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Radiation Sensitizing Effect of Estramustine is not Dependent on Apoptosis

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Abstract. *Background: Estramustine is an anti-mitotic cytostatic drug that also enhances the effect of radiotherapy. The mechanism of radiosensitization is not thoroughly known. Since both radiotherapy and estramustine induce apoptosis in prostate cancer cells, we conducted an experiment to show whether radiosensitization is mediated by apoptosis. Materials and Methods: DU-145 human prostate cancer cells were xenografted to nude mice and treated with estramustine for 2 weeks and external radiation for 3 to 6 days (18 to 36 Gy). Tumor regression was measured mechanically and the rate of apoptosis defined by the amount of low molecular weight DNA fragmentation. Follow-up time was 1 to 18 days. Results: The tumor size regressed in the group of mice receiving both radiotherapy and estramustine. Four weeks after the treatment, apoptosis was accentuated in the tumors treated with estramustine or radiation but not with their combination. Conclusion: Estramustine potentiates radiotherapy, but not by enhancing radiation-induced apoptosis.*

Estramustine phosphate (EMP) has long been used in the treatment of advanced hormone-refractory prostate cancer, and it may also be of use against breast cancer (1), glioma (2) and other malignancies. The use of EMP or its active metabolite estramustine as a radiation sensitizer has been investigated in many oncologic centres (3-5). The malignancies against which the radiosensitizing ability of estramustine has been demonstrated include prostate cancer (6-8), breast cancer (8), renal cancer (9) and glioma (8). Most treatment studies have been done on cultured or xenografted cancer cells. Clinical trials have generally shown acceptable levels of side-effects and encouraging preliminary results on effectiveness (10, 11). EMP is administered for a period starting weeks before and continuing throughout the period of external radiation therapy. It may be beneficial to combine estramustine with other anticancer drugs such as vinblastine. EMP has also been combined with strontium-89 to treat bone metastases of prostate cancer (3).

Estramustine inhibits advancement of malignant tumors in many ways. The best known mechanism of action of estramustine is that it inhibits mitosis by binding to tubulin, causing its depolymerization and preventing the formation of microtubules, or decreasing the kinetic ability of the microtubules (12), which are necessary to complete cell division (13). Mitosis is arrested in the G2/M-phase.

EMP has also been shown to induce apoptosis in human gliomas and cultured glioma cells (2). This effect was not seen in normal brain tissue (14). Apoptosis may be an important factor in the antitumor effect of EMP (2, 15). Other modes of action have been suggested, including the inhibition of invasion by suppression of matrix metalloproteinase-2 and collagenase activity (16). Both these actions and the induction of apoptosis may be consequences of the action on microtubules.

Cells in the G2/M-phase are the most sensitive to radiation (8). Systemic treatment with estramustine for a period longer than the cell cycle will cause more cells to be in that radiosensitive state (1, 11). This is thought to be the basis for the radiosensitizing effect of EMP, which, in a clinical situation, will be combined with the antitumor effect of the drug.

Radiotherapy also causes apoptosis in prostate cancer cells (17) and in other cancer cells. This study was performed to determine the significance of apoptosis in the radiosensitizing effect of estramustine. We used quantification of low molecular weight DNA fragmentation to define whether the enhancing effect of estramustine on radiotherapy is mediated by an increased apoptotic rate,

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either by the additional effects of the two treatment modalities or the ability of EMP to potentiate radiation-induced apoptosis.

Materials and Methods

Tumor xenografts. The study plan was approved by the ethical committee of the hospital and local experimental animal authorities. Human prostate cancer cells of the line DU-145 were cultured and synchronized to an exponential growth phase. Two million cells were inoculated intracutaneously into each flank of 90 male Balb/c nude mice. The mice, weighing an average of 18 g at the beginning of the experiment, had free access to food and water and were kept in an isolated room, in cages equipped with air filters. The tumors were allowed to grow for three weeks, reaching diameters of 3 to 15 mm. Most mice had two tumors, one on each side; only one tumor per mouse was taken as a specimen for tissue analysis.

Treatment with estramustine. Estramustine phosphate (Estracyt®) (EMP) was purchased from Pharmacia & Upjohn GmbH, Erlangen, Germany. The substance was diluted with a glucose-containing balanced electrolyte solution to a concentration of 1 mg/ml. A daily dose of 0.2 mg EMP was injected intraperitoneally for two consecutive weeks to each mouse randomized to receive the treatment; the other mice received a daily injection of the same amount of the solution without EMP.

Radiotherapy. During the second week of estramustine therapy, one half of the group receiving EMP and one half of the control group were submitted to a three-day or a six-day course of radiotherapy (18 and 36 Gy, respectively). The mice were placed in cylindrical plastic tubes with conical ends so that the skin of the area to be treated rested congruently against the inner surface of the tube. The tubes were then placed into tightly fitting holes in a polymethylmetacrylate (PMMA) phantom to obtain adequate immobilization of the mice and to get a sufficient build-up layer for the superficial tumors. The phantom consisted of five cylindrical cone-ended holes to irradiate five mice at a time with a field size of 28 x 4.5 cm. The mice were irradiated with a daily dose of 6 Gy with 6 MV photons produced by a Varian Clinac 600 C linear accelerator. The field of radiation was limited to the caudal part of the animals, covering both of the tumors and the testes. The 6 Gy dose was calculated to a depth of 2.5 cm in the phantom, which was the average depth of the tumors, at a source-phantom distance of 100 cm and a dose rate of 1.9 Gy/min. The testicle dose at the average depth of 4 cm was 5.6 Gy per fraction. The variation of dose within the tumors was ±5%.

Measurement of tumor size. The three dimensions of the tumors were measured (tolerance of the gauge *ca.* 0.5 mm) at the beginning of the EMP treatment, and repeatedly during the following four weeks. The change in tumor size was calculated by multiplying the length of a tumor by its width and depth and dividing the result by the outcome of the respective measurements in the same tumor at the beginning of the treatment. The average relative tumor size was calculated from these relative values.

Southern blot analysis of apoptotic DNA fragmentation. To assess the presence of apoptosis, samples of one tumor and one testis per mouse were examined. The tissue samples were snap-frozen in liquid nitrogen and stored at –70˚C until DNA isolation. Genomic DNA was extracted using the Apoptotic DNA Ladder Kit (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instructions, with some modifications. Briefly, the carcinoma and testis samples were homogenized and incubated for ten min at room temperature in a binding/lysis buffer (6 M guanidine-HCl, 10 mM urea, 10 mM Tris-HCl, 20% TritonX-100, pH 4.4). The samples were then mixed with isopropanol (final proportion of isopropanol 25%), loaded into polypropylene tubes and centrifuged for one min at 8000 rpm. The tubes were washed twice with washing buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5), and the bound DNA was eluted from the tubes with 10 mM Tris, pH 8.5. Finally, the samples were incubated with RNase (2.5 μg/ml, Roche Molecular Biochemicals) for 20 min at room temperature. After quantification, the DNA samples were 3'-end-labelled with digoxigenin-dideoxy-UTP (Dig-dd-UTP; Roche Molecular Biochemicals) by the terminal-transferase (Roche Molecular Biochemicals) reaction, subjected to electrophoresis on 2% agarose gels, and blotted onto nylon membranes overnight. Next day, the DNA was crosslinked to the membranes by UV irradiation. The membranes were then washed and blocked with 1% Blocking reagent (Roche Molecular Biochemicals) in maleic buffer (100 mmol/L maleic acid, 150 mmol/L NaCl, pH 7.5) for 30 min at room temperature. The 3'-end-labelled DNA on the membranes was localized with alkaline phosphatase-conjugated anti-digoxigenin

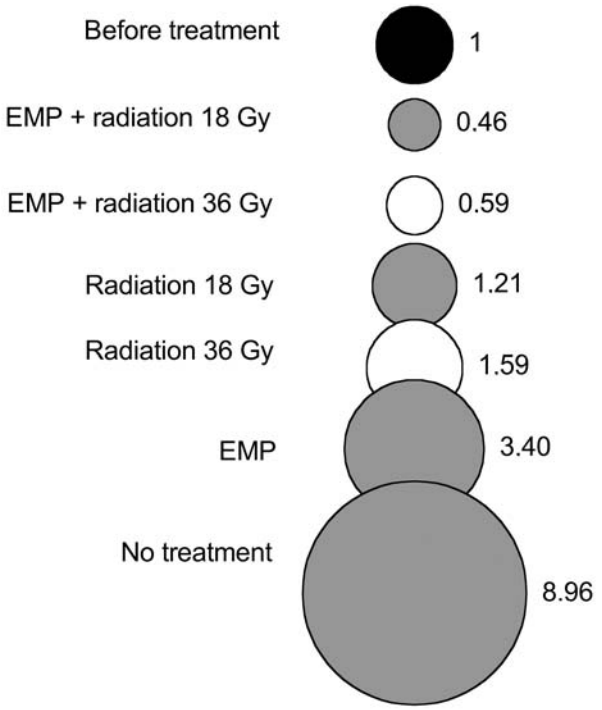

Figure 1. *Average change in tumor size in relation to the size before treatment. The area in the diagram represents the relative volume of the tumors before treatment (black) and at four weeks from the onset of treatment (gray). The white circles represent tumors that received the higher dose (36 Gy) of radiation and were measured earlier (3 weeks) because of side-effects. EMP=estramustine phosphate.*

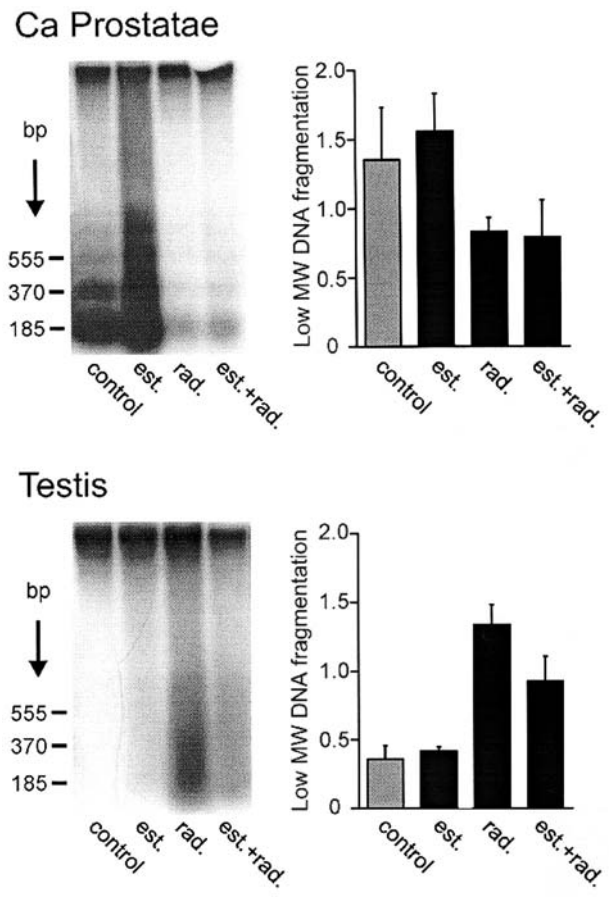

Figure 2. *The amount of apoptosis in the tumors and testes two weeks after the beginning of the different treatment regimes (radiation 18 Gy). The images on the left are examples of the electrophoresis films, showing accumulation of fragmented DNA on the sites of three molecular weights (in base pairs, bp) typical of apoptosis.*

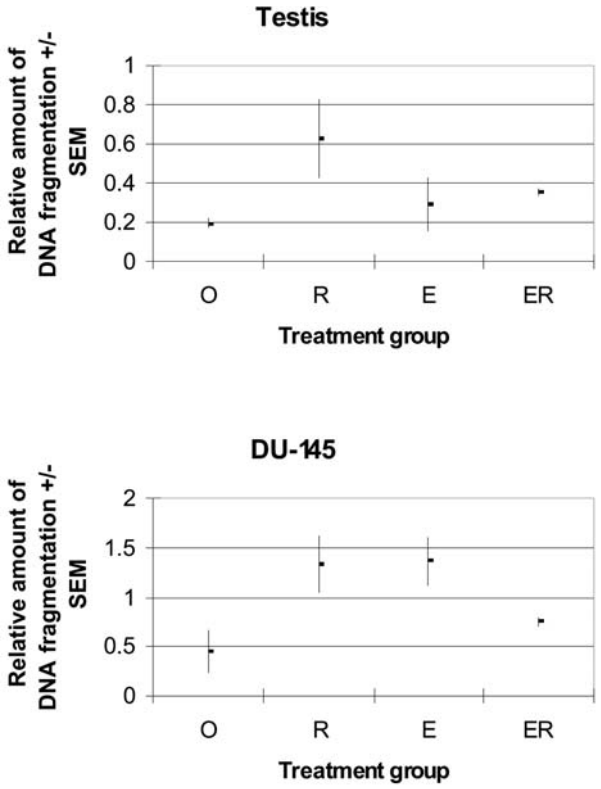

Figure 3. *The amount of apoptosis 24 hours from the end of treatment . O=no treatment, R=radiotherapy, E=estramustine, ER=estramustine and radiotherapy. Apoptosis measured by low molecular weight DNA fragmentation and shown on the vertical axis in relation to a standard sample. The scales on different charts are not directly comparable.*

antibody (Anti-Digoxigenin-AP; Roche Molecular Biochemicals), and the bound antibody was detected by the chemiluminescence reaction (CSPD, Roche Molecular Biochemicals). The X-ray films exposed to chemiluminescense were scanned with a tabletop scanner (Hewlett Packard ScanJet 6300C, Palo Alto, CA, USA) and the digital image was analyzed with Scion Image beta 4.0.2 (Scion Corporation, Frederick, MA, USA) analysis software. The digitized quantification of the low molecular weight DNA fragments (<1.3 kB) of the samples were expressed in relation to a standard amount (20 ng) of a commercial DNA marker (DNA Phix, Amersham, Buckinghamshire, UK).

Results

Unwanted effects of treatment. The untreated mice gained about 10% of weight during the follow-up. The mice in the six-day radiation groups had diarrhea starting on the fourth to fifth day of irradiation. They lost 25% of their weight rapidly and several of them died, leading to early decapitation of the rest of the mice in the six-day radiation groups, and to the conclusion that a 36 Gy total dose is too high in this setting. About half of the mice in the three-day radiation group had mild diarrhea, and they lost on average 10 % of their weight, but all survived.

Tumor size. At four weeks from the beginning of the experiment, the size of an untreated tumor was on average 8.96 (±10.75) times the original size. Tumors treated with EMP only were 3.40 (±3.58) times the original size. Those treated with radiation only (18 Gy) had grown to 1.21 (±0.61) times, while those treated with EMP and radiation (18 Gy) had diminished to 0.46 (±0.54) times the original size. The higher dose of radiation (36 Gy) was associated with high mortality and the tumor sizes in these groups were measured three weeks after the beginning of the trial: the sizes were 1.59 and 0.59 (±0.13) times the original sizes for the radiation only and the EMP and radiation groups, respectively (Figure 1).

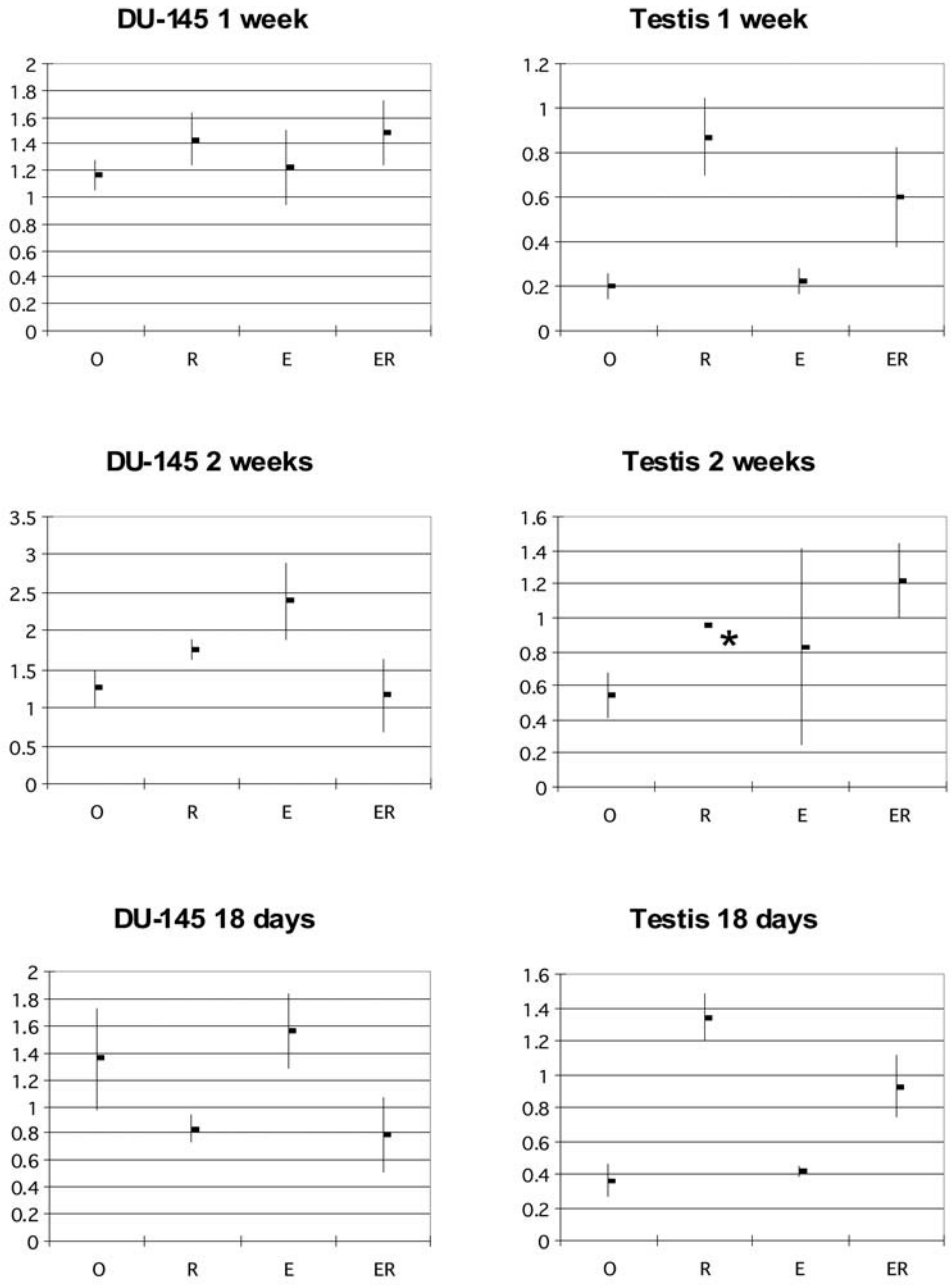

Figure 4. *The amount of apoptosis 1 week to 18 days from the end of treatment. O=no treatment, R=radiotherapy, E=estramustine, ER=estramustine and radiotherapy. Apoptosis measured by low molecular weight DNA fragmentation and shown on the vertical axis in relation to a standard sample. The scales on different charts are not directly comparable. (*Value obtained from a single measurement)*

Apoptosis. A typical DNA fragmentation image is shown in Figure 2. After 24 hours, the relative amount of low molecular weight fragments of DNA, consistent with apoptosis, was significantly higher in the DU-145 tumors treated with radiation only or EMP only than in untreated tumors or those treated with the combination of EMP + radiation. In the testes, treatment with radiation only was associated with a significantly higher level of DNA fragmentation than in all other groups (Figure 3).

After one week, the amount of DNA fragmentation in the tumors of all groups was about the same and, thereafter, the EMP group seemed to demonstrate higher levels, followed later by the untreated group. In the testes, the initial proneness to DNA fragmentation in the

radiation-treated group still persisted 18 days after therapy, and the group treated with EMP + radiation showed rising levels from one week after treatment (Figure 4). The testis two weeks after radiation only was obtained from a single mouse, and thus the value is not of statistical significance.

Discussion

Studies on EMP-induced apoptosis have had follow-up times of 4 to 96 hours. This seems practical, since the plasma half-life of EMP in humans is 10-20 hours. The possible long-term apoptotic effect of EMP has not been studied. In this study, we extended the follow-up time from 24 hours to 18 days from the end of all treatments to find out longer-term effects on apoptosis and to be able to verify the radiosensitizing effect of EMP by tumor regression.

The ability of EMP to potentiate the effect of radiation on prostate cancer xenograft growth has been demonstrated earlier in a similar setting (6). Our results are in accordance with the previous findings: the tumors that were treated with the combination of EMP and radiation tended to diminish in size, while radiation alone seemed only to retard growth and EMP alone had little effect.

DNA fragmentation analysis was used as an indicator of apoptosis. In apoptosis, the DNA is divided by a process in the cell itself into fragments with a typical molecular weight distribution. This study used quantitative analysis of the typical molecular weight sequence. The measured values were compared to a standard sample, while the measurements in the charts could only be compared within the same chart, and not so reliably between different charts and time-points. For this reason, an untreated control group was included in the study.

As seen in Figure 2, the amount of DNA fragmentation 24 hours from the end of treatment was high in DU-145 tumors that were treated with either radiation or EMP alone. The amount was lower in tumors treated with the combination of EMP and irradiation than after a single-treatment regimen, although a greater diminution of tumors was noted after 2 weeks in the combined treatment group. This is in opposition to our hypothesis of an increase in apoptotic rate with combined treatment, or an additive or potentiating effect on the separate apoptotic effects of radiotherapy and EMP. Instead, both EMP and radiation seem to prevent apoptosis caused by the other treatment modality. Therefore, the radiosensitizing effect of EMP must be due to enhancement of some other mechanism of action of radiotherapy. Ischemia, due to damage to small blood vessels, is a known effect of radiotherapy. Hypoxia, on the other hand, decreases radiosensitivity. EMP has been shown to increase blood flow in tumors (18) and, theoretically, this could temporarily reverse radiation-induced ischemia and return the cancer cells into a well-oxygenated, more radiosensitive state for the following irradiation sessions. This, however, is inconsistent with our finding of reduced apoptosis after the combined treatment. The mechanism of radiosensitization may be based on other cellular level effects of radiation. As stated earlier, the mitotic arrest of the dividing cancer cells seems to play a role in radiosensitization.

The longer-term levels of DNA fragmentation in all groups of tumors were similar, with a rise in the EMP-treated group after 2 weeks and the untreated group after 18 days. These results, showing no clear pattern, are probably of no greater significance.

In the testis, radiotherapy had a significant increasing effect on DNA fragmentation from 24 hours to 18 days from treatment. EMP, while causing fragmentation in the tumors, did not have this effect on healthy testes. This is consistent with previous findings in malignant gliomas and healthy brain tissue (14). In the testes, as in the tumors, the combination of EMP and radiation reversed the supposed apoptotic effect of both treatment modalities. In the longer-term follow-up, however, the testes treated with EMP + radiation showed DNA fragmentation levels comparable with those of radiation. This is contrary to the observation in the tumors. The reason for this difference is not clear.

The amounts of DNA fragmentation between 7 to 18 days from the end of treatment are comparable with the amounts after the first day and show considerable variation. This may be due to biological diversity in the tumors, but may also have an impact on the long-term growth or regression of tumors. Long-term effects on apoptosis should be taken into account in further studies and follow-up carried over a longer period than 1 to 3 days, even in cell culture and xenograft studies.

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