies, as well as those of the apoptosis studies, were quantified in a blinded fashion by two independent reviewers two different times.

Western Blotting

For Western blotting analysis, samples were reconstituted in 3× Laemmli sample buffer, boiled, and analyzed by SDS-PAGE. Protein lysates were electrophoretically blotted onto Immobilon-P membranes (Millipore, Bedford, MA) and blocked with 5% nonfat milk in Tris-buffered saline [10 mM Tris (pH 8.00) and 0.9% NaCl] containing 0.1% Tween 20 (Tris-buffered saline-T) for 1 h. Blots were incubated for 1 h at room temperature with primary antibodies. The blots were then incubated with secondary antimouse horseradish peroxidase conjugate antibody for 1 h at room temperature. Detection of antibodies was performed using an enhanced chemiluminescence-Plus system (Amersham Life Science).

RESULTS

Combined Administration of High-dose Chemotherapy and Antiangiogenic Treatment Failed To Improve Survival but Reduced Glioma Volume *in Vivo*. Treatment was started 15 days after implantation of tumor cells. At this time, four animals in each group were sacrificed, and the mean tumor volume measured in the brain of these animals was 13 ± 2.5 mm^3 (maximal diameter, 3.5 mm; and minimum diameter, 2.5 mm).

[4] The abbreviations used are: RD, real dose; MTD, maximum tolerated dose; OCT, ornithine carbamyl transferase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling.

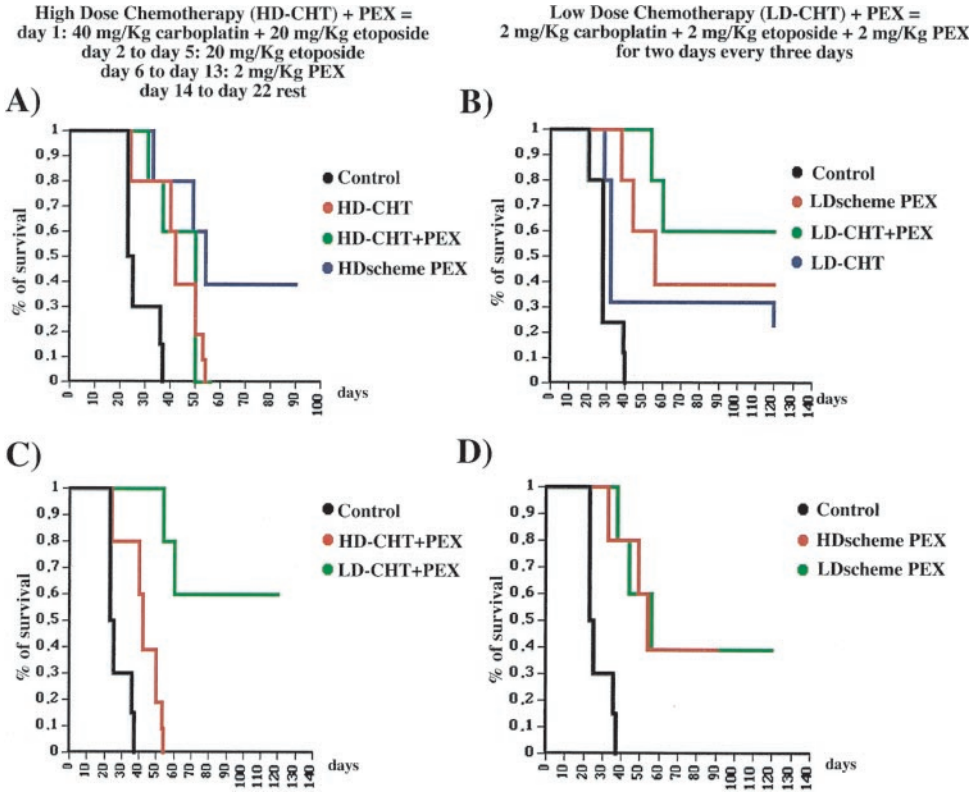

Fig. 1. Kaplan-Meyer survival curves in animals submitted to combined treatment with high-dose chemotherapy or low-dose and semicontinuous chemotherapy and PEX. Details of the chemotherapeutic regimens are reported in the figure. *A*, probability of survival of animals submitted to high-dose chemotherapy. Survival of animals treated with high-dose chemotherapy and PEX (*HD-CHT + PEX*) did not differ from that of those submitted to high-dose chemotherapy only (*HD-CHT*) or to PEX alone and administered according to what scheduled in this regimen (*HD scheme PEX*), *i.e.*, 8 days of treatment over 3 weeks. In all of these groups, the probability of survival was higher than that of the controls. *B*, probability of survival of animals submitted to low and semicontinuous chemotherapy. Survival of animals receiving low-dose chemotherapy and antiangiogenic treatment with PEX (*LD-CHT + PEX*) had the longest survival (>50% at 140 days), and four animals of this group were still alive after 140 days from implantation. Low and semicontinuous chemotherapy alone (*LD-CHT*) did not produce a significant improvement of survival. Animals treated with PEX alone (*LD scheme PEX*) administered according to what was scheduled in this regimen (2 days of treatment every 3 days) performed better. *C*, combined treatment with low-dose chemotherapy and PEX produced a longer survival than that of high-dose chemotherapy and PEX. *D*, treatment with PEX administered according to what was scheduled in the high-dose chemotherapy regimen (*HD scheme PEX*; 8 days of treatment over 3 weeks) and that in the low regimen group (2 days of treatment every 3 days) did not significantly differ in term of survival.

Animals treated with vehicle only (control group) had a 22-day mean survival rate from implantation, and all of the animals were sacrificed or died within the 35th day of implantation. At this stage, most of the animals had large tumors, with a protruding mass outside the open craniotomy. The mean tumor volume in this group reached 20.1 ± 3.3 mm^3.

The conventional high-dose chemotherapy regimen was able to improve survival of human glioma-implanted mice (50% survival rate at 45 days *versus* 22 days of the control group; Fig. 1*A*). Combination of high-dose chemotherapy and antiangiogenic treatment did not result in a significant improvement in survival in comparison with the group treated with high-dose chemotherapy only (50% survival rate at 45 days and 53 days; Fig. 1*A*). Both regimens produced similar side effects, consisting of weight loss, skin discoloration, and limited mice motility, mainly occurring during the first week after each cycle of high-dose chemotherapy. All of the mice of these two groups were sacrificed or died within the second month from implantation, and no animals were able to complete more than two cycles of treatment. Gross anatomy analysis showed enlarged liver in five to six animals in the combined treatment group and in all of the animals in the group treated with high-dose chemotherapy alone. The mean tumor volume in animals receiving antiangiogenic plus chemotherapy was significantly lower than that of the group treated with chemotherapy alone (3.35 ± 1.43 *versus* 7.6 ± 2.39; Fig. 2).

PEX administered alone using the same scheme used in the first group (8 days of treatment over 3 weeks for each cycles) produced a similar effect in survival as observed in the previous two groups (50% survival rate at 56 days from implantation), but in this group, three animals were still alive at 3 months from implantation and no treatment side effects were registered. When these animals were sacrificed at 120 days of implantation (after 3 single weeks of treatment upon 3 months), in two animals the mean tumor volume reached 0.4 ± 0.05 mm^3, whereas in the remaining animals, it was 2 mm^3. In this case, the animals started to experience mild neurological deficits. Macroscopic analysis of body organs did not show any sign of distress.

Combined Administration of Low-dose and Continuous Chemotherapy Plus Antiangiogenic Treatment Improved Survival, Reduced Glioma Growth *in Vivo*, and Was Associated with No Chemo-related Side Effects. Treatment was started 15 days from implantation. At this time, four animals in each group were sacrificed, and the mean tumor volume measured in the brain of these animals reached 12 ± 3.5 mm^3 (maximal diameter, 3.2 mm; and minimum diameter, 2.6 mm).

Animals belonging to the control group and treated with vehicle only had a 50% survival rate of 25 days, and all of the animals were sacrificed or died within 40 days of implantation. At the time of sacrifice, all of the animals had a large tumor mass with some protruding outside the skull through the craniotomy.

Animals receiving a low chemotherapy regimen associated with angiogenic treatment had the longest survival (50% survival rate longer than 120 days), and four animals were still alive after 120 days of implantation (Fig. 1*B*). Analysis of the brain of these animals revealed small tumors in three animals and a tumor of 0.7 mm of maximal diameter in the remaining animal (Fig. 2). No side effects during the treatment or macroscopic signs of distress at any organs were seen at gross analysis. Low regimen chemotherapy alone was not able to produce a significant improvement of survival in comparison with the control group, although three animals were still alive after 120 days. At this stage, all of the animals showed tumors in which the mean tumor volume reached 4.8 ± 1.06 mm^3. Treatment was not associated with any side effects, and no pathological changes were seen at gross macroscopical analysis at the time of sacrifice. PEX administered alone after the scheme used in the first group (every 3 days for 2 days) was able to improve survival (50% survival rate, 55 days), and at 120 days, three animals were still alive. Treatment was not asso-

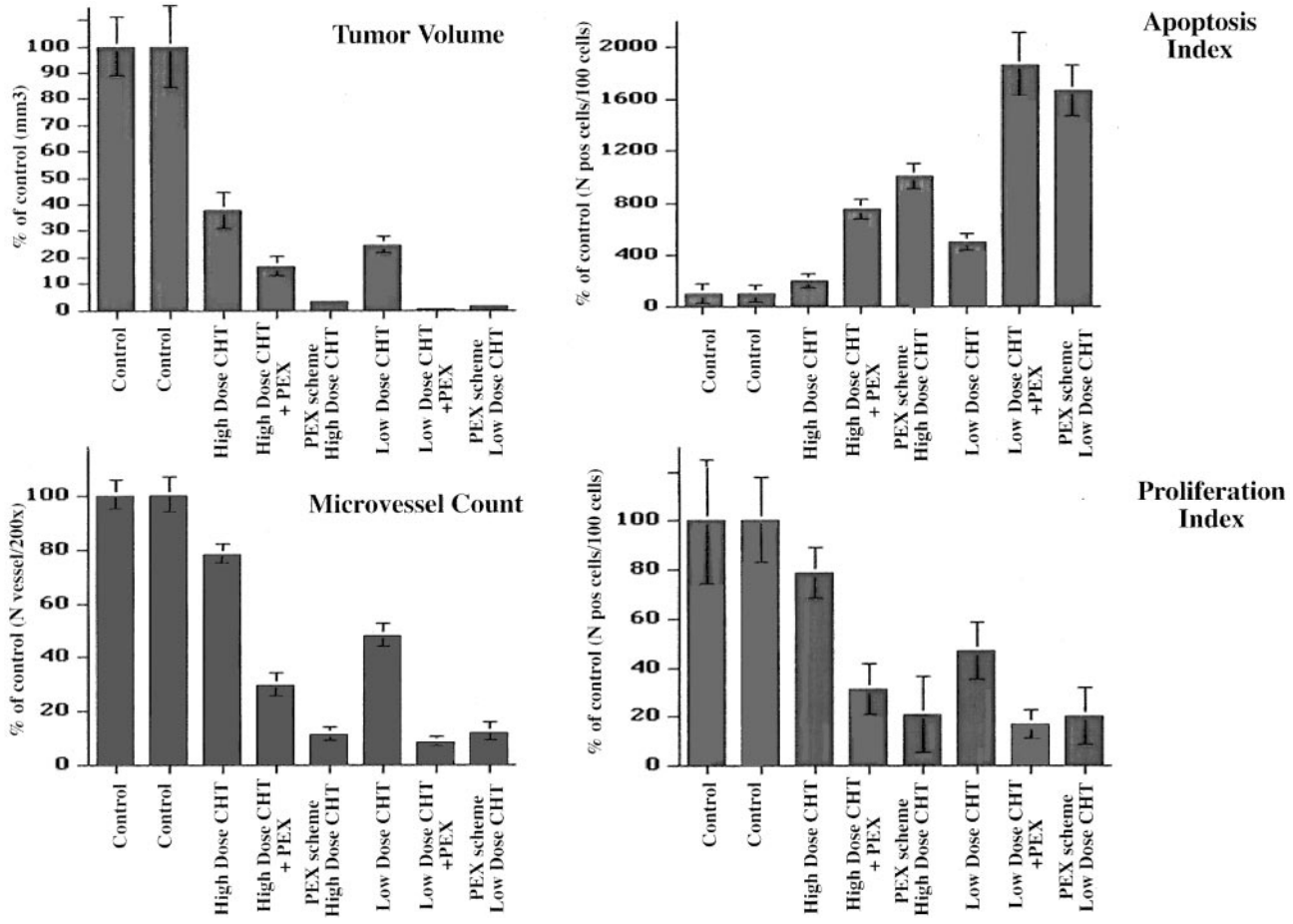

Fig. 2. Tumor volume, vascularity, apoptosis, and proliferation indices in intracranial glioma xenografts submitted to the different regimens of therapy. Data are normalized to the controls and presented as a percentage of the controls ± SE. High-dose chemotherapy + PEX regimen = 5 days of high-dose chemotherapy (carboplatin + etoposide the first day plus etoposide for 4 days) followed by 8 days of PEX and a week of rest. Low-dose chemotherapy + PEX regimen = low-dose carboplatin and etoposide plus PEX for 2 days every 3 days).

ciated with any side effects, and at the time of sacrifice no signs of distress were seen at macroscopical analysis of organs.

Comparison between low and high regimen chemotherapy showed a significant improvement of survival when low-dose chemotherapy was used in combination with antiangiogenic treatment (Fig. 1, *A* and *C*).

Interestingly, no significant difference in survival was noticed when PEX was administered alone using the scheme performed in the high-dose chemotherapy regimen or in that used in the low-dose regimen group (Fig. 1, *A* and *D*).

Histopathological Analysis. Sections of brain removed from each group of treatment were stained with H&E for routine histological examinations and with anti-CD31 and anti-Ki67 antibodies to determine microvessel counts and proliferation rate, respectively. Other sections were submitted to TUNEL assay to measure the apoptotic rate.

On histological examination, no significant difference in morphology was documented between tumors of the control group and those of the group of animals submitted to chemotherapy alone or combined with antiangiogenic treatment. Measurement of angiogenic indices showed some differences among the various groups of treatment (Fig. 2). Tumors belonging to animals of the control group showed the highest microvessel count. Those from animals submitted to chemotherapy alone had intermediate values, whereas the lowest counts were measured in tumors from animals that received combined chemotherapy and antiangiogenic treatment or antiangiogenic treatment alone. Analysis of vessel morphology showed that tumors from animals submitted to combined treatment were characterized by small capillaries and a lack of the large teleangietactic vessels or glomeruloid structures seen in tumors from the control groups or from animals submitted to chemotherapy alone. These last tumors were also characterized by the less apoptotic and the highest proliferative indices. No difference in apoptotic and proliferative indices were seen between tumors from animals submitted to chemotherapy alone and those from mice belonging to the control group. Tumors from animals that received the combined treatment or antiangiogenic treatment alone showed the less proliferative and the highest apoptotic indices, although no difference were seen between these two groups.

DISCUSSION

Various chemotherapy schemes are currently available for the treatment of malignant gliomas in humans (1–5). Most of them usually produce only a limited improvement of survival and are generally considered as a palliation reserved to patients in which other treatments were limited or failed. The majority of these regimens are also associated with considerable side effects (5). Many studies (7, 10) have been conducted to search for alternative modalities of treatment. Antiangiogenesis, for instance, seems a promising approach for the treatment of gliomas. A strategy combining continuous low-dose chemotherapy and antiangiogenesis has been developed recently (14, 15, 18) and used for the treatment of experimental tumors such as lung carcinomas, retinoblastomas, lymphoma, and leukemia. The combi-

nation of low-dosage chemotherapy and antiangiogenic treatment suppressed the growth of these tumors more effectively than conventional chemotherapy alone, even when the tumors were made drug-resistant before therapy. The occurrence of side effects was also considerably lower.

The present study was designed to evaluate whether the association of antiangiogenic treatment and chemotherapy was able to improve survival of nude mice implanted with human glioma cells. As chemotherapeutic drugs, we used carboplatin and etoposide, those that are currently in use in our institutions for the treatment of malignant astrocytomas (2–4). We specifically addressed two hypotheses. In the first, we evaluated whether the association between the standard chemotherapy regimens currently in use followed by a limited antiangiogenesis treatment was able to improve survival in comparison with the standard regimen alone. In the second, we studied the effect of the association between a low and semicontinuous chemotherapic regimen and an antiangiogenic treatment.

To design the first scheme of treatment, we moved from the hypothesis that the antivascular effects produced by the antiangiogenic treatment could hamper and delay the repair of the damages inflicted on dividing cells by the MTD-based chemotherapy during the long rest periods normally associated with these intermittent schemes of therapy (12, 14, 15). Moreover, this effect could also be targeted on endothelial cells, although, because of their lower rate of cell division, the replication of these cells is only weakly disrupted by episodic regimens of high-dose chemotherapy protocols (12). This last consideration seemed also to restrict the potentially higher efficacy of the simultaneous administration of standard chemotherapy and antiangiogenic treatment. To design the second scheme of treatment, we hypothesized that the concurrent administration of an antiangiogenic treatment could amplify the antivascular effect of low-dose chemotherapy (14, 15). The simultaneous exposure to antiangiogenesis treatment could also enhance the inhibitory effect on endothelial cell motility, invasion, and vessel remodeling produced by the low-dose chemotherapy itself (15). Moreover, the metronomic regimen of administration could limit the repair of the slowly proliferating tumor endothelial cells, abrogating their capability to repair during the usual extended rest periods (12) and questioning the potentially higher efficacy of a regimen combining low-dose chemotherapy followed by an antiangiogenic treatment.

Our data showed that the association between high or standard chemotherapy regimen and antiangiogenic treatment did not produce a significant improvement of survival, in comparison with high chemotherapy alone. Both regimens were characterized by a comparable incidence of side effects, substantially attributable to the chemotherapic treatment. The combination of antiangiogenesis and standard chemotherapy produced a decrease in tumor volume, microvessel counts, and proliferative rate and an increase of apoptosis. Animals submitted to antiangiogenic treatment alone administered according to what was scheduled in this regimen did not experience any side effects. The same animals had the longest survival, and the treatment produced a decrease of tumor volume, proliferative indices, and angiogenic indices as in the previous group. These modifications were not documented in tumors from animals submitted to chemotherapy alone, suggesting that they were probably attributable to the antiangiogenic treatment.

The longest survival was documented in animals submitted to low and semicontinuous chemotherapic regimen and antiangiogenic treatment. This treatment was also associated with the more pronounced reduction in tumor volume, a decrease in angiogenic and proliferative indices, and an increased apoptosis. The treatment was very well tolerated by the animals as demonstrated by the absence of any side effects during the entire period of treatment. The administration of low and semicontinuous chemotherapy alone did not produce a comparable improvement of survival, although it was accompanied by no side effects and reduced tumor volume. Antiangiogenic treatment alone administered according to what was scheduled in this regimen improved survival, reduced tumor volume, angiogenic indices, and proliferative indices, and increased apoptosis, and it was not associated with any side effects. Interestingly, three animals of this group were still alive after 120 days from implantation, similar to those in the group submitted to antiangiogenic treatment administered according to the schedule of the high chemotherapy regimen. These two groups were comparable in terms of survival, although the tumors from animals belonging to the group submitted to the semicontinuous regimen showed slightly lower tumor volumes, reduced angiogenic and proliferative indices, and an increased apoptosis. Probably a longer period of treatment is needed to observe a more pronounced difference between these two groups, and we are currently performing experiments in this direction.

In summary, our data suggest that the combination of low and semicontinuous chemotherapy with antiangiogenic drugs is effective in the treatment of experimental human malignant gliomas, because of the improvement of survival and the minimization of side effects. More studies are needed to evaluate the optimal chemotherapeutic and antiangiogenic drugs doses and scheduling characteristics in these tumors.

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REFERENCES

1. Nicholas, M. K., Prados, M. D., and Larson, D. A. *In:* P. M. Black and J. Loeffler (eds.), Malignant Astrocytomas in Cancer of the Nervous System, pp. 464–491. Oxford, United Kingdom: Blackwell Publishers, 1997.
2. Boiardi, A., Silvani, A., Milanesi, I., Botturi, M., and Broggi, G. Primary glial tumor patients treated by combining cis-platin and etoposide. J. Neuro-oncol., *11:* 165–170, 1991.
3. Buckner, J. C., Brown, L. D., Cascino, T. L., Gerstner, J. B., Krook, J. E., Westberg, M. W., Wiesenfeld, M., O'Fallon, J. R., and Scheithauer, B. Phase II evaluation of infusional etoposide and cisplatin in patients with recurrent astrocytoma. J. Neuro-oncol., *9:* 249–254, 1990.
4. Jeremic, B., Grujicic, D., Jevremovic, S., Stanisavljevic, B., Milojevic, L., Djric, L., and Mijatovic, L. Carboplatin and etoposide chemotherapy regimen for recurrent malignant glioma: a Phase II study. J. Clin. Oncol., *10:* 1074–1077, 1992.
5. Stewart, D. J. A critique of the role of the blood-brain barrier in the chemotherapy of human brain tumors. J. Neuro-oncol., *20:* 121–139, 1994.
6. White, L., Sterling-Levis, K., Fisher, R., and Tobias, V. Response of brain tumors to chemotherapy, evaluated in a clinically relevant xenograft model. J. Neuro-oncol., *25:* 39–47, 1995.
7. Kirsch, M., Strasser, J., Allende, R., Bello, L., Zhang, J., and Black, P. M. Angiostatin suppresses malignant glioma growth *in vivo*. Cancer Res., *58:* 4654–4659, 1998.
8. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell, *88:* 277–285, 1997.
9. Tanaka, T., Manome, Y., Wen, P., Kufe, D. W., and Fine, H. A. Viral vector-mediated transduction of a modified platelet factor 4 cDNA inhibits angiogenesis and tumor growth. Nat. Med., *3:* 437–442, 1997.
10. Nguyen, J. T., Wu, P., Clouse, M. E., Hlatky, L., and Terwillinger, E. F. Adeno-associated virus-mediated delivery of antiangiogenic factors as an antitumor strategy. Cancer Res., *58:* 5673–5677, 1998.
11. Auguste, P., Gursel, D. B., Lemiere, S., Reimers, D., Cuevas, P., Carceller, F., Di Santo, J., and Bikfalvi, A. Inhibition of fibroblast growth factor/fibroblast growth factor receptor activity in glioma cells impedes tumor growth by both angiogenesis-dependent and -independent mechanisms. Cancer Res., *61:* 1717–1726, 2001.
12. Hanahan, D., Bergers, G., and Bergsland, E. Less is more, regularly: metronomic dosing of cytotoxic drugs can target tumor angiogenesis in mice. J. Clin. Investig., *105:* 1045–1047, 2000.

13. Kakeji, Y., and Teicher, B. A. Preclinical studies of the combination of angiogenic inhibitors with cytotoxic agents. Invest. New Drugs, *15:* 39–48, 1997.
14. Klement, G., and Kerbel, R. The concept of continuous low dose (metronomic) anti-angiogenic chemotherapy. Proceeding 11th NCI-EORTC-AACR Symposium, 2000.
15. Klement, G., Baruchel, S., Rak, J., Man, S., Clark, K., Hicklin, D. J., Bohlen, P., and Kerbel, R. S. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J. Clin. Investig., *105:* 1–17, 2000.
16. Schiller, J. H., and Bittner, G. Potentiation of platinum antitumor effects in human lung tumor xenografts by the angiogenesis inhibitor squalamine: effects on tumor neovascularization. Clin. Cancer Res., *5:* 4287–4294, 1999.
17. Teicher, B. A., Sotomayor, E. A., and Huang, Z. D. Antiangiogenic agents potentiate cytotoxic cancer therapies against primary and metastatic diseases. Cancer Res., *52:* 6702–6704, 1992.
18. Vacca, A., Iurlaro, M., Ribatti, D., Minischetti, M., Nico, B., Ria, D., Pellegrino, A., and Dammacco, F. Anti-angiogenesis is produced by nontoxic doses of vinblastine. Blood, *94:* 4143–4155, 1999.
19. Deroanne, C. F., Hajitou, A., Calberg, C. M., Nusgens, B. V., and Lapiere, C. M. Angiogenesis by fibroblast growth factor 4 is mediated through an autocrine up-regulation of vascular endothelial growth factor expression. Cancer Res., *57:* 5590–5597, 1997.
20. Waltenberg, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C. H. Different signal transduction properties of KDR and flt-1, two receptors for VEGF. J. Biol. Chem., *269:* 26988–26995, 1994.
21. McKeage, M. J., Morgan, S. E., Boxall, F. E., Murrer, B. A., Hard, G. C., and Harrap, K. R. Lack of nephrotoxicity of oral ammine/amine platinum (IV) dicarboxylate complexes in rodents. Br. J. Cancer, *67:* 996–1000, 1993.
22. Takahashi, I., Maehara, Y., Kusumoto, H., Kusumoto, T., Baba, H., Kohnoe, S., and Sugimachi, K. Interaction of cis-diamminedichloro-platinum (II) and its analogues cis-1,1-cyclobutanedicarboxylato(2R)-2-methyl-1,4-butanediammineplatinum(II)and cis-diammine (glycolato)platinum with hyperthermia *in vivo*. Oncology (Basel), *53:* 68–72, 1996.
23. Uchida, N., Kasai, H., Takeda, Y., Maekawa, R., Sugita, K., and Yoshiota, T. Synergy of the combination of nedaplatin with etoposide in murine and human lung carcinoma. Anticancer Res, *18:* 247–252, 1998.

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