RADIATION-INDUCED APOPTOSIS IN HUMAN SARCOMA AND GLIOMA CELL LINES

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Six human soft-tissue sarcoma and 14 glioma cell lines, exhibiting considerable differences in radioresponsiveness and histological grade of differentiation of the parental tumour, were examined with respect to apoptosis development after irradiation with ^{60}Co γ-rays. After test doses of 6 and 25 Gy, significant changes characteristic of apoptosis occurring within 6 to 30 hr were exhibited by only 2 differentiated sarcoma cell lines, EL7 and ESS2. The characteristic internucleosomal fragmentation of DNA was detected as early as 6 hr after exposure of subconfluent monolayer cultures to 6 Gy. It was limited to cells that had detached from the culture plate, whereas adherent cells showed random degradation of DNA, namely after higher doses (25Gy) or longer incubation times (30 hr). As assessed by fluorescence microscopy of unfixed cultures stained with Hoechst 33342 and propidium iodide, the proportion of cells showing apoptotic bodies in non-irradiated controls was <0.1% and 0.3% for EL7 and ESS2, respectively. The dose-response relationship for apoptosis was determined at 9 hr post-irradiation. After 2 Gy, the percentage of apoptotic cells was elevated to 3.4% in EL7 and 4.5% in ESS2 cultures. Saturation was obtained above 6 Gy, with 8.4% apoptosis in EL7 and 15% in ESS2 after 25 Gy. Taken together, rapid ionizing-radiation-induced apoptosis seems to be limited to a subgroup of sarcomas and is unlikely to occur in gliomas.

Apoptosis, also referred to as interphase death, is an active, genetically programmed mode of cell death with characteristic morphological and biochemical features that distinguish the process from necrosis (Wyllie *et al.,* 1980). These features are, *e.g.,* chromatin condensation, DNA cleavage into oligonucleosome-sized fragments, considered to be the result of endogenous endonuclease activity (Arends *et al.,* 1990), and cytoplasmic blebbing. Finally, the cell separates into apoptotic bodies that are phagocytosed by macrophages or neighbouring cells.

Ionizing-radiation-induced apoptosis is well documented to occur in lymphoid cells (Yamada and Ohyama, 1988; Warters, 1992), and also in some murine tumours (Wyllie *et al.,* 1980; Stephens *et al.,* 1991, 1993). We wished to investigate whether, besides reproductive cell death, apoptosis plays a role in radiation-induced cell deletion in non-lymphoid human tumour-cell lines. Therefore, post-irradiation apoptosis development was examined by both morphological and biochemical methods in 6 soft-tissue sarcoma and 14 glioma cell lines. These cell lines differ markedly with respect to radioresponsiveness, histological differentiation and grading of the parental tumour (Stuschke *et al.,* 1992; 1993). The experimental conditions chosen span a broad range with respect to applied doses and incubation time, *i.e.,* irradiation with 6 or 25 Gy ^{60}Co γ-rays, followed by incubation for 6, 12, 24, or 30 hr, and should be suitable for the detection of apoptosis, even if it occurs slowly or after completion of mitosis (Radford *et al.,* 1994).

Changes in nuclear morphology and the ladder-like electrophoretic pattern of DNA extracted from the cells served as markers for radiation-induced apoptosis. To improve the analysis of minimal amounts of apoptotic DNA fragments, total cellular DNA was labelled by nick translation (Suschek *et al.,* 1993) with digoxygenin-dUTP prior to electrophoresis, transferred to nylon membranes, and detected with a commercially available immunoluminescence assay.

To evaluate apoptosis on a cell-to-cell basis, the appearance of apoptotic bodies was examined using the Hoechst-PI method (Ormerod *et al.* 1993). This method is based on the fact that Hoechst 33342 penetrates intact cell membranes and fluoresces blue under 365 nm-UV radiation after binding to DNA, whereas propidium iodide (PI) fluoresces red and is taken up only by cells with damaged plasma membranes.

MATERIAL AND METHODS

Cell lines

The establishment and radiobiological characterization of the human sarcoma cell lines EL7, EF8, EAS1, ESS2, ENF3, and EPG1 have been described elsewhere (Stuschke *et al.,* 1992, 1993). The cell lines A7, EA3, EA7, EA9, EA10, EA12, EA14, EO1, HTZ17, U373 and U87 stemmed from malignant human astrocytomas or oligodendrogliomas (EO1). U87 and U373 were obtained from the ATCC, Rockville, MD. A7 was from Dr. L.E. Gerweck, Massachusetts General Hospital, Boston, and HTZ17 was a gift from Dr. U. Bogdahn, Department of Neurology, University of Würzburg (Germany). The properties of the human low-grade glioma cell lines EAII1, EAII2 and EPF1 have been described (Stuschke *et al.,* 1993).

Thymocytes were obtained from BALB/c mice killed by cervical dislocation. The thymus was excised and disintegrated by passage through a sieve. The cell suspension was washed twice with cold RPMI-medium (GIBCO-BRL, Paisley, UK), adjusted to 10^6 cells per ml and stored on ice until irradiation. Viability was greater than 95% as assessed by Trypan-blue exclusion.

Cell culture and irradiation experiments

Cells were grown in Eagle's minimal essential medium supplemented with 10% FCS, non-essential amino acids, penicillin (100 units/ml; all from GIBCO-BRL) and 5% Seru-max serum extender (Sigma, Munich, Germany) under an atmosphere of 5% CO_2/95% air at 37°C. All cell lines were free of Mycoplasms, as tested by the 6-methylpurine desoxyriboside method (Boehringer Mannheim, Germany). Passage numbers were below 20 for the low-grade glioma cell lines EAII1, EAII2 and EPF1, or ranged from 30 to 50, with the exception of A7, HTZ17, U87 and U373. For irradiation experiments, cells were harvested from exponentially growing monolayers and seeded into 25-cm^2 flasks (Falcon, Becton-Dickinson, Heidelberg, Germany) to obtain about 90% confluent monolayers within 3 to 4 days. Cultures were irradiated with standard doses of 6 or 25 Gy at room temperature using a ^{60}Co source at 1.4 Gy/min. Controls were mock-irradiated. Then they were returned to the incubator and culture was continued for 6, 12, 18 or 30 hr. Thereafter, the medium was withdrawn and monolayers were washed with Hanks' BSS without Ca^{++}/Mg^{++} and treated with trypsin/EDTA solution (both from GIBCO-BRL). To include DNA of cells detached from the substratum after irradiation, medium and wash solution were centrifuged (300 *g*, 5 min) and the pellet was combined with trypsinized cells. Viability of control cultures

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was characteristically greater than 98% as determined by Trypan-blue dye exclusion and phase-contrast microscopy.

Morphological analysis of apoptotic cells

To visualize apoptosis in unfixed monolayer cultures, the bis-benzimidazole dye Hoechst 33342 (Sigma) was added to the culture medium to give 1 μg/ml final concentration, then, 15 min later, PI solution was included to 5 μg/ml and monolayers were observed with a Zeiss inverted fluorescence microscope at 400× magnification. For quantificative analysis, monolayer cells were trypsinized, combined with cells floating in the culture medium and kept on ice before being counted in a Neubauer chamber. More than 500 cells were counted at each point.

DNA fragmentation assay

For DNA extraction, cells were exposed to lysis buffer (50 mmol/l TRIS, pH 7.8, 10 mmol/l EDTA, containing 1% N-lauryl sarcosine and 0.5 mg/ml Proteinase K (Boehringer Mannheim) and incubated at 37°C overnight. After digestion with RNase A (Sigma; 0.5 mg/ml, heat-treated to remove DNase) for 3 hr at 37°C, samples were extracted twice with phenol/chloroform/isoamyl alcohol and DNA was precipitated in sodium acetate/ethanol overnight. The DNA was pelleted by centrifugation, washed twice with 70% ethanol, air-dried, redissolved in 30 μl 10 mmol/l TRIS, 1 mmol/l Na_2 EDTA, pH 7.5 and loaded into a 1.5% agarose gel. DNA fragments were separated by electrophoresis (5V/cm) in 0.5 × TBE running buffer (TBE = 90 mM TRIS, 90 mM boric acid, 4 mM EDTA) containing 0.5 μg/ml PI. DNA fragments from bacteriophage λ digested with HindIII or BstEII served as size markers. After completion of electrophoresis, gels were viewed by transillumination with UV light and photographed.

Nick translation assay

Nick translation mixture consisted of 10 μg DNA; 4.5 μmol/l digoxygenin-dUTP; 3 μmol/l each of dGTP, dCTP and dATP; 1U Kornberg polymerase (all components from Boehringer Mannheim); 50 mmol/l Tris-HCl pH 7.5; 5 mmol/l $MgCl_2$ in a total volume of 20 μl. After 30 min incubation at room temperature, the reaction was stopped by addition of EDTA solution to achieve 30 mmol/l final concentration. The samples were loaded into 1.5% agarose gels and electrophoresed as described above. Digoxygenin-labelled fragments of pBR328 DNA, cleaved separately with BglI and HinfI, served as molecular-weight markers (154 to 2176 bp; Boehringer Mannheim). After alkaline transfer to DNA to nylon membranes, digoxygenin-labeled DNA was detected by means of the DIG luminescent detection kit of Boehringer Mannheim.

Statistical methods

Using the maximum likelihood criterion, the dose-dependent percentages of apoptotic cells were fitted by a Gompertz function, defined by $p = a \cdot \exp(-b \cdot D)/b - a/b + c$ (Procedure Nonlin, SAS Institute, 1989) (p, percentage of apoptotic cells; D, radiation dose (Gy); a, b, c, parameters of the Gompertz curve). The percentages of apoptotic cells in irradiated and control groups were compared using a χ^2-test.

RESULTS

Of the 20 human sarcoma and glioma cell lines tested in our study, only the 2 soft-tissue sarcoma cell lines EL7 and ESS2 underwent morphological changes and internucleosomal DNA fragmentation characteristic of apoptosis after exposure to ionizing irradiation. The results obtained with these 2 cell lines are described below.

As examined by phase-contrast microscopy, a wave of cells started to dissociate off the culture plate at 4 hr post-irradiation. They rounded up and showed many enclosed blebs. In the case of ESS2, 6 hr after application of 6 and 25 Gy, 5 and 10% of the cells, respectively, were floating in the culture medium. They excluded Trypan blue. At 30 hr post irradiation, 12 and up to 35% of the cells, respectively, had left the monolayer, and now readily took up Trypan blue, indicating membrane damage. With EL7, the effect was less pronounced, reaching maximally 20% detached cells at 30 hr after application of 25 Gy.

On excitation with 365 nm-UV radiation of cultures stained with vital dye Hoechst 33342 and propidium iodide (PI), the nuclei of adherent monolayer cells and chromosomes of mitotic cells fluoresced blue, whereas the nuclei of necrotic cells fluoresced red due to the uptake of PI and were sometimes swollen. In both cases nuclei were ovoid and outlined and fluorescence intensity was evenly distributed (Fig. 1*a*). The cells dissociating from the monolayer exhibited bright blue, fluorescing masses of chromatin that abutted at the nuclear membrane or, at later stages, showed bright blue, fluorescing spherical bodies (Fig. 1*b*) and were therefore identified as apoptotic (Ormerod *et al.*, 1993; Harmon *et al.*, 1990). At >18 hr post-irradiation, fluorescence of these particles turned to light reddish-blue, indicating increased membrane fragility of the apoptosing cells. The characteristics of non-irradiated ESS2 cells were as follows (weighted means of percentages, range of observed percentages in brackets): apoptosis, 0.3 (0.0–2.0); mitosis 0.7 (0.4–1.2); necrotic/dead, 0.8 (0.4–1.5). For EL7, ≤ 0.1, 3.9 (3.3–4.7) and 3.3 (2.5–4.7), respectively, were counted. Irradiated cultures never displayed mitotic cells, as scored by the appearance of chromosomes or metaphase plates.

The dose-response characteristics for the development of apoptosis in ESS2 and EL7 cells are presented in Figure 2 and Table I. For purposes of comparison, the latter also includes data from some of the sarcoma and glioma cell lines not exhibiting radiation-induced apoptosis. Nine hours post-irradiation was chosen as the time point, because then apoptosis development was maximal, with a minimum of apoptotic cells taking up PI. The incidence of apoptotic cells was significantly increased above control level after application of 2Gy ($p < 0.05$; Table I). With 1.4 ± 0.4% and 1.9 ± 0.2% apoptosis per Gy for ESS2 and EL7, respectively, the initial slope a of the dose-response curve was similar for both cell lines. At doses above 6 Gy, saturation was obtained.

In gel electrophoretic analysis of EL7 and ESS2 DNA, faint bands of oligonucleosome-sized fragments appeared at >6 hr after irradiation of monolayer cultures with 6 and 25 Gy, respectively (Fig. 3*a*). After labelling by nick translation of total cellular DNA with digoxygenin-dUTP, these fragments were readily detected on Southern-blot luminograms (Fig. 3*b*). Oligonucleosomal DNA fractionation was limited to those cells that had dissociated from the substratum after irradiation, whereas the adherent cells showed random degradation of DNA, namely after 25 Gy (data not shown). In parallel, the proportion of PI taking up necrotic ESS2 or EL7 cells was doubled within 9 hr after application of 25 Gy, indicating radiation-induced necrosis in both cell lines.

In experiments with the other 4 sarcoma and 14 glioma cell lines tested in this study, no significant morphological changes typical of apoptosis occurring post-irradiation were discovered. Southern blots of nick-translation-labelled DNA did not reveal evidence of apoptosis-like DNA fragmentation (Fig. 4). Only a slight increase of random-sized DNA fragments was commonly observed in samples incubated for 30 hr after application of 6 or 25 Gy.

DISCUSSION

The biochemical and morphological methods used in this study on radiation-induced apoptosis in human tumour cells are suitable for the identification and quantification of apoptotic cells (Ormerod *et al.*, 1993) and make it possible to detect

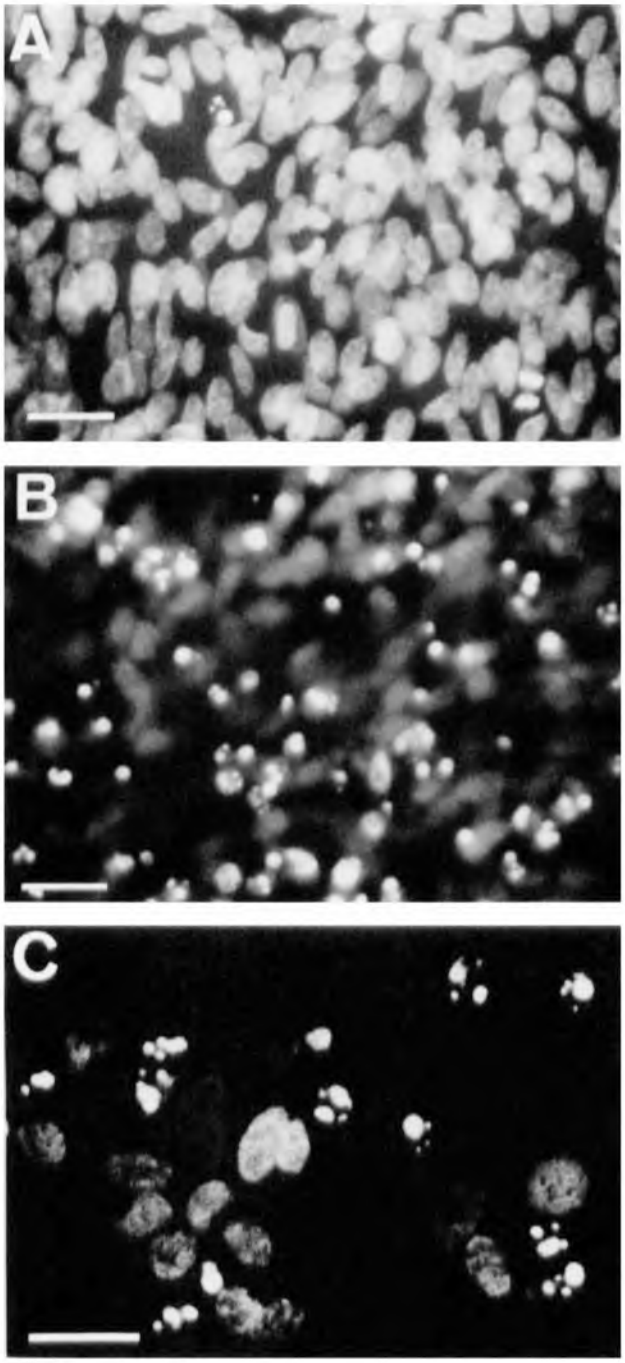

FIGURE 1 – Detection of apoptotic cells by fluorescence microscopy. ESS2 monolayer cultures were stained with Hoechst 33342 and propidium iodide (PI) and nuclear morphology was examined under UV-light. Nuclei of adherent cells were commonly oviform. Cells that had detached from the substrate excluded PI. Instead of nuclei they showed Hoechst-33342-stained spherical bodies and were therefore identified as apoptotic. *(a)* Non-irradiated controls; *(b)* 9 hr after 25 Gy, apoptotic cells (focal plane) are floating above the adherent cells; *(c)* cytocentrifuged cells, 9 hr after 25 Gy. Scale bars, 20 μm.

minimum amounts of apoptotic DNA fragments (Suschek *et al.,* 1993).

The following observations are consistent with rapid apoptotic cell death occurring in ESS2 and EL7 cultures after

TABLE I – PERCENTAGES OF APOPTOTIC CELLS FOR RADIATED AND UNIRRADIATED SARCOMA AND GLIOMA CELL LINES

Cell line	Induced apoptosis (%)			
	) Gy	2 Gy	6 Gy	25 Gy
ESS2	0.3 (0.0–2.0)	4.5* (0.8–7.2)	5.1* (4.7–6.2)	15.2* (6.0–28.1)
EL7	≤0.1 (0.0–0.0)	3.4* (3.1–3.9)	5.7* (4.4–7.0)	8.4* (5.6–9.8)
EPG1	1.9 (1.7–2.5)	N.D.	2.0 (1.6–2.9)	5.1*[1] (4.4–5.6)
EF8	≤0.1	N.D.	≤0.1	≤0.1
EAII 1	1.0 (0.0–2.5)	N.D.	0.6 (0.4–0.9)	1.9[1] (1.4–2.2)
A7	≤0.1 (0.0–0.0)	N.D.	0.2 (0.0–0.3)	0.3[1] (0.1–0.4)
EA14	≤0.1	N.D.	≤0.1	≤0.1

Apoptosis was evaluated by fluorescence microscopy of monolayers stained with Hoechst 33342 and propidium iodide 9 hr post-irradiation. Results are expressed as weighted means of the observed percentages of apoptotic cells, ranges are given in brackets.–*Significantly different from non-irradiated controls ($p < 0.05$; χ^2-test).–[1]All apoptotic nuclei were stained by propidium iodide, indicating secondary necrosis.

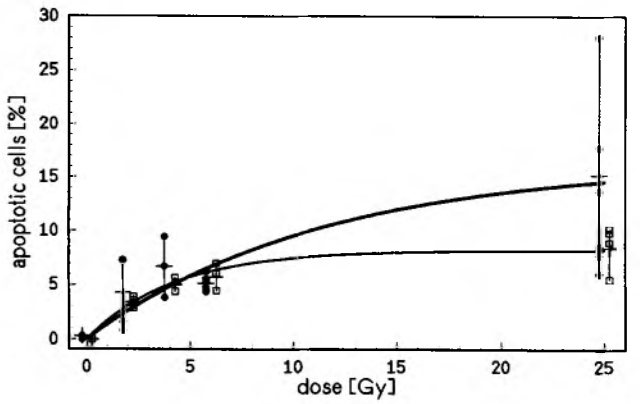

FIGURE 2 – Dose response for the development of apoptosis in ESS2 and EL7 monolayers 9 hr after application of single doses. Apoptosis was evaluated by fluorescence microscopy. The regression curves are maximum likelihood approximations of the Gompertz function to all individual data points. Closed symbols, ESS2; open symbols, EL7. Results of 2 experiments run at different dose levels in triplicate are shown.

exposure to ^{60}Co-γ-rays: (1) The changes characteristic of apoptosis, *i.e.,* development of apoptotic bodies subsequent to chromatin margination, and internucleosomal cleavage of DNA, were restricted to one and the same subpopulation of cells—those that detached from the substrate (Wyllie *et al.,* 1980). (2) These changes preceded Trypan blue and propidium iodide uptake, indicating that membrane damage was secondary to nuclear changes. (3) Time and dose-response characteristics of apoptosis, *i.e.,* rapid onset within 6 hr after doses of as little as 2 Gy and saturation above 6 Gy, were similar to those obtained with lymphoid cells or with responding murine tumours (Meyn *et al.,* 1993; Stephens *et al.,* 1993). (4) Although it has been reported that apoptosis is not limited to non-dividing cells (Warters, 1992; Stephens *et al.,* 1993), ESS2 and EL7 cells, like thymocytes (Yamada and Ohyama, 1988), did not enter mitosis before they apoptotically died. This is suggested by the finding that, in contrast to sham-irradiated controls, irradiated cultures displaying apoptotic cells were essentially free of dividing cells, due to radiation-evoked G_2 block (Elkind *et al.,* 1963).

After electrophoretic separation, the DNA of irradiated murine lymphocytes appeared to be completely degraded into

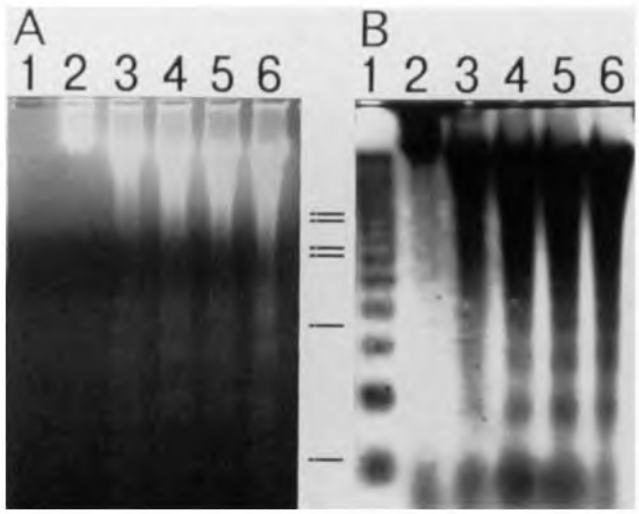

FIGURE 3 – Analysis by gel electrophoresis and nick translation of radiation-induced DNA fragmentation in ESS2 sarcoma cells. *(a)* DNA stained with propidium iodide; *(b)* staining of DNA by nick translation. Lane 1 includes 50 ng of mouse thymocyte DNA isolated 6 hr after application of 9 Gy. Lanes 2–6 each include 10 μg of ESS2 DNA; lane 2: sham-irradiated; lanes 3–6: 6, 12, 18 and 30 hr after application of 6 Gy, respectively. The positions of bacteriophage λ DNA/BstEII fragments are indicated (from bottom to top: 224, 702, 1264, 1371, 1929, 2323 bp, respectively).

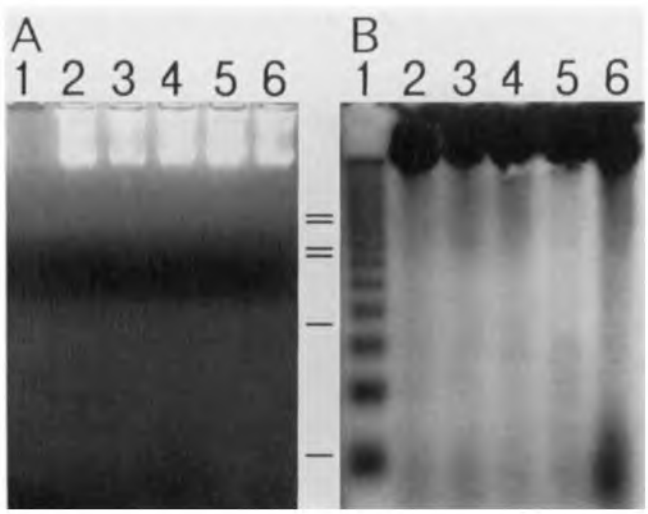

FIGURE 4 – Absence of radiation-induced DNA fragmentation in EA14 glioma cells. DNA of EA14 glioma cells irradiated with 6 Gy was analysed by gelectrophoresis *(a)* and nick translation *(b)* as in legend to Figure 3. No ladder-like pattern of DNA fragments was observed.

oligonucleosomal fragments. EL7 and ESS2, however, showed a ladder of mono- to pentanucleosome units as well as a smear of higher-molecular-weight DNA, similar to ST4 mouse lymphoma cells (Radford, 1991). At longer incubation times, EL7 and ESS2 gels displayed maxima of oligonucleosomal size in front of a continuous background signal. This may be due to radiation-induced necrosis occurring in parallel to apoptosis, as is also indicated by the elevated proportion of necrotic nuclei taking up PI in irradiated cultures.

A major result of this study is that none of the 14 human glioma, and only 2 of 6 soft-tissue sarcoma cell lines responded significantly with apoptosis within 30 hr post-irradiation. In a previously published study on radiation-induced apoptotic death in mouse lymphoid and myeloid cell lines, even the slowest of the reacting cell lines showed the ladder-like pattern of DNA fragments at around 30 hr after irradiation with 6 Gy (Radford *et al.,* 1994). Heterogeneity in the development of apoptosis was also observed with murine tumours, the time courses and dose responses being similar in the responding tissues: given a dose of 2.5 up to 25 Gy, the percentage of apoptotic cells was maximal at 3–6 hr and declined thereafter (Stephens *et al.,* 1991, 1993; Meyn *et al.,* 1993). Probably, the non-reacting cell lines predominantly die slowly via necrosis, as suggested by increased random-degradation of DNA observed in all cell lines 30 hr after exposure to 6 or 25 Gy.

There may be as many explanations for the absence of apoptosis development in 18 of the 20 cell lines tested here, as biochemical factors appear to control the apoptotic response in irradiated cells. Among these are, for example, different growth factors and cytokines, the tumour-suppressor p53 and several cellular proteins that act as inhibitors of apoptosis, for example bcl-2, or inducers of apoptosis such as c-myc, E1A, and bcl-2 associated proteins (reviewed by Szumiel, 1994). Thus, the heterogeneity in radiation-induced apoptosis could be due to mutations of the corresponding genes or differences in their expression.

In the comparative study with murine tumours mentioned above, Meyn *et al.* (1993) found a correlation between radiation-induced apoptosis and tumour radioresponse measured by tumour-growth delay and tumour radiocurability. Among the 6 sarcoma cell lines tested here, the 2 responding lines, EL7 and ESS2, belong to the more radiosensitive ones, as determined by the spheroid control assay, whereas EF8 is relatively radioresistant and does not develop apoptosis (Table I; Stuschke *et al.,* 1993). Furthermore, EL7 and ESS2 are derived from grade-II sarcomas, while EF8 stems from an undifferentiated sarcoma (Stuschke *et al.,* 1993). For that reason, there might be a correlation between radiation-induced apoptosis, tumour differentiation and radioresponsiveness of human sarcomas.

Whether differentiation agents may modulate the propensity of a cell line to undergo apoptosis after irradiation, as has been reported for some cell types (Langley *et al.,* 1994), is currently under investigation. Differentiation modulators or substances directly acting on the apoptotic signaling pathway could open new possibilities for combined chemoradiotherapy in the future.

Taken together, the results indicate that therapeutic doses of ionizing radiation can indeed induce rapid apoptotic cell deletion in differentiated human sarcoma lines, but not in glioma cell lines.

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