Apoptotic Tumor Cell Death Induced by Estramustine in Patients with Malignant Glioma

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ABSTRACT

Estramustine phosphate (EMP), a cytotoxic drug used in the treatment of prostatic carcinoma, is metabolized and exerts specific cytotoxic effects in malignant glioma in vitro and in vivo. In the present study, we have evaluated the cytotoxic effect of EMP in the clinical situation with regard to appearance of DNA damage and its correlation to the uptake of estramustine (EaM) in human malignant astrocytoma tissue. Ten patients were given 280 mg of EMP p.o. 12 h before surgery. Specimens from brain tumor tissue were collected during surgery and used for detection of fragmented DNA, a hallmark of apoptosis, with in situ end labeling (ISEL) and agarose gel techniques. The main metabolite of EMP in glioma tissue, EaM, was analyzed with gas chromatography. It was demonstrated that EMP induced clusters of ISEL-positive tumor cells and fragmentation of DNA on agarose gels in patients treated with EMP. In the same patients, a significant uptake of EaM in tumor tissue was demonstrated. In control patients, who were not treated with EMP, and in two EMP-treated patients with no uptake of EaM, no signs of fragmented DNA and only a few scattered ISEL-positive cells were seen in the tumor tissue. Signs of apoptosis were also seen in two different experimental models, i.e., in vitro cell cultures of rat glioma cells and an in vivo rat glioma model. It is suggested that EaM can induce apoptosis by a direct effect on a subpopulation of glioma cells in human brain tumors in the clinical situation.

INTRODUCTION

EaM, a fusion molecule between 17β-estradiol and non-nitrogen mustard, has been shown to exert specific cytotoxic effects in human glioma cells in vitro (1, 2) and in a rat glioma model (3). The effects seem to involve the microtubuli system, resulting in an arrest of glioma cells in G2-M phase of the cell cycle (1, 4, 5). In addition, other cytotoxic effects involving cell membrane-coupled events (6–8) and DNA (2, 8) are involved. The phenomena of apoptosis, programmed cell death, has gained increased interest in experimental tumor research, and recently, it was suggested that EaM causes programmed cell death in an experimental glioma model (9). In the present study, we have been able to demonstrate that EaM induces apoptosis in malignant glioma in the clinical situation.

MATERIALS AND METHODS

Patients. Eighteen patients with supratentorial astrocytoma grade III-IV operated on at the Department of Neurosurgery were included in the study. The mean age was 54 years (range, 21–77 years), and the male:female ratio was 11:7. Ten patients were given 280 mg of EMP p.o. The time was chosen from earlier pharmacokinetic studies (10), and the dose of EMP is routinely used in the treatment of patients with advanced prostatic carcinoma. Exclusion criteria were cardiac failure, angina pectoris, and impaired liver or renal function. Eight patients served as controls and did not receive EMP. All patients routinely received 8 mg of betamethason i.v. 12 and 2 h before surgery. The study was approved by the local ethical committee, and informed consent was obtained individually from each patient.

At surgery, specimens of tumor tissue were collected for routine histopathological analysis and for the analysis described below. The interval between administration of EMP and sample taking was approximately 12 h. Specimens for analysis were removed with special attention to avoid denaturation. The samples were immediately fixated in formalin or frozen and stored at −70°C until analyzed.

In Vitro Experiments. A nitrosourea-induced rat glioma cell line BT4C (kindly provided by Prof. Rolf Bjerkvig, University of Bergen, Bergen, Norway) was used for this study to evaluate direct effects on single cells. The tumor cells were cultured in DMEM (Flowlab, Glasgow, United Kingdom) supplemented with 10% FCS and 60 mg/liter gentamicin at 37°C with 5% CO2 for at least 3 days, until the cell growth was exponential. The medium was changed every second or third day. EMP (Estracyt; Kabipharma, Berger, Sweden) was dissolved in sterile water and diluted in DMEM to give the concentration 20 μg/ml. The cells were exposed to EMP for 1, 2, 3, 4, 8, 24, and 96 h before DNA integrity analysis. In addition, cells treated as described above were spun down and analyzed using the ISEL technique.

Rat Glioma Model. A rat glioma model using inbred BD-IX rats and the BT4C glioma cell line was set up. The cells were grown as a monolayer for 1 week before implantation. Cells growing in log phase were harvested and trypsinized.
Table 1  Uptake of EaM, the main metabolite of EMP, in 10 patients with astrocytoma grade III-IV and the percentage of tumor cells positive for ISEL and immunohistochemistry (p53, bcl-2, and c-myc)

<table>
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<tr>
<th>Patient no.</th>
<th>Tumor EaM (ng/g)</th>
<th>Serum EaM (ng/g)</th>
<th>T:S EaM*</th>
<th>ISEL (%)</th>
<th>p53 (%)</th>
<th>bcl-2 (%)</th>
<th>c-myc (%)</th>
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Mean 52.3 2.9 17.5

* T:S, tumor:serum ratio; *, not applicable.

before being spun down and diluted in MEM supplemented with 5% BD-IX rat serum to give 20,000 cells/μl. The rats, 8–14 weeks old, were anesthetized by i.p. administration of 1.8 ml/kg of a 1:1:1 mixture of fluanisonum (10 mg/ml), fentanyl (0.2 mg/ml), and midazolam (5 mg/ml) (Dormicum). The cells were transplanted under stereotactic conditions in the caudate nucleus. EaM was dissolved in ethanol and castor oil to a concentration of 10 mg/ml. Twenty-four days after implantation, tumor samples were taken out 0.5, 1, 2, 4, 8, 24, and 96 h after administration of EaM (20 mg/ml, i.p.). Samples of tumor and normal brain were treated as described for each method. The study was approved by the local ethical committee.

**ISEL.** Samples of tumor tissue from each patient and rat glioma tissue were fixed in formalin and embedded in paraffin. **In situ** detection of apoptotic cells using nucleotides of which one is labeled [ATP, dGTP, dCTP, and biotin dUTP (Boehringer Mannheim, Mannheim, Germany)] and DNA-polymerase I (Sigma, St. Louis, MO) was performed according to the protocol by Wijsman et al. (11). For details, see Vallbo et al. (9). Normal rat prostate 3 days after castration was used as a positive control, because about 4% of the epithelial cells are known to be apoptotic at this time (12, 13). The number of ISEL-positive cells was quantified in the light microscope. Five randomly chosen areas (5 × 10^−3 cm²) in each section were counted. Sections were also processed for routine staining with H&E.

**Analysis of DNA Integrity.** Paraffin sections of human glioma specimens were deparaffinized and rehydrated according to standard histological procedures. The tissue fragments were then suspended in digestion buffer and treated with DNase-free RNase. The samples were loaded into agarose gel containing ethidium bromide. Electrophoresis was performed, and the gels were viewed by transillumination with UV light and photographed. λ-HindIII standard was used as molecular size standard. The analysis was performed according to the method described previously (9, 14).

**Immunohistochemistry (p53, bcl-2, and c-myc).** The deparaffinated sections were heated in a microwave in 0.01 M citrate buffer (pH 6), and incubation with the primary antibodies [p53 (Ab6; clone do-b; Oncogene Science, Uniondale, NY), bcl-2 (clone 124; Cambridge Biotechnology, Cambridge, MA), and c-myc (clone 9E10; Cambridge Biotechnology)] was conducted overnight. After incubation with a biotinylated secondary antibody, the sections were incubated with avidin-biotin complex reagents and peroxidase substrate for development. The
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Fig. 3 DNA integrity analysis for detection of apoptosis in normal brain and glioma tissue in rats treated with EaM and controls. Lanes 1 and 2, 0.5 h after EaM; Lanes 3 and 4, 1 h after EaM; Lanes 5 and 6, 2 h after EaM; Lanes 7 and 8, 4 h after EaM; Lanes 9 and 10, 8 h after EaM; Lanes 11 and 12, 24 h after EaM; Lanes 13 and 14, 96 h after EaM; Lanes 15 and 16, untreated controls; Lanes 17 and 18, rats treated with drug vehicle only. For all lanes: odd numbers, normal tissue; even numbers, tumor tissue.

sections were counterstained with Mayer’s hematoxylin solution. Quantification of immunostained tumor cells was made in light microscopy and expressed as the percentage of the total number of cells in five randomly chosen areas. Positive controls were from human lymphoid tissue and normal prostatic cells.

Analysis of EaM. EaM, the main metabolite of EMP, was analyzed in tumor specimens and serum, using gas chromatography according to the method of Andersson et al. (15, 16). Tissue samples were homogenized in methanol, the extracts were evaporated, and the residues were dissolved in water, extracted with hexane, and then separated on an aluminum column. The samples were dissolved in xylene and quantified by gas chromatography with NP detection (HP 5890; Hewlett Packard).

RESULTS

Apoptosis was detected in 8 of 10 patients who were given EMP before surgery. Analyzed using the ISEL technique, clusters of apoptotic cells were seen in the tumors (Fig. 1). The size of the clusters varied from 100 to 500 positive cells per cluster, and the percentage of stained cells varied from 5 to 50% in tumor tissue (Table 1). In 2 of 10 patients treated with EMP, we could not detect any ISEL-positive cells. However, in these two patients, no EaM was detectable in the tumor tissue. In the control group, which had not been treated with EMP, we showed clusters of ISEL-positive cells in their tumors. The results of the immunostaining of p53, bel-2, and c-myc are presented in Tables 1 and 2. The analysis of EaM in tumor tissue is summarized in Table 1. In eight patients, high levels of EaM were detected in tumor tissue as well as in the tumor tissue. The most striking feature was the high EaM level in tumor tissue relative to serum. In two patients (patients 1 and 8), no drug could be detected in the tumor tissue or in the serum. Notably, these two patients were also negative for ISEL, with no signs of fragmented DNA.

In the rat glioma model, EaM induced time-dependent apoptosis in tumor tissue, but the normal brain was without signs of an apoptotic cell death (Figs. 2 and 3).

In vitro, EaM caused clearly visible signs of DNA fragmentation, analyzed by both DNA integrity and ISEL methods, at 4 and 8 h but not at 1, 2, 3, 24, 48, or 96 h after treatment (Fig. 4).

DISCUSSION

The present study demonstrates that EaM is able to induce apoptosis in a subpopulation of cells in malignant human gli-

Fig. 4 DNA integrity analysis for detection of apoptosis in cultured rat glioma cells treated with EaM. Lane 1, 1-h treatment with EaM; Lane 2, 2-h treatment with EaM; Lane 3, 3-h treatment with EaM; Lane 4, 4-h treatment with EaM; Lane 5, 8-h treatment with EaM; Lane 6, 24-h treatment with EaM; Lane 7, 48-h treatment with EaM; Lane 8, 96-h treatment with EaM; Lane 9, control.
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oma. This finding is strengthened by the observation that in the same patients in which apoptosis was induced, a significant uptake of EaM was demonstrated. To our knowledge, this is the first report on induction of apoptosis in malignant brain tumors in a clinical setting. The probability of EaM-induced apoptosis was also supported by an earlier study in a rat glioma model that demonstrated a time-dependent response without any signs of inflammatory reaction or necrosis (9). In glioma culture, the same time-dependent induction of apoptosis was found.

Fragmentation of genomic DNA into low molecular weight DNA (<1000 bp) is considered a sign of apoptosis (13, 17). ISEL is thought to facilitate a proper detection of this fragmented DNA in tissue (11), using the fact that double-stranded DNA fragments produce 3' hydroxy ends of terminal deoxyxynucleotides during programmed cell death. These ends can be labeled with biotinylated deoxynucleotides and can thus be identified (11, 18, 19). The pattern of DNA degradation with stereotypic nucleosomal-sized fragment together with the demonstration of ISEL-positive cells strengthens the finding that a significant fraction of the cells in the treated human tumors die of apoptosis. Our finding is further supported by a previous report on fragmentation of DNA after exposure to EaM (20). Other effects on DNA, such as hampering of synthesis after EaM exposure, have also been demonstrated (2).

Because EaM has the capacity to induce signs of apoptosis in vitro, it is plausible to propose direct effects on individual cells to be involved. In the present study, EaM penetrates the blood-tumor barrier and also accumulates in tumor tissue in the majority of patients. Therefore, a direct influence of glioma cells, even in the clinical situation, may be assumed. It is noteworthy that in two patients in our study without any uptake of EaM in tumor tissue, no signs of apoptosis were detected. The reason for this lack of uptake is unclear, but it may be due to a concomitant intake of calcium-containing food and/or antacid (21, 22).

Another significant finding was that normal brain tissue had no signs of apoptosis after EaM treatment in a rat glioma model. In this context, it is of interest to recall that EaM affects microtubule integrity and displayed toxicity only in glioma cells and not in normal astrocytes (23). This selective sensitivity in glioma cells may be explained by the high expression of a specific binding protein, EaM-binding protein, in glial tumors compared to normal brain tissue (1, 24, 25).

Obviously, EaM caused apoptosis only in a subpopulation of glioma cells in both the present clinical situation and in rats. An implication from this, observed clusters of apoptotic cells, can be attributed to the well-known heterogeneity and chemoresistance of malignant glioma. Hence, a subpopulation of glioma cells sensitive to chemotherapeutics such as EaM may in some sense be primed to undergo apoptosis. They just need to be exposed to the agent. Consistent with this, a significant number of tumor cells may be resistant to chemotherapy, that is, the lack of or altered signal pathway leading to apoptosis, and thus contribute to the poor prognosis in patients with malignant glioma. The cluster appearance of apoptotic cells might also be due to an uneven blood supply and penetration of EaM in the tumor tissue. This tumor heterogeneity regarding the induction of apoptosis needs to be further investigated.

Earlier studies with EaM have shown a complex and diverse pattern of cytotoxic effects in glioma cells that, in the light of current knowledge, may be features of the controlled process of programmed cell death. Effects on membrane-coupled events such as a rapid influence on ion fluxes (6, 7, 20) and formation of bleb-like projections have been observed (6). Bleb formation has been correlated with loss of cell viability and may be related to lipid peroxidation via free oxygen radicals (26, 27). The observation of a rapid induction of free oxygen radicals after EaM treatment (8) must also be encountered, because free radicals are proposed to be involved in the late steps in the pathway leading to apoptosis (28).

It has been reported that p53 is frequently overexpressed in human astrocytoma of all grades (29, 30), and in half of these cases, the wild-type p53 was present (31). If a mutant form of p53 is present, it will indirectly stimulate an increase of uncontrolled growth. Using immunohistochemistry, p53 is detected mainly in its mutant form (30), which must be considered when evaluating the present results. In patients not treated with EMP, we found a clear expression of p53 (cut-off level, >10%) in only two patients and in none of the patients treated with EMP. The number of patients was limited, however, and the findings do not exclude the presence of wild-type p53 and subsequently are not contradictory to the demonstrated induction of apoptosis.

In conclusion, the antimitotic drug EaM was detected at high levels in malignant glioma tissue in a majority of patients. In tumors with a demonstrated uptake of EaM, visible damage of DNA indicative of apoptosis was encountered. This induction of apoptosis might primarily be a membrane- or cytoskeleton-triggered programmed cell death rather than a direct interaction with the DNA and thus does not necessarily contradict the earlier proposed mechanism of action of EaM, which involves the microtubular system. This finding of induced apoptosis in the clinical setting further increases the interest in EaM and similar drugs in the treatment of malignant glioma.

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