Apoptosis as a Predictor of Paclitaxel-induced Radiosensitization in Human Tumor Cell Lines

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ABSTRACT

Paclitaxel is a determinate with antitumor activity against a variety of human neoplasms. Paclitaxel cytotoxicity is thought to derive mainly from a stabilization of microtubules as a result of enhanced tubulin polymerization that leads to an accumulation of cells in the mitotic (M) phase of the cell cycle. Because cells in this phase of the cell cycle are known to be radiosensitive, it was thought that paclitaxel, in addition to its direct toxicity, may also sensitize tumor cell populations to radiation. Studies evaluating the radiosensitizing potential of paclitaxel in cultured cells have been equivocal, with only ~50% of the tested cell lines showing radiosensitization. To explain this variability, we advanced the hypothesis that the ability of paclitaxel to radiosensitize cells may be inversely correlated to the efficacy with which it induces apoptosis. To test this hypothesis, we studied paclitaxel-induced apoptosis and radiosensitization in seven human tumor cell lines. Approximately one-half of these cell lines showed radiosensitization that was associated with a low apoptotic index (<20% after a 48-h treatment with 10 or 20 nM paclitaxel). The results suggest that the level of apoptosis, after paclitaxel treatment, may predict for paclitaxel-induced radiosensitization, and that it could be introduced as a parameter for the optimization of combined treatment protocols.

INTRODUCTION

Paclitaxel is being evaluated for clinical use in a variety of tumor types including ovarian cancer, breast cancer, lung cancer, head and neck cancer, testicular carcinoma, and malignant melanoma (1–5). Despite the wide application in the clinic, understanding of the mechanism(s) of action of paclitaxel is incomplete, but clearly, microtubules represent a critical target. Unlike other antimicrotubule drugs, such as Vinca alkaloids, which induce the disassembly of microtubules, paclitaxel promotes the polymerization of tubulin and interferes with microtubule depolymerization, an essential step in cell division and other cellular functions. (6–13). The binding of paclitaxel to tubulin is strong and stoichiometric and targets the NH2-terminal 31 amino acids of the β-tubulin subunit (14, 15). It leads to polymerization, even in the absence of GTP or microtubule-associated proteins, and occurs at temperatures that normally inhibit in vitro polymerization (11, 16). The microtubules formed in the presence of paclitaxel are extraordinarily stable and dysfunctional and probably cause cell death by disrupting the normal microtubule dynamics required for cell division. This leads to arrest in the mitotic phase of the cell cycle (12) but also causes disruption of other vital interphase processes.

Excellent responses in tumor growth after treatment with paclitaxel do not necessarily translate into cures. Therefore, combined treatment with radiation and other antitumor agents is increasingly considered. When multiple agents are rationally combined to treat tumors, the resulting response is expected to be either additive or synergistic. Of particular importance in the clinic is the combination of agents that act synergistically. Because cells at mitosis are known to be exquisitely radiosensitive (17, 18), it was theorized that paclitaxel may be a potent radiosensitizing agent by virtue of its ability to arrest cells in this phase of the cell cycle (6) and, therefore, to act synergistically when combined with radiation. However, laboratory studies have shown evidence for both additive and synergistic cytotoxicity for combined treatment with paclitaxel followed by exposure to radiation. For example, paclitaxel has been shown to cause the expected radiosensitization in squamous carcinoma cells lines of the head and neck (19), in the astrocytoma cell line G18 (20, 21), in a series of glioma cell lines (22), the leukemia cell line HL60 (23), the breast carcinoma cell line MCF-7 (24), in a series of ovarian cancer cell lines (24–26), and in some prostate carcinoma cell lines (27). Other reports, however, show only additive or subadditive toxicity for combined paclitaxel-radiation treatments (21, 24, 28–32), despite the fact that paclitaxel alone generally induced the expected arrest at mitosis. The reasons for these differences in response are not completely understood, but it has been suggested that if cells arrested at mitosis are doomed to die from the cytotoxic action of paclitaxel alone, radiosensitization may not ensue (29). Because the combination of paclitaxel with radiation in the clinic will benefit from knowledge of the type of interaction between the two treatment modalities, we searched for parameters that could predict the mode of this interaction. Here, we report that the level of apoptosis after treatment of cells with paclitaxel alone may predict for additive or synergistic effect in combined treatment applications.
MATERIALS AND METHODS

Cell Lines and Cell Culture Conditions and Treatments. Seven human tumor cell lines were used for experiments. They were of diverse origin including cervical carcinoma (HeLa), human lung carcinoma (A549), human colon carcinoma (HT29), human astrocytoma (G18), and human prostate carcinoma (PC3, DU-145, and LNCaP). All cell lines were maintained at 37°C in a humidified incubator in an atmosphere of 5% CO2 and 95% air. HeLa cells were grown in S-MEM medium supplemented with 5% bovine calf serum (Intergene), A549 cells in McCoy’s 5A medium supplemented with 5% bovine calf serum (Intergene), HT29 cells in McCoy’s 5A medium supplemented with 10% fetal bovine serum (Sigma Chemical Co.), G18 cells in MEM supplemented with 15% fetal bovine serum (Sigma), and PC3, DU-145, and LNCaP cells in MEM supplemented with 10% fetal bovine serum (Sigma). Growth media were supplemented with penicillin-streptomycin. For experiments, cells were plated in 60-mm tissue culture dishes with 5 ml of culture medium and allowed to grow for 48 h before paclitaxel treatment.

Cell survival after exposure to paclitaxel and/or radiation was determined by measuring colony-forming ability. For this purpose, cells were treated as required by the experimental protocol, trypsinized, and plated, aiming to 20–200 colonies per 100-mm dish. Induction of apoptosis after exposure of cells to paclitaxel was measured by morphological evaluation after a 48-h treatment at the indicated drug concentrations. Cells were collected by trypsinization, making sure to include detached cells, and were fixed in 70% ethanol. Before analysis, they were resuspended in 1 ml of DAPI3 solution (0.1 M Tris, 0.1 M NaCl, 5 mM MgCl2, 0.05% Triton X-100, and 2 µg/ml DAPI; 5 × 105 cells/ml) and scored under a fluorescence microscope. Two hundred nuclei were scored from each of the samples, and results were expressed as a percentage of cells undergoing apoptosis, as determined by pyknotic and fragmented nuclei. The distribution of cells through the cycle after treatment with paclitaxel was determined by flow cytometry. Cells were collected as described above, resuspended in DAPI solution, and analyzed using an Epics Elite flow cytometer (Coulter). The percentage of cells in the various phases of the cell cycle was calculated using the Multicycle Analysis program (Multicycle-San Diego).

Paclitaxel (concentration 6 mg/ml) was obtained from Bristol Myers Squibb (Princeton, NJ) and stored in a sterile container at a refrigerated temperature of 4°C. From this stock, appropriate dilutions into culture medium were made immediately prior to use. Radiation exposures were carried out using an X-ray machine (Pantak) operating at 320 kV, 10 mA, with a 2-mm aluminum filtration. The effective photon energy was ~90 keV.

Western Blot Analysis and DNA Binding Assays. The status of p53, in the cell lines used in the experiments described here, was confirmed by Western blot analysis and DNA binding assays. For this purpose, cells grown under conditions similar to those used in the toxicity experiments were exposed to 5 Gy of X-rays and returned to 37°C for 2 h. Subsequently, cells were trypsinized, collected, and washed twice in PBS. They were resuspended in 1 ml of buffer A [8 µM zinc acetate, 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, 10 mM NaPO4 (pH 7.0), 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 2 µg/ml pepstatin A, 1 µM wortmannin, 0.1 µM staurosporine, 0.1 µM Na3VO4, and 15 mM NaF], incubated in ice for 10 min, spun, and resuspended in 0.25 ml of buffer A. Cells were disrupted by two cycles of freeze-thaw and spun at 10,000 rpm for 1 min. The supernatant was carefully aspirated and kept as the cytoplasmic fraction. The pellet was resuspended in 1 ml of buffer A, spun at 14,000 rpm for 2 min, and the supernatant was discarded. The pellet was resuspended in 0.15 ml buffer B [8 µM zinc acetate, 20 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.3 M KCl, 0.2 mM EDTA (pH 8.0), 25% glycerol, 10 mM NaPO4 (pH 7.0), 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride, 2 µg/ml pepstatin A, 1 µM wortmannin, 0.1 µM staurosporine, 0.1 µM Na3VO4, and 15 mM NaF], incubated for 1 h at 4°C, and spun at 14,000 rpm for 5 min, and the supernatant (nuclear extract) was collected. Protein concentration was determined in cytoplasmic and nuclear extracts by the Bradford assay (Bio-Rad). For Western blots, 6 µg of nuclear extract were loaded onto an 12% SDS-polyacrylamide mini-gel (Bio-Rad) and run at 180 V for 50 min. Proteins were then transferred to a polyvinylidene difluoride-plus transfer membrane and incubated for 1 h at room temperature in PBS containing 0.1% Tween 100 and 5% nonfat milk. For detection, an anti-p53 monoclonal antibody (Ab-6; Calbiochem) was used at a concentration of 0.1 µg/ml. After extensive washing, membranes were incubated with a peroxidase-conjugated rabbit anti-mouse antibody for 1 h, and signal was detected by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham). For DNA binding assays, nuclear extracts were assayed in a reaction containing the Ep21 consensus binding site (CCC-GAACATGTCCT-CACA-TGTGG-GGG) as well as nonspecific single- and double-stranded oligonucleotides (33). Each strand of Ep21 was labeled separately with T4 polynucleotide kinase (Life Technologies, Inc.) in the presence of [γ-32P]ATP (10 µCi/µl; Amersham). Binding reactions were performed at room temperature for 20 min in a volume of 15 µl containing 10 mM Tris (pH 8.0), 1 mM DTT, 5% glycerol, 50 mM NaCl, 0.1 mM zinc acetate, 0.1 µg of single-stranded nonspecific oligonucleotide, 0.67 µg of double-stranded, nonspecific oligonucleotide, 3–5 × 105 cpm of labeled probe, 2 µg of nuclear extracts, and 100 ng of Ab-6 antibody. All reaction mixtures were electrophoresed in a 5% Tris/Glycine polyacrylamide gel, which was prerun at 115 V for 1.5 h. The gel was fixed for 1 h in 30% MeOH/10% AcOH and was then washed in distilled H2O for 15 min. Subsequently, the gel was dried and autoradiographed.

RESULTS

To facilitate comparison of the results obtained with different cell lines, we carefully standardized the conditions of treatment. Preliminary studies had shown a strong dependence of paclitaxel effects on cell density. Therefore, we conducted experiments to define the levels of paclitaxel-induced cytotoxicity in a range of cell concentrations. We uniformly observed a strong dependence of paclitaxel cytotoxicity on cell concentra-

3 The abbreviation used is: DAPI, 4’,6-diamidino-2-phenylindole.
tion, with the toxicity rapidly decreasing as the density of cells in the culture increased (results not shown). This phenomenon could be explained only in part by a gradual reduction in the rate of cell progression through the cycle with increasing cell concentration, suggesting that other effects also contribute to this response. To minimize such effects, all cell lines were treated with paclitaxel at a density of $1.0 \pm 0.3 \times 10^6$ cells/60-mm tissue culture dish. The experiments were designed to characterize, first, paclitaxel toxicity in terms of cell cycle arrest, growth inhibition, cell killing, and induction of apoptosis, and subsequently, use the information to interpret the results of paclitaxel-induced radiosensitization. All cell lines included in the study were tested along these lines in a family of practically identical experiments. Here, we show detailed results obtained with two representative cell lines and summarize results of the remaining cell lines with the purpose of evaluating whether paclitaxel-induced radiosensitization can be predicted from the levels of apoptosis observed after treatment with the drug alone.

Fig. 1 summarizes the results of treatment of HeLa cells with paclitaxel (†). Growth inhibition was calculated by dividing the number of cells at the end of the treatment by the number of cells at the beginning of the treatment. ▼, percentage of cells in G2-M phase as measured by flow cytometry after a 24-h incubation with the indicated concentrations of paclitaxel. ●, percentage of cells at mitosis, as measured by staining with DAPI and observation under the microscope, in cells treated for 24 h with different concentrations of paclitaxel. ○, percentage of cells with apoptotic morphology, measured by staining with DAPI and observation under the microscope, in cells treated for 24 h with various concentrations of paclitaxel. △, as in A, but for cells treated for 48 h with different concentrations of paclitaxel. Flow cytometry data are not shown. C, survival of cells treated for 24 h with different concentrations of paclitaxel as measured by colony formation. D, radiation sensitivity of HeLa cells treated for 24 h with 0 nM (●), 5 nM (○), 10 nM (▼), and 20 nM (▲) paclitaxel, exposed to graded doses of radiation and plated for colony formation immediately thereafter. The results obtained are shown normalized to the survival levels measured with cells treated with paclitaxel alone. Bars, SE.

We observed a 50% reduction in growth at 7 nM and an essentially complete inhibition at concentrations above 15 nM. Parallel flow cytometry indicated that the drastic reduction in growth was accompanied by an accumulation of cells in the G2-M phase, such that nearly 90% of cells were found in this stage of the cell cycle 24 h after treatment at concentrations >10 nM. Direct microscopic examination of the same cell populations indicates that arrested cells are in their majority at mitosis, rather than in G2, as expected by the mechanism of paclitaxel action (see “Introduction”). Apoptosis induction is, at this time, relatively low and does not exceed the 10% level at all concentrations examined. Thus, HeLa cells exposed to paclitaxel for 24 h show a strong inhibition in growth, an arrest at mitosis, and little evidence for apoptosis.

The results in Fig. 1B demonstrate that cell growth remains strongly inhibited in cells exposed to different paclitaxel concentrations for 48 h. At the same time, the fraction of apoptotic cells increases dramatically and reaches 80% at concentrations >10 nM. Concomitant with this increase in the apoptotic fraction, a decrease in the mitotic index occurs. Flow cytometry is
not informative at this stage, because extensive cell destruction by apoptosis complicates cell cycle analysis and is, therefore, not included in the results shown. Preliminary experiments showed that induction of apoptosis is at a maximum 48 h after treatment of HeLa cells with paclitaxel, and similar results were obtained with all cell lines included in the present study.

The effect of a 24-h exposure to paclitaxel on cell survival as measured by colony formation is shown in Fig. 1C. Paclitaxel effectively kills HeLa cells up to concentrations of ~10 nM, but cell killing reaches a plateau at higher concentrations, suggesting the presence of drug-resistant subpopulations. From the survival levels at this range of concentrations, it can be estimated that the drug-resistant subpopulation may not exceed 2–3% and may comprise cells that are not actively growing. The effect of pretreatment with paclitaxel on HeLa cell radiosensitivity to killing is shown in Fig. 1D. Cells were exposed first, for 24 h, to the indicated concentrations of paclitaxel and subsequently to graded doses of X-rays. The results shown have been normalized for paclitaxel-induced cell killing to allow direct evaluation of radiosensitization. It is evident that within the experimental uncertainties, cells surviving treatment with paclitaxel have a radiation sensitivity comparable with that of untreated cells. Because >85% of the cells arrest at mitosis after exposure to paclitaxel (Fig. 1A), we expected a significant radiosensitization, provided that the arrested cells were not destined to die, by apoptosis or other mechanisms. The high incidence of apoptosis in HeLa cells, combined with the lack of radiosensitization, suggests that the majority of cells arrested at mitosis as a result of paclitaxel treatment are destined to die. Thus, high incidence of apoptosis after treatment with paclitaxel may predict for reduced levels of radiosensitization or no radiosensitization at all.

Experiments carried out with the PC3 human prostate carcinoma cell line provided evidence for the other extreme, i.e., that low levels of apoptosis after exposure to paclitaxel may predict for paclitaxel-induced radiosensitization. The results are summarized in Fig. 2. Similar to results with HeLa cells, exposure of PC3 cells to paclitaxel for 24 h inhibits cell growth, albeit to a slightly lower degree (Fig. 2A). As expected, this inhibition is associated with an accumulation of cells in G2-M phase. In parallel with the accumulation of cells in G2-M, an increase is observed in the fraction of mitotic cells. However, in contrast to observations with HeLa cells where accumulation of cells in G2-M and at mitosis closely follows each other, in PC3 cells there is a lag in the accumulation of cells at mitosis, such that at any concentration there is ~20% fewer cells at mitosis than in G2-M. This is equivalent to an arrest of cells in the G2 phase of the cell cycle and may be important in the response of these cells either to paclitaxel alone or to the combined treatment with paclitaxel and radiation. There is no detectable apoptosis in PC3 cells exposed to different amounts of paclitaxel for 24 h (Fig. 2A). After exposure to paclitaxel for 48 h, a reduction is observed in the fraction of cells arrested at mitosis (Fig. 2B) and a modest increase in the apoptotic cells. It is notable, however, that apoptosis never exceeds 10% at all concentrations examined. Fig. 2C shows the survival of PC3 cells

**Fig. 2** Effects of paclitaxel on different end points in PC-3 cells. Other details as in Fig. 1. In D, cells were treated with 0 nM (○), 5 nM (△), 10 nM (▽), and 20 nM (▼) paclitaxel. Bars, SE.
exposed to different concentrations of paclitaxel for either 24 h or 48 h. The overall shape of the survival curve is similar to that of HeLa cells (see Fig. 1C), suggesting that similar mechanisms are active, but PC3 cells are slightly more resistant to paclitaxel as compared with HeLa cells. The results of combined treatment with paclitaxel and radiation are shown in Fig. 2D. It is evident that exposure of cells to 5, 10, and 20 nM paclitaxel leads to significant levels of radiosensitization in support of the hypothesis that reduced apoptosis induction after treatment with paclitaxel predicts for radiosensitization.

To test the generality of the above observations and to investigate whether apoptosis is a useful indicator of paclitaxel-induced radiosensitization, we compared the level of paclitaxel-induced radiosensitization with the level of paclitaxel-induced apoptosis in the seven cell lines included in the present study. Apoptosis was measured in cells exposed to paclitaxel, at the indicated concentrations, for 48 h and is presented as the percentage of cells showing nuclear morphology characteristic of apoptosis. Two formulas were used to calculate paclitaxel-induced radiosensitization. In the first, radiosensitization was calculated at a fixed dose of radiation (2 or 3 Gy), by dividing the surviving fraction observed in untreated cells with that of cells exposed to the indicated concentrations of paclitaxel. Numbers higher than 1 indicate radiosensitization. In the second, radiosensitization was calculated at a given survival level for the untreated cells by dividing that survival value (e.g., 10%) by the survival value obtained with paclitaxel-treated cells at the same dose. Here again, numbers larger than 1 indicate radiosensitization. Numbers higher than 1 indicate radiosensitization. In the second, radiosensitization was calculated at a given survival level for the untreated cells by dividing that survival value (e.g., 10%) by the survival value obtained with paclitaxel-treated cells at the same dose. Here again, numbers larger than 1 indicate radiosensitization. Similar results were obtained at 2 Gy, as well as when the apoptotic index was measured after treatment with 20 nM paclitaxel. A hypothetical model for expected results after combined administration of paclitaxel with radiation. See text for more details.
as the level of apoptosis decreases, paclitaxel-induced radiosensitization increases. The paclitaxel enhancement factor ranges between 1 and 15, depending upon the cell line and the type of calculation for radiosensitization adopted. It is notable that radiosensitization is only observed with cell lines showing levels of apoptosis, 20% under the conditions used. Similar results were also obtained when radiosensitization was measured after treatment with 20 nM paclitaxel. Thus, apoptosis at levels, 20% may be predictive for paclitaxel-induced radiosensitization.

The status of the p53 protein, mutant versus wild-type, may contribute to the ability of cells to undergo apoptosis after treatment with paclitaxel. We therefore examined whether a correlation can be established between p53 status and paclitaxel-induced radiosensitization. As a first step toward this goal, we determined p53 status in the cell lines used in the present study by measuring induction of the protein and induction of its DNA binding activity after radiation exposure. The results obtained are shown in Fig. 4. Fig. 4A shows a Western blot using anti-p53 antibody DO-1, whereas Panel B shows binding of p53 to the p21 consensus DNA sequence. There is no evidence for p53 in HeLa or PC-3 cells before or after irradiation. There is a detectable amount of p53 that does not change after irradiation, as expected from mutant protein, in HT29, G18, and DU-145 cells. p53 is barely detectable before irradiation but clearly detectable after irradiation in A549 and LNCaP cells, as expected from the wild-type form of the protein. For the most part, the Western blot results are confirmed by the DNA binding results. There is a clear induction of DNA binding activity after irradiation in A549 and LNCaP cells. There is no evidence for DNA binding activity before or after irradiation in HeLa, HT29, PC3, and G18 cells, confirming the mutant character of the protein. Surprisingly, some DNA binding activity is observed in DU-145 cells, despite the fact the levels of the protein remained unchanged after irradiation. Because DU-145 cells have been reported to have a mutant form of p53, we conclude that the mutation only partly compromises the DNA binding activity of the protein. These results indicate that it is not possible to draw unequivocal correlations between p53 status and paclitaxel-induced radiosensitization.

DISCUSSION

The results summarized above, as well as results published previously by other investigators (see “Introduction”), suggest that treatment with paclitaxel sensitizes to radiation tumor cell populations, but that the magnitude of the effect is unpredictable and varies from no radiosensitization to significant radiosensitization. It has been suggested that radiosensitization by paclitaxel requires progression of cells through the cycle and accumulation in the G2-M phase (20, 21, 23–25). All cell lines used in the present study showed an accumulation in the G2-M phase after treatment with paclitaxel, and yet radiosensitization was observed only with some cell lines. We conclude, therefore, that accumulation of cells by paclitaxel in a radiation-sensitive phase of the cell cycle does not automatically confer increased sensitivity to radiation. Radiosensitization, as a result of cell redistribution in the cycle, requires that paclitaxel causes an accumulation of cells in G2-M phase after treatment with paclitaxel, and yet radiosensitization was observed only with some cell lines. We conclude, therefore, that accumulation of cells by paclitaxel in a radiation-sensitive phase of the cell cycle does not automatically confer increased sensitivity to radiation. Radiosensitization, as a result of cell redistribution in the cycle, requires that paclitaxel causes an accumulation of cells in G2-M phase, but also that cells blocked in G2-M are not committed to die. If cells blocked in G2-M after treatment with paclitaxel are committed to die, no radiosensitization can be expected because a cell can be killed only once.

Along these lines, Geard and Jones (28) showed that in a cervical carcinoma cell line showing no radiosensitization in response to treatment with paclitaxel, all cells reaching mitosis are not clonogenic. This is in agreement with results showing that HeLa cells blocked in mitosis by paclitaxel are unable to resume normal proliferation upon removal of the drug and develop a morphology characteristic of apoptosis (34). In the present study, we developed these observations further and examined whether the radiosensitizing potential of paclitaxel correlates with the levels of apoptosis induced after treatment with the drug alone. We inquired whether cell lines efficiently entering apoptosis upon arrest in G2-M by paclitaxel show no radiosensitization, whereas cell lines able to overcome (at least partly) the cell cycle arrest without undergoing apoptosis show the radiosensitization expected as a result of the cell cycle
redistribution in G2-M. Indeed, there is evidence that blockage at mitosis by paclitaxel does not necessarily lead to cell death (35).

The pooled results of seven cell lines suggest that reduced levels of apoptosis after paclitaxel treatment increase the probability of radiosensitization by increasing the probability that cells arrested at mitosis will survive the treatment. It is worth noting that clear radiosensitization was observed only in cell lines showing <20% apoptosis after a 48-h exposure to paclitaxel. The emergence of surviving cells under conditions of low apoptosis suggests that cell cycle deregulation rather than direct damage to cellular structures induces apoptosis in cells treated with paclitaxel. As a result, the probability that a cell will survive treatment may increase when the apoptotic pathways are inhibited, either as a result of genetic alterations, or by other means. These considerations may explain the divergence observed in paclitaxel-induced radiosensitization among the different tumor cell lines (20, 21, 29, 31, 36).

The role of p53 on paclitaxel-induced apoptosis has been studied by a number of investigators (37–42). Although radiation, DNA intercalating agents, antimetabolites, and alkylating agents efficiently induced apoptosis in cells derived from wild-type p53 animals and failed to do so in cells derived from p53-deficient animals (43), paclitaxel-induced apoptosis was frequently independent of p53 status, despite the fact that treated cells showed up-regulation of p21 (38, 41, 42, 44, 45). Furthermore, the presence of wild-type p53 did not change the sensitivity to paclitaxel in a group of human ovarian cancer cell lines, despite the fact that the levels of the protein increased and downstream genes such as p21 were induced (41). It is likely that p53-independent pathways contribute to paclitaxel-induced apoptosis. Indeed, induction of apoptosis in cells treated with paclitaxel correlates with the phosphorylation and inactivation of Bcl-2 and Bcl-xL (46–49), a modification that at least in some cells requires the activity of Raf-1 (50, 51). The lack of correlation between p53 status and paclitaxel-induced apoptosis explains why we did not observe a clear correlation between paclitaxel-induced radiosensitization and p53 status.

The results presented here generate a rational background for the use of apoptotic index as a parameter for optimization of protocols combining paclitaxel with radiation for the treatment of human tumors. Tumors showing a low level of apoptosis are expected to benefit considerably from radiation given concurrently with paclitaxel. Because tumors showing low levels of apoptosis are likely to be resistant to paclitaxel, the combination with radiation should increase the probability of local control. Because these tumors may also be radiosensitized by paclitaxel (see “Results”), the administered dose of radiation will be more effective, as required for sterilizing a high percentage of tumor cells surviving paclitaxel. For responsive tumors, the dose of radiation may be decreased without jeopardizing local control, with the purpose of reducing radiation injury to normal tissue. Tumors with high levels of apoptosis are more likely to be chemoresistant (52) and therefore responsive to paclitaxel. The combination with radiation will be expected to enhance the overall response, but only in an additive fashion. It is thus possible that evaluation of the apoptotic potential of a tumor after treatment with paclitaxel will provide a powerful parameter for customizing the treatment of individual tumors.

Our in vitro observations and conclusions are supported by studies on the response of experimental tumors in animals after combined treatment with paclitaxel and radiation. In a comprehensive set of studies, Milas et al. (39, 53–56) demonstrated that combination of paclitaxel with radiation can lead to improved therapeutic gain. These studies provide strong rationale for the combination of these agents in the treatment of human tumors. The same studies further indicate that the rationale developed from our in vitro studies holds only for tumors with a small fraction of hypoxic cells. In tumors with a sizable fraction of hypoxic cells, paclitaxel may radiosensitize by yet another mechanism. It has been observed that hypoxic tumors showing high levels of apoptosis after treatment with paclitaxel will be sensitized to radiation as a result of reoxygenation that ensues a massive cell loss (53–55).

The combination of findings from the in vivo studies outlined above and our results suggests that paclitaxel and radiation will interact in a synergistic manner in tumor cells showing low levels of apoptosis (independently of oxygenation status), mainly by piling-up surviving cells in radiosensitive phases of the cell cycle. In tumors where the level of apoptosis is high, synergistic interaction will be limited to hypoxic tumors and will be mainly the result of reoxygenation. These predictions are summarized in the hypothetical scheme shown in Fig. 3C. It will be intriguing to test these predictions in the clinical setting.

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