Cancer Chemother Pharmacol (1994) 33: 410–414

Cancer Chemotherapy and Pharmacology

Induction of apoptosis in murine tumors by cyclophosphamide

Raymond E. Meyn[1], L. Clifton Stephens[2], Nancy R. Hunter[1], Luka Milas[1]

[1] Department of Experimental Radiotherapy, [2] Department of Veterinary Medicine and Surgery, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA

Received 9 April 1993 / Accepted 24 September 1993

Abstract. Whereas there have been several recent reports of the induction of apoptosis by chemotherapy agents in cell culture systems, much less is known about the role of this mode of cell death in tumors treated in vivo. We therefore quantitated the proportion of apoptotic cells induced as a function of time and dose in two murine tumors treated with cyclophosphamide in vivo. The two tumors were a mammary adenocarcinoma, MCa-4, and an ovarian adenocarcinoma, OCa-1. The percent apoptosis was scored from stained histological sections of the tumors using a system based on the characteristic features of the apoptotic nuclei. The kinetics of apoptosis development were determined over a 5-day period following treatment of the mice with 200 mg/kg. The percent apoptosis peaked between 10–18 h in both tumors and then slowly declined to background levels by 5 days after treatment. The dose responses showed that even much lower doses, 25 mg/kg, could induce significant apoptosis and that the proportion of apoptotic cells plateaued at doses higher than 100 mg/kg. These results are compared and contrasted with our previous reports on apoptosis induction in these same tumors with ionizing radiation.

Introduction

Apoptosis, or programmed cell death, is a mode of cell death distinct from necrosis that has important roles in a variety of biological processes including embryonic morphogenesis, metamorphosis, hormone-dependent tissue atrophy, and lymphocyte maturation in the thymus [1, 4]. Spontaneous apoptosis occurs even in malignant tumors, of both rodent and human [19] origin, probably as the manifestation of an intrinsic homeostatic process to balance the cell gain by mitosis and hence restrain tumor growth. This selective, programmed process of cell deletion features chromatin condensation, DNA fragmentation, and cytoplasmic blebbing. The dying cells disintegrate into clusters of membrane-bound apoptotic bodies, which are ultimately engulfed by healthy neighboring cells and enzymatically digested [21].

In addition to its spontaneous occurrence as a manifestation of tissue homeostasis, apoptosis can be induced by a variety of exogenous agents such as ionizing radiation [14, 18, 22], chemotherapeutic drugs [2, 5, 7, 13] and glucocorticoids [3, 10]. Most investigations were performed in in vitro systems, whereas in vivo studies of induced apoptosis have been rare, especially those dealing with tumors. Recently, we have characterized the in vivo apoptotic response of a murine tumor to radiation [17], using an assay we initially developed for quantifying apoptotic cells in stained histological sections of salivary glands from irradiated monkeys [15]. We observed that about 30% of the cells in a murine ovarian carcinoma, OCa-I, became apoptotic after being exposed to about 10 Gy of radiation. The apoptosis developed within 2–3 h after irradiation, reaching a maximum between 4 and 6 h and returning to background values by 24 h.

Searle et al. [13] reported nearly 20 years ago that several different chemotherapeutic agents induced apoptosis in the normal and tumor tissues of treated mice when analyzed by nonquantitative approaches. Whereas apoptosis has previously been characterized by morphometric analysis in tumors treated in situ with radiation [6, 16, 17], hyperthermia [6] or following hormone ablation [8, 9], no information, to our knowledge, has been reported on the quantitation of the apoptotic response of cells in tumors treated with cancer chemotherapeutic agents such as cyclophosphamide (CY). We report here the magnitude and kinetics of apoptosis induced in two murine tumors, OCa-1 and MCa-4, growing in animals treated with CY.

The Work reported in this paper was supported by research grants CA-06294 and CA-16672 awarded by the National Cancer Institute, Department of Health and Human Services, and by the Katharine Unsworth Memorial Fund

Correspondence to: Raymond E. Meyn, Department of Experimental Radiotherapy, Box 66, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, Texas 77030, USA

Materials and methods

Mice. We used inbred male or female C_3Hf/Kam mice bred and maintained in our specific-pathogen-free colony. The mice were 11–13 weeks old at the beginning of experiments. Within each experiment mice of the same sex were used, and they were kept three to five to a cage.

Tumors. The two tumors, an ovarian carcinoma (OCa-I) and a mammary carcinoma (MCa-4), were originally spontaneous in C_3Hf/Sed mice. These transplantable tumors are non-immunogenic to these syngeneic hosts and produce metastases in a proportion of the animals. Both tumors are sensitive to development of radiation-induced apoptosis, which correlates with their radioresponse as measured by tumor growth delay and tumor cure [11].

Solitary tumors were produced in the muscles of the right thigh by the inoculation of 5×10^5 viable tumor cells. The viability of single-cell suspensions of tumor cells, which were prepared by enzymatic digestion of minced tumor tissue from tumor-bearing source mice using 0.025% trypsin and DNase, was greater than 90% as determined by trypan blue dye exclusion and phase-contrast microscopy examinations. When the tumors had grown to 8 mm in diameter, the mice were treated with CY, further tumor growth was monitored, and the apoptosis was quantitated at a given time. The groups contained three mice each.

CY-treatment. CY (Mead Johnson, Evansville, Ind.) was dissolved in distilled water at concentrations ranging from 5.0 to 20 mg/ml and was administered i.p. at doses of 50–200 mg/kg body weight.

Quantitation of apoptosis. The mice were killed by cervical dislocation, and the tumors were immediately excised and placed in neutral-buffered formalin. This tissue was embeded in paraffin blocks, from which 2- to 4-mm sections were cut and stained with hematoxylin and eosin (HE). The morphological features we used for histological identification of apoptosis and distinction of this process from necrosis have been described and illustrated in our earlier publications [15–17] As before, apoptosis was scored in coded slides by one of us (L.C.S.) by microscopic examination of HE-stained sections at 400× magnification. Five fields of non-necrotic areas were selected in each specimen, and in each field the number of apoptotic nuclei and mitoses were recorded as numbers per 100 nuclei and expressed as a percentage. The percentage was based on scoring 1500 nuclei, obtained from three mice per group.

Tumor growth delay. Tumor growth delay was used to assess the antitumor efficacy of CY. Tumors growing in the right hind thighs of mice were treated with CY when 8 mm in diameter. The growth of non-treated and CY-treated tumors was determined by measuring three mutually orthogonal tumor diameters with a Vernier caliper. The measurements were performed at 2- to 3-day intervals until tumors reached at least 17 mm in diameter.

Results

The magnitude and kinetics of apoptosis induction in 8-mm OCa-I and MCa-4 tumors treated with 200 mg/kg CY are shown in Fig. 1. This dose of CY is commonly used in studies of the antitumor activity of CY (12), and is slightly below the maximum tolerated dose in mice. Whereas the background values of apoptosis were 4.5% for OCa-I and 3.5% for MCa-4, the data in Fig. 1 show that there was a significant increase in the percentage of apoptosis in both tumors even by 1 h after treatment with CY. Apoptosis increased further with time, reaching a peak value at about 16 h for the OCa-I tumor and 8–10 h for the MCa-4 tumor. Thereafter, the level of apoptosis slowly decreased, reaching background levels by 3–5 days after treatment. In general, both the magnitude and the kinetics of CY-induced apoptosis were similar for the OCa-I and MCa-4 tumors. The histology of CY-treated and untreated tumors is presented in Fig. 2.

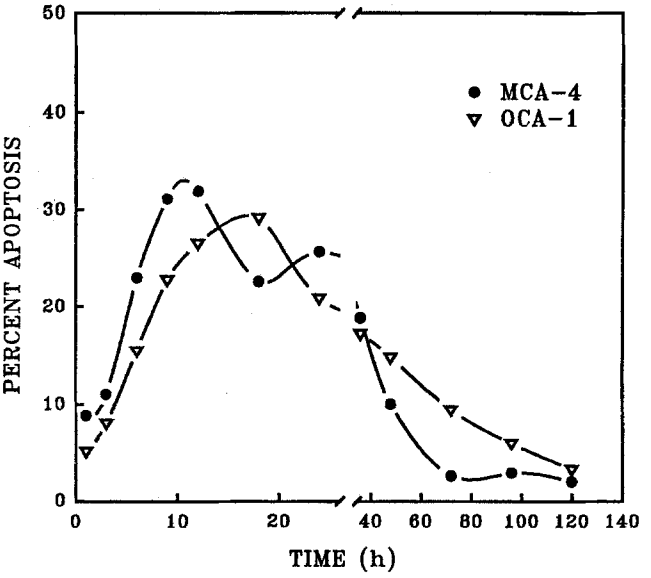

Fig. 1. The kinetics of apoptosis development in MCa-4 and OCa-1 tumors in mice treated with a single dose of 200 mg/kg i.p. given at time zero. Mice were sacrificed at different times following treatment, and apoptosis scored from stained histological sections of the tumors

The dependence of induction of apoptosis on the dose of CY was determined by treating animals bearing 8-mm OCa-I or MCa-4 tumors with 0, 50, 100, or 200 mg/kg of CY and scoring the percent apoptotic cells at 24 h after treatment (Fig. 3). Both tumors showed dramatic apoptosis development as a function of dose of CY. The lowest dose of CY, 50 mg/kg, produced measurable apoptosis in both OCa-I and MCa-4 tumors. Further increases in the dose resulted in increased apoptosis; however, it appeared that, in general, lower doses were more effective per unit dose for inducing apoptosis than higher doses and the dose-response relationships appeared to be leveling off at doses of 100 mg/kg and above. Consistent with the data in Fig. 1, the MCa-4 tumor had a somewhat greater response to CY than did the OCa-1 tumor.

CY-induced apoptosis was associated with significant antitumor efficacy of CY as measured by the delay in tumor growth (Fig. 4). The delay was more pronounced as the dose of CY was increased. There was clear regression of both tumors within 2–4 days after CY treatment at the higher doses.

Discussion

The results presented in this article show that the alkylating agent CY strongly induces apoptosis in murine tumors. Both the extent and kinetics of apoptosis were quantitated by scoring apoptotic nuclei in histological sections of tumors, a method we have used previously to assess radiation-induced apoptosis in normal tissues [15] and tumors

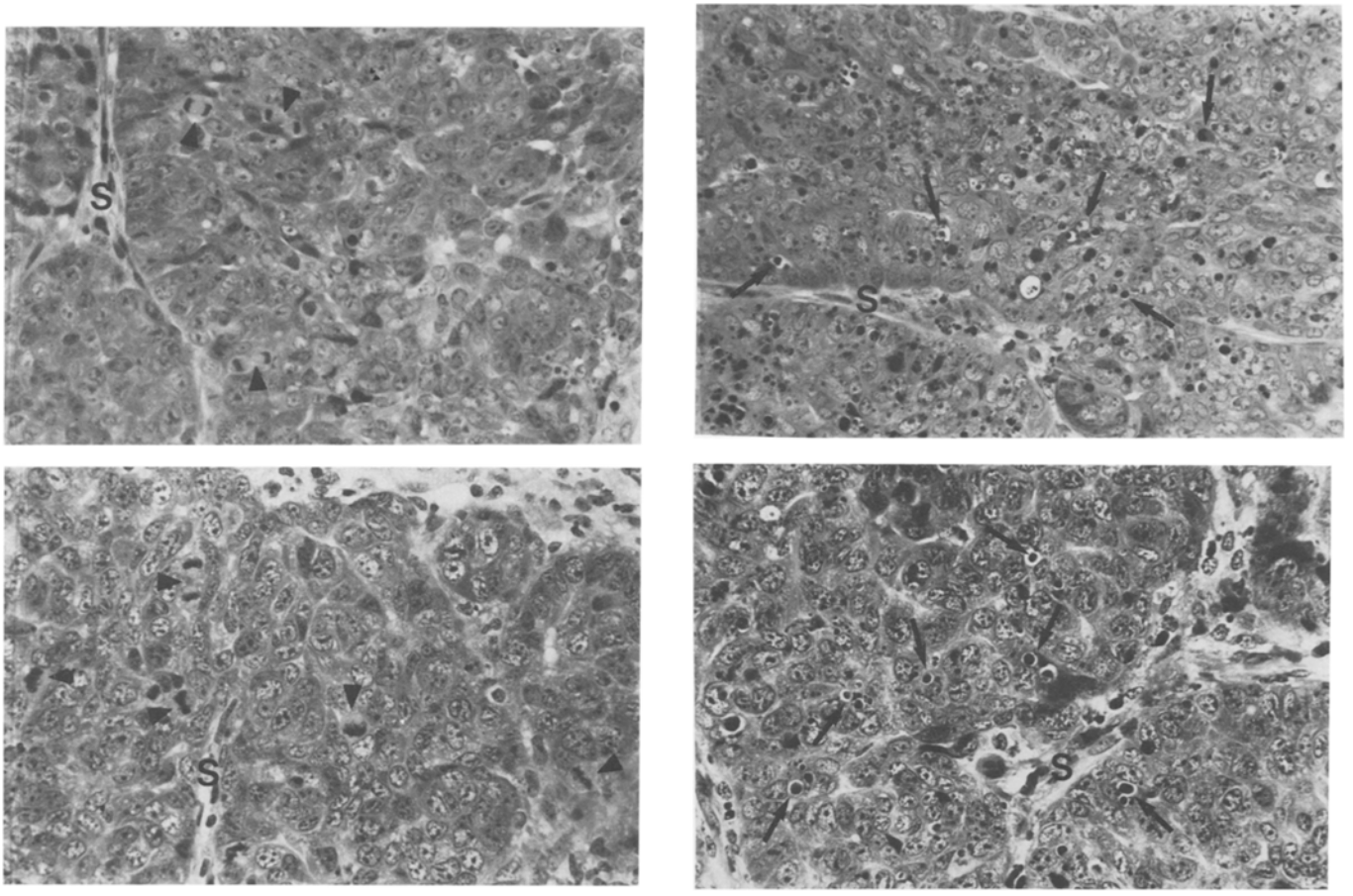

Fig. 2. Photomicrographs of stained histological sections of MCa-4 (*top panels*) and OCa-1 (*bottom panels*) tumors from untreated mice (*left panels*) or mice that received 200 mg/kg 12 h (*right panels*) before being sacrificed. Stroma (*S*) is identified. *Arrows* point to examples of mitotic cells in the *left-hand panels* and apoptotic cells in the *right-hand panels*. HE, 400×

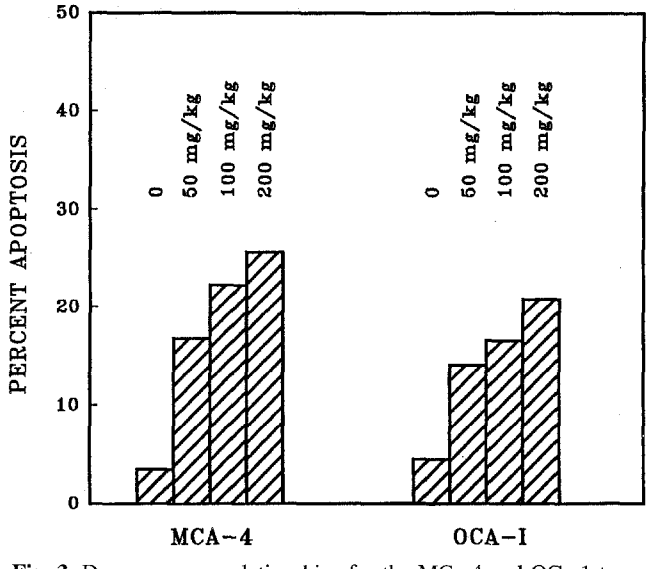

Fig. 3. Dose-response relationships for the MCa-4 and OCa-1 tumors treated with different doses of cyclophosphamide and scored for the percent apoptosis at 12 h

[11, 16, 17]. The endpoint of DNA fragmentation has also been used to characterize apoptosis in numerous studies involving cells treated in vitro (e.g. [2, 7, 11, 14, 18]) and in some in vivo investigations [8, 9]. However, morphometric analysis of the type used here may be of advantage when the goal of the study is to determine the proportions of tumor cells dying by apoptosis in response to the treatment and the tumors under investigation (such as those used here) contain significant stromal cells (Fig. 2).

It has been shown in previous studies that apoptosis can be induced by a number of chemotherapy drugs [2, 5, 7, 13], but these studies were either limited to tissue culture systems or did not quantitate the apoptosis present. A unique value of our study is that it assessed the kinetic parameters of apoptosis induction in tumors treated with CY in situ. The time course of apoptosis induction (Fig. 1) shows that apoptosis begins within a few hours after treatment with CY and that it peaks between 8 and 12 h in MCa-4 tumors and between 12 and 18 h in OCa-I tumors. Thereafter, the percentage of apoptosis declines, returning to the background values at 3 days in MCa-4 and 4 days in OCa-I tumors. This time course of apoptosis is different from that seen after ionizing radiation in the OCa-I tumor [17]. Radiation-induced apoptosis appeared within 1 h following tumor exposure to radiation, peaked at 4 h, and was essentially back to background levels by 24 h. At least two factors could account for this difference.

First, Barry et al [2] recently reported that the time required for development of apoptosis after treatment with chemotherapeutic drugs or hyperthermia may depend on the agent used. Using DNA fragmentation as an indicator, they found that the time for the induction of apoptosis in

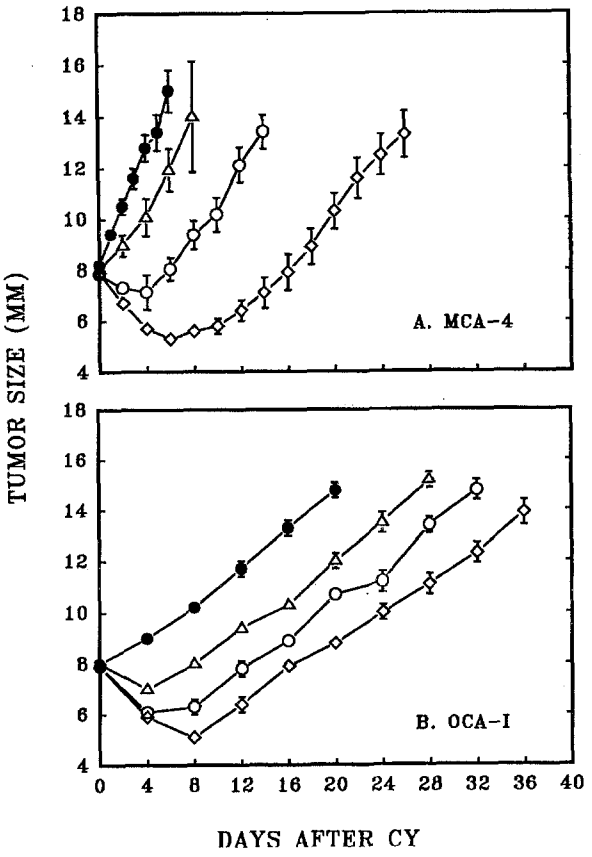

Fig. 4. The kinetics of tumor growth delay for the MCa-4 and OCa-1 tumors treated with different doses of cyclophosphamide. Tumors were treated when 8 mm in diameter. The different symbols represent: 0 dose (*closed circles*); 50 mg/kg, (*open triangles*); 100 mg/kg, (*open circles*); and 200 mg/kg, (*open diamonds*)

vitro was 30 min for hyperthermia, 18 h for methotrexate, and 2–3 days for a number of other chemotherapeutic agents. These times were obtained at the drug concentrations causing 90% cell lethality. Lower doses resulted in a further delay in the appearance of apoptosis. They further suggested that there are two distinct signaling pathways for apoptosis induction [2]. One pathway involves induction of some unknown events in the G2/M phase cells, upon which the affected cells die slowly. The other pathway is cell cycle independent and results in rapid cell death. The dynamics of development of CY-induced apoptosis in OCa-I and MCa-4 tumors suggest that CY could have acted through a combination of both of these pathways. However, the dynamics of radiation-induced apoptosis [17] suggests that radiation acts through the cell-cycle-independent mechanism only.

Second, pharmacokinetic factors are also likely to participate in the observed kinetics of CY-induced apoptosis. In order to be active against tumor cells, CY first needs to be activated in the liver and then reach the tumor, where its access to tumor cells widely depends on anatomical distribution and physiological functioning of tumor microvasculature. Since it may take several hours for activated CY to reach tumor cells, it is logical to anticipate that the peak of apoptosis would be delayed compared with that after irradiation and that the increase in apoptosis would be less steep and its duration longer. In contrast, during tumor irradiation all tumor cells receive radiation within the same, short, time; therefore, the subset of cells that respond by apoptosis probably enter the apoptotic process as a synchronous wave, resulting in the kinetics characteristic to this particular agent.

The CY-induced apoptosis in both OCa-I and MCa-4 tumors was dose dependent in that as the dose of CY was increased the percentage of apoptosis increased. However, the increase in apoptosis at the lowest CY dose used (50 mg/kg) was higher on a per unit dose basis than that at the highest CY dose used (200 mg/kg). This is consistent with a similar pattern seen with radiation [17], where we hypothesized that the dose-response relationship levels off at about 30% apoptosis induced due to a heterogeneity of tumor cell propensity for this mode of cell death. The mechanisms in tumors responsible for this heterogeneity seen in response to both CY and radiation are not well understood at the present time. However, Wyllie [20] has proposed that the pattern of oncogene expression of the tumor cells may be very important.

Macroscopically, both tumors responded to CY with tumor growth delay, which was also more pronounced as the dose of the drug was increased. It seems reasonable to suggest that apoptosis must play a role in the tumor growth delay but the extent of its participation in the overall manifestation of this delay is unclear. The apoptosis is probably responsible for the shrinkage of the tumors that occurred within 2 days after treatment with high doses of CY. At lower doses of CY, tumors did not regress but rather continued to grow after only a slight delay, even though significant apoptosis was induced. It is possible that the effect of apoptosis was offset at the low doses by the proliferation of clonogenic tumor cells that survived CY treatment and that the apoptosis is manifested only at the higher doses, when more clonogenic cells are killed. Obviously, ultimate elucidation of the role of apoptosis in tumor response must await additional understanding of the factors/mechanisms that regulate the propensity of tumor cells for the apoptotic mode of cell death.

References

1. Arends MJ, Wyllie AH (1991) Apoptosis: mechanisms and roles in pathology. Int Rev Exp Pathol 32: 223
2. Barry MA, Behnke CA, Eastman A (1990) Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. Biochem Pharmacol 40: 2353
3. Cohen JJ, Duke RC (1984) Glucocorticoid activation of calcium-dependent endonuclease in thymocyte nuclei leads to cell death. J Immunol 132: 38
4. Duvall E, Wyllie AH (1986) Death and the cell. Immunol Today 7: 115
5. Eastman A (1990) Activation of programmed cell death by anticancer agents: cisplatin as a model system. Cancer Cells 2: 275
6. Falkvoll KH (1991) Quantitative histological changes in a human melanoma xenograft following exposure to single dose irradiation and hyperthermia. Int J Radiat Oncol Biol Phys 21: 989
7. Kaufmann SH (1989) Induction of endonucleolytic DNA cleavage in human actute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anti-cancer drugs: a cautionary note. Cancer Res 49: 5870

8. Kyprianou N, English HF, Isaacs JT (1990) Programmed cell death during regression of PC-82 human prostate cancer following androgen ablation. Cancer Res 50: 3748
9. Kyprianou N, English HF, Davidson NE, Isaacs JT (1991) Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. Cancer Res 51: 162
10. McConkey DJ, Nicotera P, Hartzell P, Bellomo G, Wyllie AH, Orrenius S (1989) Glucocorticoids activate a suicide process in thymocytes through an elevation of cytosolic Ca++ concentration. Arch Biochem Biophys 269: 365
11. Meyn RE, Stephens LC, Ang KK, Hunter N, Milas L, Peters LJ (1993) Heterogeneity in apoptosis development in irradiated murine tumors. Int J Radiat Biol (in press)
12. Milas L, Ito H, Hunter N (1983) Effect of tumor size on S-2-(3-aminopropylamino) ethylphosphorothioic acid and misonidazole alteration of tumor response to cyclophosphamide. Cancer Res 43: 3050
13. Searle J, Lawson TA, Abbott PJ, Harmon B, Kerr JFR (1975) An electron-microscope study of the mode of cell death induced by cancer chemotherapeutic agents in populations of proliferating normal and neoplastic cells. J Pathol (Lond) 116: 129
14. Sellins KS, Cohen JJ (1987) Gene induction by γ-irradiation leads to DNA fragmentation in lymphocytes. J Immunol 139: 3199
15. Stephens LC, Schultheiss TE, Small SM, Ang KK, Peters LJ (1989) Response of parotid organ culture to radiation. Radiat Res 120: 140
16. Stephens LC, Ang KK, Schultheiss TE, Milas L, Meyn RE (1991) Apoptosis in irradiated murine tumors. Radiat Res 127: 308
17. Stephens LC, Hunter NR, Ang KK, Milas L, Meyn RE (1993) Development of apoptosis in irradiated murine tumors as a function of time and dose. Radiat Res 135: 75
18. Story MD, Stephens LC, Tomasovic SP, Meyn RE (1992) A role for calcium in regulating apoptosis in rat thymocytes irradiated in vitro. Int J Radiat Biol 61: 243
19. Wyllie AH (1985) The biology of cell death in tumors. Anticancer Res 5: 131
20. Wyllie AH (1992) Apoptosis and the regulation of cell numbers in normal and neoplastic tissues: an overview. Cancer Metast Rev 11: 95
21. Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68: 251
22. Yamada T, Ohyama H (1988) Radiation-induced interphase death of rat thymocytes is internally programmed (apoptosis). Int J Radiat Biol 53: 65