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ORIGINAL ARTICLE

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Comparison of apoptotic, necrotic and clonogenic cell death and inhibition of cell growth following camptothecin and X-radiation treatment in a human melanoma and a human fibroblast cell line

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Abstract *Purpose:* We evaluated apoptotic, necrotic and clonogenic cell death and inhibition of cell growth in a human melanoma cell line (Sk-Mel-3) and a normal human fibroblast cell line (AG1522) following treatment with camptothecin (CPT) or with concurrent CPT and X-radiation. *Materials and methods:* Apoptotic and necrotic cell death was determined morphologically by dual-staining (propidium iodide, acridine orange). Inhibition of cell growth was determined from the number of cells remaining in the culture dish following treatment. *Results:* In Sk-Mel-3 cells: (a) after treatment with CPT alone, both apoptotic and necrotic cell death increased significantly ($P < 0.05$) relative to untreated controls; (b) after concurrent CPT and radiation treatment, however, only the increase in necrotic cell death was significant ($P < 0.05$) relative to cells receiving radiation alone; and (c) all assays of cellular effects/cytotoxicities were consistent in showing that CPT, given alone or with radiation, led to a substantial increase in cell kill. In contrast, in AG1522 cells: (a) there were no significant increases in apoptotic or necrotic cell death following either CPT alone or concurrent CPT and radiation; and (b) the clonogenic assay measured substantially higher cytotoxicities than the other assays. *Conclusions:* Necrotic cell death was more important than apoptotic cell death during concurrent CPT and radiation treatment in Sk-Mel-3 cells, but not in AG1522 cells.

Keywords Apoptosis · Necrosis · Clonogenic cell death · Camptothecin · X-radiation

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Abbreviations *CPT:* camptothecin · *MM:* metastatic melanoma · *mt:* mutant · *wt:* wild type

Introduction

There are several pertinent issues to consider when treating human melanoma. First, melanomas have a wide range of resistance to radiation. Second, melanomas have a propensity to metastasize quickly. Third, metastatic melanoma (MM) is characteristically unresponsive to conventional chemotherapy [32]. Recently, numerous clinical trials have demonstrated that chemoradiation treatment (in particular, concurrent chemotherapeutic and radiation treatment) given to tumor at various sites is associated with significantly increased patient survival relative to treatment with either drug or radiation alone [9, 25, 31]. Chemoradiation treatment has two principal objectives: (a) enhancement of local tumor control through the interaction of the two cytotoxic modalities, and (b) reduction of treatment failure caused by distant metastases through the action of the drug on sites that are not irradiated. Thus chemoradiation treatment can potentially enhance the response of nonresectable primary melanomas to treatment and reduce the incidence of metastases resulting from local therapeutic failure. Chemoradiation may also have a role in the treatment of various MM (dermal, subcutaneous, lymph node, brain).

The reason for the radioresistance of some melanomas is unknown. The melanin content of the cells may be important, but this issue is controversial [16, 36]. The reason why MM is refractory to chemotherapeutic agents is also unknown. However, there is recent evidence that melanoma cells may be resistant because of a reduced ability to undergo apoptosis following treatment [19, 20, 32]. It is well established that two important forms of cell death, apoptosis and necrosis, occur in response to treatment with drugs and radiation [4, 10, 11]. Apoptosis is characterized by distinct cellular changes such as chromatin condensation, membrane blebbing,

cell shrinkage, and, frequently, formation of DNA ladders [4]. Apoptosis typically occurs at protracted times (can be up to several days later) following the inducing event and apoptotic cells are frequently found in the floating fraction of cells growing in culture following treatment with drugs and radiation [10]. Conversely, necrotic cell death is associated with an absence of the cellular characteristics described for apoptosis (i.e. no chromatin condensation, detection of DNA smearing rather than laddering, cell swells rather than shrinks, cell dies soon after necrosis-inducing event) [4].

We and others [5, 8, 26, 38] have previously shown that camptothecin (CPT) is a useful adjunct to radiation. CPT is a compound derived from the oriental plant *Camptotheca acuminata* (tree of joy) and is a specific inhibitor of DNA topoisomerase (topo) I. CPT analogs are already being used in cancer therapy. We have reported that CPT and radiation interact synergistically in plateau-phase cultures (which model non-actively proliferating cells) of radioresistant human melanoma cells [27]. Conversely, CPT and radiation interact additively in exponential-phase cultures (which model actively proliferating cells) of these cells [30]. In this study, we evaluated the relevance of the different forms of cell death in these radioresistant human melanoma cells following treatment with CPT alone or with concurrent CPT and radiation. This was done to test the hypothesis that apoptotic and necrotic cell death are important following combined treatment with CPT and radiation.

Additionally, we extended our studies to a normal human fibroblast cell line. Although the normal counterpart of melanoma cells is the melanocyte, there is little evidence that its response is germane to late radiation effects. Late effects of radiation (fibrosis, tissue necrosis), which often limit radiation dosage, are correlated with the response of fibroblasts [6, 7, 15]. Thus the response of this cell type to combined CPT and radiation is potentially relevant from a clinical perspective.

Materials and methods

Cell lines and culture

Sk-Mel-3 is a very radioresistant, human melanoma cell line originally established from a lymph node metastasis (ATCC) [27]. AG1522 is a human skin fibroblast cell line that is non-tumorigenic, is contact-inhibited and has been previously described [28]. Cells were cultured in a 1:1 mixture of Dulbecco's modified essential medium/F12 Nutrient Mixture (Sigma Chemical Co., St Louis, Mo.) supplemented with 10% (v/v) fetal bovine serum (Gibco Life Technologies, Mississauga, ON, Canada), 0.1 m*M* non-essential amino acids (Sigma), 20 m*M* Hepes (Boehringer Mannheim) and 10 m*M* $NaHCO_3$ (Sigma) in a humidified atmosphere of air containing 2% CO_2 at 37°C. Cultures in the exponential phase of growth were obtained by seeding 2×10^5 viable cells in 25-cm^2 flasks on day 0. Cells were used for experiments on day 3.

Drug exposures and irradiation

CPT (Sigma) was dissolved in DMSO and appropriate drug concentrations were added to the culture medium covering the cells as previously described [27]. All CPT exposures were for 1 h at 37°C. For concurrent CPT and X-radiation treatments, CPT was added and cells were irradiated at room temperature on a 250 kVp X-ray system (Pantak, Ct.) at a dose rate of 150 cGy/min. Flasks were immediately returned to the incubator at 37°C for the remainder of the hour-long CPT exposure.

Colony-forming assay

Immediately following treatment with CPT or CPT and radiation, cells were rinsed twice with isotonic citrate saline, trypsinized (0.2% trypsin/2.5 m*M* EDTA for 5 min at 37°C) and counted with an electronic particle counter. Cell suspensions were plated to yield about 50 colonies per 60-mm dish after 14 days in a humidified atmosphere of air containing 2% CO_2. Dishes were stained with methylene blue and colonies containing more than 50 cells were scored to assess relative colony formation. Radiation survival curves were fitted with the linear quadratic model $S = exp(-\alpha D - \beta D^2)$. Both radiation and CPT curves were fitted using Sigma Plot software (SPSS).

Inhibition of cell growth/attachment assay

Cells were treated with DMSO (i.e. control), 2 μ*M* CPT, or 25 μ*M* CPT. The medium was changed at the end of the exposure. Cells were returned to the incubator and kept at 37°C in air containing 2% CO_2 for various times up to 96 h. At the appropriate times, medium was collected and floating cells counted on an Elzone 80 cell counter (Particle Data, Elmhurst, Ill.). Attached cells were trypsinized and counted following a wash with phosphate-buffered saline (PBS).

Determination of apoptotic, necrotic and live cells

For the treatment with CPT alone, cells were treated with DMSO (i.e. control), 2 μ*M* CPT, or 25 μ*M* CPT. At the end of the exposure, the medium was changed (after rinsing twice with non-drug/DMSO-containing medium). The cells were incubated at 37°C in air containing 2% CO_2 for various times up to 120 h. For concurrent CPT and X-radiation treatments, cells were irradiated in the presence of CPT (as described above) and returned to the incubator for 48 h. At this time, the cells were rinsed with PBS and trypsinized (0.2% w/v, 2.5 m*M* EDTA, 5 min, 37°C). Both detached and attached cells were pooled for determination of apoptotic, necrotic and live cells.

The relative percentages of apoptotic, necrotic and live cells were determined using a well-established assay [35]. Briefly, cells were simultaneously stained with 5 μg/ml propidium iodide (PI) and 50 μg/ml acridine orange (AO) in PBS. The morphologies of the cell nuclei were viewed under a fluorescent microscope. Apoptotic and live cells excluded PI because of their intact membranes and were stained green by AO. However, apoptotic cells were clearly distinguishable by their condensed or fragmented chromatin and cell membrane blebbing. Conversely, necrotic cells were stained red by PI because of the compromised integrity of their cellular membranes. At least 200 cells were counted in each field.

Statistics

All experiments were repeated at least three times. Within each experiment, either duplicate or triplicate plates were scored for each dose for the clonogenic assays. The results are presented as means ± SEM. For the determination of increase in apoptotic or necrotic cells following treatment, comparisons were made against the untreated (for CPT-alone treatments) or irradiated-alone (for concurrent CPT and X-radiation treatments) controls using the one-tailed Student's *t*-test. For all statistical tests, *P*-values < 0.05 were considered significant.

Results

Relative colony formation

Sk-Mel-3 cells were significantly more resistant to CPT than AG1522 cells (Fig. 1). Although the difference in relative colony formation (RCF) was small at a dose of 2 μ*M* CPT, the difference was about threefold at a dose of 25 μ*M* CPT. The presence of sensitive subpopulations in these two cell lines was clearly shown by the shapes of the curves. The sensitive subpopulation, which comprised about 40% of Sk-Mel-3 cells, agrees well with our previous finding that S-phase cells form about 35% of exponential-phase cultures of this cell line [29]. The sensitive subpopulation of about 80% of AG1522 cells, however, was much larger than the S-phase subpopulation which comprise about 33% (unpublished data) of this cell line.

Figure 2 shows the RCF levels following radiation alone and concurrent CPT and X-radiation treatments. The CPT dose (25 μ*M*) was chosen to be in the plateau regions of the survival curves of both cell lines (see Fig. 1). The addition of CPT to X-radiation significantly increased the killing of both cell lines without substantially changing the shapes of the radiation survival curves. This confirmed our previous findings that the interaction between CPT and radiation was essentially additive in exponential cultures of these cells. Sk-Mel-3 cells were again more resistant than AG1522 cells to both X-radiation alone and concurrent CPT and X-radiation. At a RCF level of 0.1, this difference was about 1.8-fold for X-radiation alone and about 2.5-fold for CPT and X-radiation.

Inhibition of cell growth/attachment assay

There was a significant decrease in the number of Sk-Mel-3 cells that remained attached to the culture vessel after treatment with CPT and subsequent incubation of the treated cells at 37°C for up to 96 h in the absence of CPT (Fig. 3a). More cells were lost or released into the medium following a high (25 μ*M*, chosen to be in the plateau region of the survival curve of Fig. 1) than a low (2 μM, chosen to be in the initial part of the survival curve of Fig. 1) dose of CPT. Conversely, most AG1522 cells remained attached to the culture vessel under the same conditions of treatment and post-treatment incubation, and this was true for both concentrations (2 and 25 μ*M*) of CPT (Fig. 3b).

To determine whether the treatments were cytotoxic or cytostatic, we expressed the number of attached cells at various times following treatment with CPT on the basis of the initial number of attached cells (normalized to 100%). Relative to the untreated control, 2 μ*M* CPT prevented an increase in the number of Sk-Mel-3 cells whereas 25 μ*M* CPT actually caused a substantial reduction to fewer than the initial number of cells by 96 h after treatment (Fig. 4a). However, both these doses were actually cytotoxic, not cytostatic, since they caused a detachment of cells into the medium especially at times more than 48 h (Fig. 3a). In contrast, both concentrations of CPT clearly prevented an increase in numbers of AG1522 cells (Fig. 4b) and were mainly cytostatic, not cytotoxic, since few AG1522 cells were released into the medium under these treatment conditions (Fig. 3b).

Apoptotic versus necrotic cell death

Because of the obvious differences in detachment and release of dead or dying cells into the surrounding

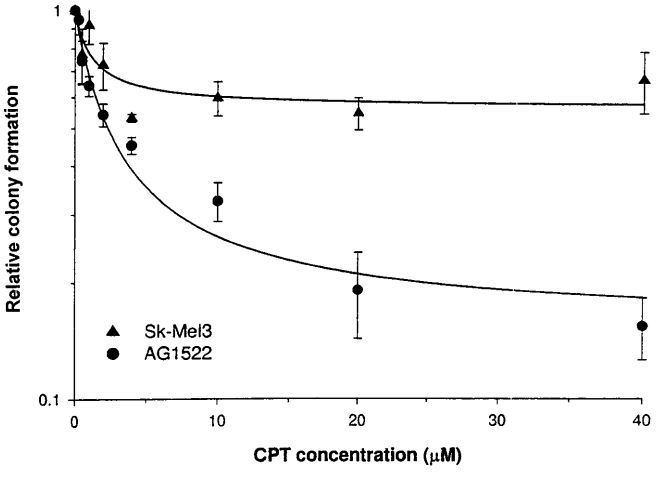


Fig. 1 Relative colony formation curves for Sk-Mel3 and AG1522 cells following treatment with CPT (1 h, 37°C)

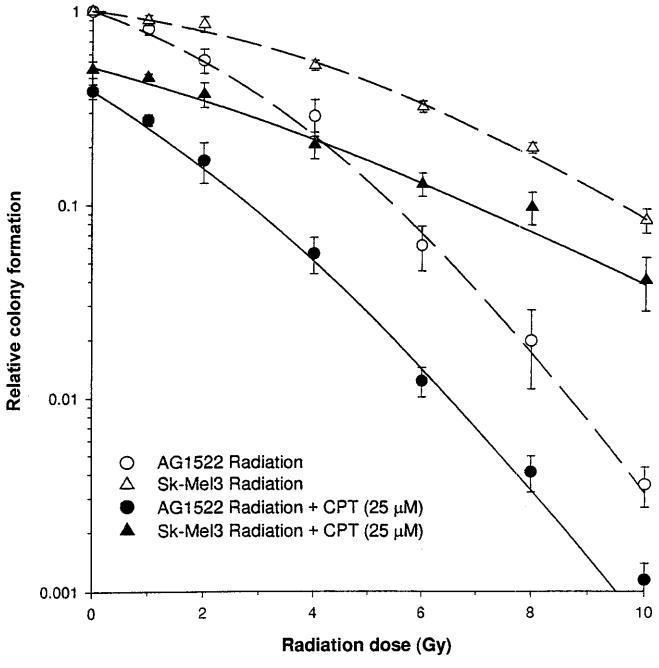


Fig. 2 Relative colony formation curves for Sk-Mel-3 and AG1522 cells following radiation alone or concurrent CPT and radiation. CPT exposure was 1 h at 37°C

medium after treatment manifested by these two cell lines, we evaluated the total number of cells (i.e. attached plus floating in medium) in subsequent experiments. Figure 5 shows the time-course of CPT-induced cell death. Relative to untreated controls, there were significant increases ($P < 0.05$) in both apoptotic and necrotic Sk-Mel-3 cells after treatment with 25 μM, but not 2 μM, CPT (Fig. 5a). For the latter (2 μM CPT), only the increase in necrotic death at 96 h was significant. Conversely, there were no significant increases in either form of cell death in AG1522 cells after treatment with CPT alone (Fig. 5b).

After concurrent CPT and X-radiation treatment in Sk-Mel-3 cells, there were again increases in the number of apoptotic and necrotic cells relative to the cells receiving irradiation alone (Fig. 6a). However, only the increases in necrotic cells were significant ($P < 0.05$) and this was true for both 2 and 25 μM CPT given with X-radiation. There were small increases in apoptotic AG1522 cells following both 2 and 25 μM CPT with X-radiation, but these increases were statistically insignificant (Fig. 6b). Thus there were no significant increases in either form of cell death for both concentrations of CPT given concurrently with radiation in AG1522 cells.

Necrotic Sk-Mel-3 cells were more frequent than apoptotic Sk-Mel-3 cells after both CPT and concurrent CPT and X-radiation (Fig. 5a, Fig. 6a) whereas the reverse was true for AG1522 cells (Fig. 5b, Fig. 6b).

Discussion

The incidence of human melanoma is increasing at a rate that exceeds that of all other solid tumors [2]. Although surgery is usually curative when melanomas are detected at an early stage, the outlook for melanoma that has metastasized beyond the regional lymph nodes remains bleak (median survival is in the order of months). Further, metastases are challenging to treat because they frequently involve multiple sites (including the skin, lung, liver, bone, and brain) [2]. Radiation is being re-evaluated as a treatment modality in primary locoregional management of melanomas because some

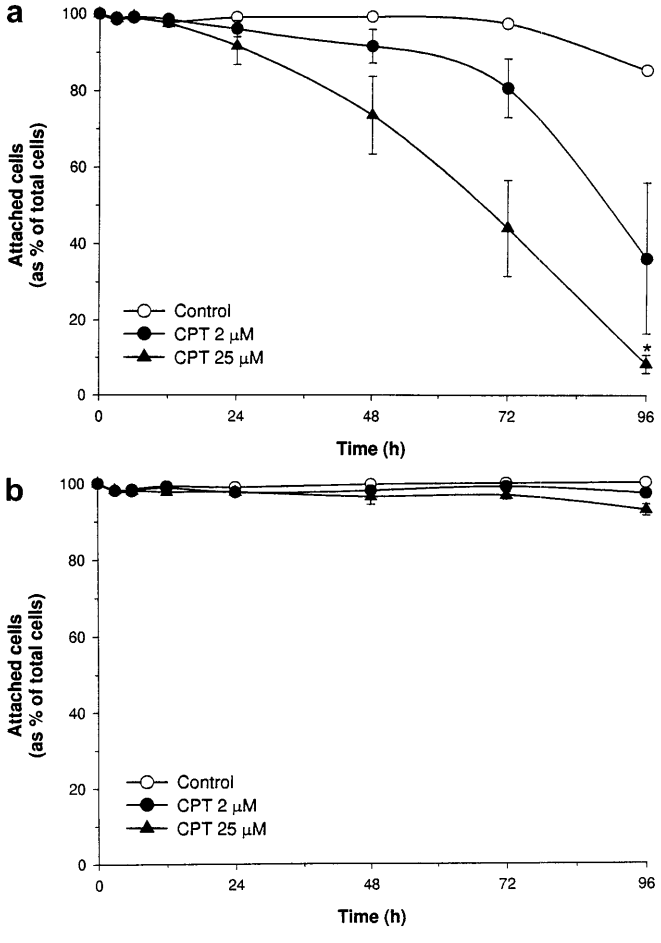
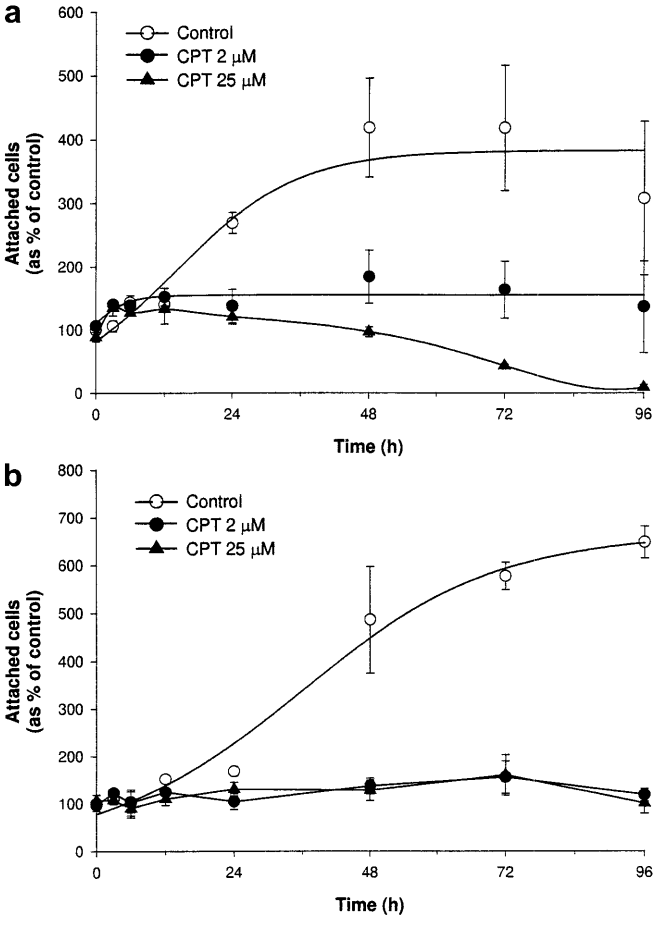


Fig. 3a, b The number of cells (**a** Sk-Mel-3, **b** AG1522) that remain attached following treatment with CPT (1 h, 37°C) and post-treatment incubation at 37°C for various times up to 96 h. The numbers of attached cells are expressed as a percentage of the total (i.e. attached plus floating in medium) number of cells for both cell lines



Fig, 4a, b Effect of treatment with CPT (1 h, 37°C) and post-treatment incubation at 37°C for various times up to 96 h on the growth of cells (**a** Sk-Mel-3, **b** AG1522)

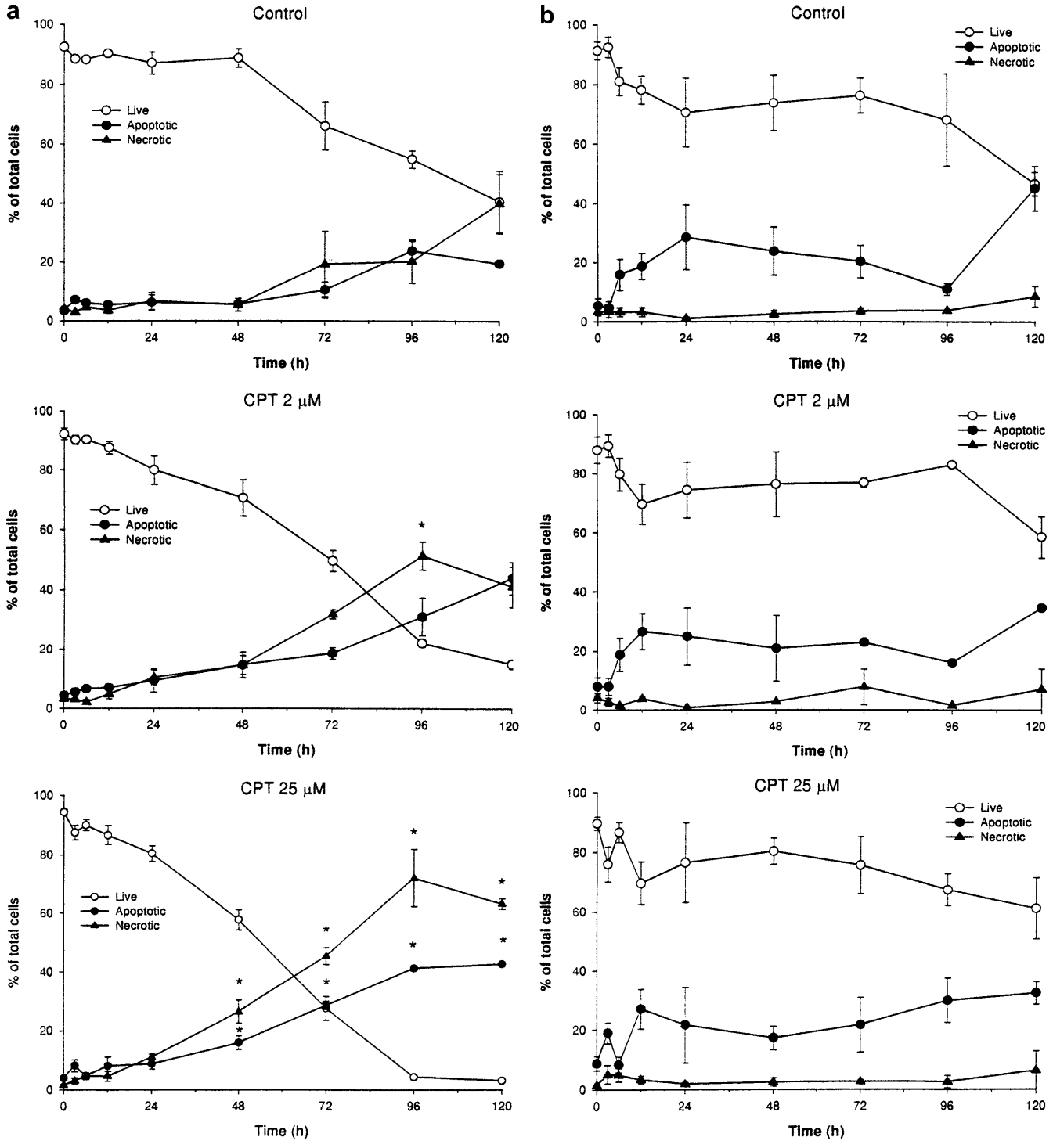


Fig. 5a, b The relative number of apoptotic, necrotic and live cells (**a** Sk-Mel-3, **b** AG1522) following treatment with CPT (1 h, 37°C) and post-treatment incubation for various times up to 120 h. Attached cells were pooled together with floating cells in these experiments. *Top panels* control cells treated only with DMSO (*control*), *center panels* 2 μ*M* CPT, *bottom panels* 25 μ*M* CPT. $^*P < 0.05$ vs control

melanomas do respond to radiation clinically [1]. Chemoradiation, acting either through a synergistic or additive interaction between the drug and radiation, may therefore be potentially useful for decreasing the incidence, and/or for the treatment, of MM.

There is strong evidence for the genetic basis of apoptosis with some genes being regarded as proapoptotic (e.g. wt p53, bax) [21, 22, 24, 37] and others as antiapoptotic (p21/waf1/cip1, bcl-2) [13, 14]. More specifically, adenovirus-driven ectopic expression of p21/waf1/cip1 substantially protects against p53-dependent apoptosis in human melanoma cells [13]. Recently, it has been claimed that some proto-oncogenes (Bcl-2/Bcl-xL)

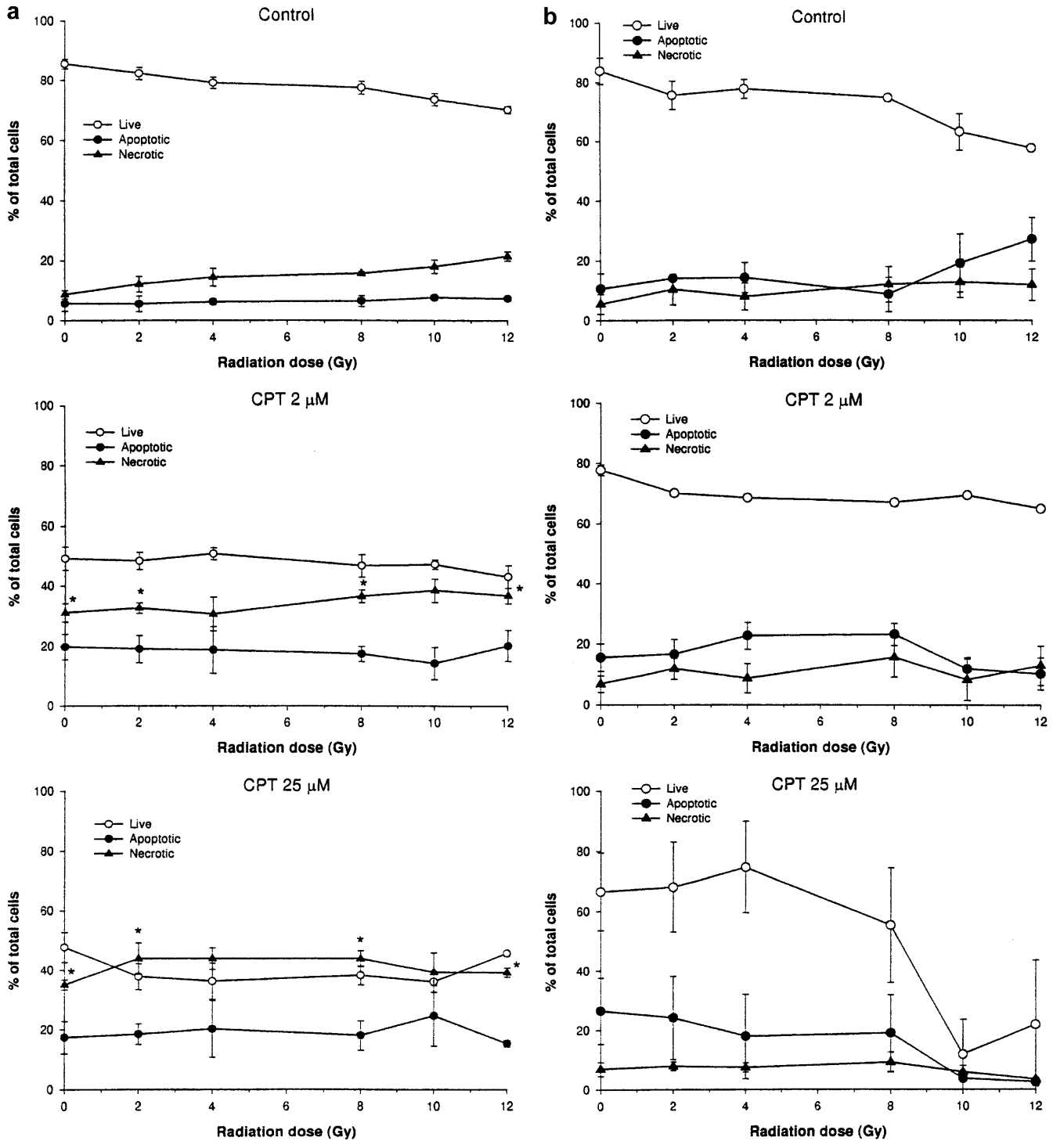


Fig. 6a, b The relative number of apoptotic, necrotic and live cells (**a** Sk-Mel-3, **b** AG1522) as a function of radiation dose assayed 48 h after treatment with concurrent CPT and X-radiation. CPT exposure was for 1 h at 37°C. Attached cells were pooled with floating cells in these experiments. *Top panels* control cells treated with irradiation and DMSO, *center panels* 2 μ*M* CPT with radiation, *bottom panels* 25 μ*M* CPT with radiation. $*P < 0.05$ vs irradiated-alone control

and ICE-like proteases may modulate both apoptotic and necrotic cell death, indicating that apoptosis and necrosis may share some common pathways and/or mediators [12, 33]. This further suggests that the relationship between apoptotic and necrotic death may be important in manifesting cytotoxic stresses due to chemoradiation treatments. Modulation of apoptotic and necrotic cell death on a genetic basis may ultimately permit enhanced killing of melanoma cells and decreased cytotoxicity to normal cells, thereby leading to an increase in clinical therapeutic index. Our intention in this study therefore was to examine the relevance of both

these forms of cell death following combined CPT and radiation treatment. It is noteworthy that: (a) although recent studies have documented the importance of the apoptotic form of death to melanoma cells treated with CPT [19, 20], there are very few data in regard to the relative importance of apoptotic and necrotic cell death in human melanoma and human normal fibroblast cells treated with combined CPT and radiation, and (b) the concentrations of CPT used in this study were higher than typical clinical exposures.

The main findings of this study were: (a) in Sk-Mel-3 cells, both necrotic and apoptotic cell death were important following treatment with CPT alone, but necrotic death was more important following CPT combined with X-radiation, (b) in AG1522 cells, both apoptotic and necrotic cell death were insignificant after treatment with CPT alone or combined with X-radiation, and (c) in Sk-Mel-3 cells, all four methods of determining treatment-related cellular effects (apoptotic, necrotic and clonogenic cell death, inhibition of cell growth) were consistent in so far as demonstrating that CPT given alone, or with X-radiation, led to a substantial increase in cytotoxicity, but this was not the case with AG1522 cells. Thus our present studies provided evidence that necrotic cell death may be more important than apoptotic cell death during combined CPT and radiation treatment in Sk-Mel-3, but not AG1522, cells.

The apparent discrepancy in the AG1522 results merits further consideration. In this cell line, three assays (necrotic and apoptotic cell death, inhibition of cell growth) were consistent in so far as showing that these treatments (2 and 25 μM CPT) had minimal toxicity to these cells. Curiously, the clonogenic surviving fraction assay showed that 25 μM CPT in particular had killed a substantial proportion (about 80%) of the AG1522 cells. Similarly, the clonogenic assay also detected substantially higher cytotoxicity following 25 μM CPT and radiation as compared to the apoptotic/necrotic assays. It should be noted, however, that the clonogenic assay detected the loss of clonogenic potential from all possible causes, including apoptosis and necrosis, occurring over a longer period (14 days) after treatment than the apoptotic/necrotic and growth inhibition assays (120 h for CPT alone treatment or 48 h for CPT combined with radiation). This, therefore, suggests that either the AG1522 cells were dying in the period after the completion of the apoptotic/necrotic assays or that the additional steps of trypsinizing and replating the cells for the clonogenic assay might be related to the apparent discrepancy in the perceived killing of these cells. Future studies are required to determine which was the case. Interestingly, while CPT and its analogs can induce p53-dependent apoptosis in human ovarian carcinoma cell lines, these drugs can also induce p53-independent cell death as measured by the clonogenic assay [23]. Whether this was also happening in the AG1522 cells (i.e. p53-independent cell death being measured by the clonogenic assay) is presently unknown.

One factor that might have potentially confounded the evaluation of the relative importance of apoptotic to necrotic death in the present study is the possibility of apoptotic cells undergoing a subsequent secondary necrosis in the absence of phagocytosis occurring in vitro. (*Note:* even in the presence of phagocytosis, there might be an increase in necrotic cells if the rate constant for the conversion of apoptotic to necrotic cells is larger than that for the formation of apoptotic cells.) Secondary necrosis might artificially increase the proportion of necrotic relative to apoptotic cells that was detected by the apoptotic/necrotic assay. However, secondary necrosis was unlikely to have been important in AG1522 cells under our experimental conditions. This is because both apoptotic and necrotic deaths were very low in this cell line following both types of treatment (CPT with or without radiation).

While it is possible that secondary necrosis might have made some contribution at later time-points (>72 h after CPT alone) in Sk-Mel-3 cells, it is also unlikely to have been important at the earlier time-points. Specifically, whereas apoptosis was still relatively low, necrosis was already significantly increased at both 48 and 72 h after treatment with 25 μM CPT (Fig. 5a). In particular, the actual increase in apoptotic cells relative to untreated controls was very small and statistically insignificant (about 8%) for the 48-h point after treatment with 25 μM CPT; in contrast, the relative increase in necrotic cells was larger (about 20%) and statistically significant (Fig. 5a). Similarly, at 48 h following combined CPT and radiation treatments, there were already significant increases in necrotic deaths in Sk-Mel-3 cells occurring in the absence of significant increases in apoptotic death (Fig. 6a). Hence, secondary necrosis was also unlikely to have been important after combined CPT and X-radiation in Sk-Mel-3 cells under our experimental conditions.

Various cellular mechanisms have been proposed as potentially pertinent to the expression of resistance to chemotherapeutic agents by melanoma cells. These mechanisms include alterations in the expressions of the multidrug resistance associated protein (MRP), glutathione and related enzymes, the target enzymes (i.e. DNA topo I and II) of the DNA topo-targeting drugs, and various genes (N-ras, bax/bcl-2, p53, Apaf-1) involved in the expression of cell death [3, 32, 34]. There has been particular recent interest in elucidating the roles of p53 in human melanoma because: (a) p53 is apparently commonly overexpressed in MM but not in primary melanoma [17, 18], and (b) mutations of the p53 gene occur in 25–30% of MM and cultured melanoma cell lines [32]. Thus mutation of the p53 gene may be a useful indicator for drug resistance in this disease. Consistent with this view, melanoma cells expressing wt p53 are much more sensitive to treatment with CPT relative to those expressing mt p53 [19]. Further, melanoma cells overexpressing mt p53 show reduced sensitivity to CPT and lower (two- to threefold) CPT-induced apoptosis [20]. Note that p53 was upregulated, especially

significantly at the later time-points (about 96 h), after treatment with CPT in AG1522 but not in Sk-Mel-3 cells (data not shown). Further, Sk-Mel-3 cells also have hardly detectable endogenous amounts of p53, suggesting that this cell line has dysfunctional wt p53 whereas the AG1522 cell line has functional wt p53 (data not shown). Interestingly, a recent study has indicated that loss of Apaf-1, a cell death effector that acts with cytochrome *c* and caspase 9, is responsible for abrogating p53-dependent apoptosis in MM cells [34].

In summary, our present study demonstrated that necrotic cell death may be more important than apoptotic cell death in Sk-Mel-3, but not AG1522, cells being treated with combined CPT and radiation.

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