POLYPLOID GIANT CELLS PROVIDE A SURVIVAL MECHANISM FOR p53 MUTANT CELLS AFTER DNA DAMAGE

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The relationships between delayed apoptosis, polyploid ‘giant’ cells and reproductive survivors were studied in p53-mutated lymphoma cells after DNA damage. Following severe genotoxic insult with irradiation or chemotherapy, cells arrest at the G2-M cell cycle check-point for up to 5 days before undergoing a few rounds of aberrant mitoses. The cells then enter endoreduplication cycles resulting in the formation of polyploid giant cells. Subsequently the majority of the giant cells die, providing the main source of delayed apoptosis; however, a small proportion survives. Kinetic analyses show a reciprocal relationship between the polyploid cells and the diploid stem line, with the stem line suppressed during polyploid cell formation and restituted after giant cell disintegration. The restituted cell-line behaves with identical kinetics to the parent line, once re-irradiated. When giant cells are isolated and followed in labelling experiments, the clonogenic survivors appear to arise from these cells. These findings imply that an exchange exists between the endocyclic (polyploid) and mitotic (diploid or tetraploid) populations during the restitution period and that giant cells are not always reproductively dead as previously supposed. We propose that the formation of giant cells and their subsequent complex breakdown and subnuclear reorganization may represent an important response of p53-mutated tumours to DNA damaging agents and provide tumours with a mechanism of repair and resistance to such treatments.

INTRODUCTION

The major aim of applying irradiation and cytotoxic drugs in eradicating malignant disease is to permanently inhibit the reproductive ability of stem cells (Mackillop et al., 1983). Increasing evidence has led to the hypothesis that sensitive tumours respond to these DNA damaging agents by rapidly undergoing apoptosis. In contrast, resistant tumours do not readily undergo apoptosis and respond only partially to treatment, displaying delayed death and ultimately surviving the genotoxic insult (Stellar, 1995; Graeber et al., 1996; Rupnow et al., 1998). One of the factors which determines a cell’s apoptotic propensity is its p53 status, which is known to be an important predictor of treatment outcome for haemopoietic tumours, although its role in solid tumours is more controversial (Brown and Wouters, 1999).

After genotoxic insult, cells commonly undergo cell cycle arrest prior to initiating apoptosis. This

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ABBREVIATIONS: BL, Burkitt’s Lymphoma; PI, Propidium Iodide; PM, pycnotic mitoses; FISH, Fluorescent in situ Hybridization; TUNEL, Terminal dUTP Nucleotide End Labelling

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arrest occurs predominantly at the G₁ and G₂ cell cycle checkpoints (Hartwell and Kastan, 1994). Arrest at these checkpoints prevents further DNA replication in the presence of an unrepaired DNA template. The p53 protein is intimately involved in these processes and is critical in evoking arrest and/or apoptosis at the G₁ checkpoint. Cells possessing mutated p53 fail to arrest or undergo apoptosis at G₁ and instead arrest at the G₂ checkpoint (Kastan et al., 1991; Kuérbitz et al., 1992; Lowe et al., 1994).

A role for p53 has also been established in the coupling of S and M phases in the cell cycle (Waldman et al., 1996). Cells lacking wild-type p53 function exhibit gene amplification (Yin et al., 1992) and an enhanced rate of polyploid giant cell accumulation (Livingstone et al., 1992; Olive et al., 1996). Polyploid giant cell formation is further delayed following irradiation in p53 mutated cells (O’Connor et al., 1993; and our own unpublished observations). TK6 and WI-L2-NS human B-lymphoblast cell lines were derived from the same WI-L2 isolate, are near diploid and have stable, indistinguishable karyotypes. TK6 are p53 wild-type and WI-L2-NS p53-mutated and have been previously described (Amundson et al., 1995). All cell lines were maintained in culture medium described below at 37°C in a 5% CO₂ humidified incubator. Cells were maintained in log phase of growth for at least 24 h prior to experimental studies.

Culture materials
All cell lines were cultured in RPMI 1640 medium (Gibco), supplemented with antibiotics and 10% Myoclon plus fetal calf serum (FCS; Gibco).

Irradiation of cells
All cell lines were irradiated using a Phillips 5 Mv linear accelerator with photons at a dose rate of 3.0 Gy/min.

Apoptosis detection and cell cycle analysis using flow cytometry
Samples were analysed essentially as per the method of Nicoletti et al. (1991) with minor modifications. Briefly, samples of 0.5–1 × 10⁶ cells were taken at relevant time points and centrifuged for 5 min at 500 × g. Samples were then washed once in PBS, resuspended in hypotonic fluorochrome solution [50 µg/ml propidium iodide (PI), 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton X-100] and stored in the dark at 4°C overnight. Analysis of samples was performed on a FACSscan flow cytometer (Becton Dickinson), using a 488 nm argon laser for excitation and a 560 nm dichroic mirror and 600 nm band pass filter (bandwidth 35 nm) for detection. Samples were represented as DNA histograms using LYSIS II software (Becton Dickinson) and the distribution of cells in the G₁, S, G₂-M and polyploid phases of the cell cycle calculated. Apoptosis was quantified by measuring...
the proportion of cells with sub-G₁ levels of DNA and was confirmed by microscopy as described below.

**Light and electron microscopy**

For light microscopy, cytospins were prepared, fixed with ethanol/aceticone (1:1) for >30 min at 4°C and air-dried. Slides were then treated with 0.1 N HCL at 4°C for 10 min, washed in distilled water and stained with 0.05% Toluidine blue in citrate-phosphate McIlvain buffer pH 5 for 10 min. After gentle rinsing, the slides were blotted, dehydrated in butanol, passed through Histoclear and embedded into DPX.

These preparations were used for cytological studies and counts of apoptotic, mitotic, segmenting and micronucleating cells. 1000–2000 cells were counted per sample. For electron microscopy (EM) lymphoma cells were fixed with 3% glutaraldehyde in 0.1 m cacodylate buffer (pH 7.2) containing 2 mM CaCl₂ for 2 h at room temperature. Cells were then washed several times in cacodylate buffer. Post-fixation was carried out in 1% buffered osmium tetroxide and aqueous uranyl acetate, followed by embedding in Spurr resin. Ultra thin sections were then prepared and contrasted with lead citrate.

**TUNEL (terminal dUTP nucleotide end labelling) assay for apoptosis**

TUNEL analysis was carried out on cytospin cells, which were fixed in methanol/aceticone (1:1) at −20°C for 10 min and then air-dried. The subsequent reaction was carried out according to the method of Gavrieli et al. (1992) with the proteinase K pretreatment omitted. Briefly, the reaction was performed on slides under a cover slip by addition of 25 µl of the end-labelling buffer (30 mM Tris/HCl, pH 7.2, 1% BSA, 140 mM Na-cacodylate, 1 mM CoCl₂) containing 2.5 µM dNTP with the biotin-conjugated dUTP, and 7.5 U terminal transferase (Boehringer Mannheim Corp., U.K.) at 37°C, for 1 h. This was followed by treatment with Avidin-peroxidase conjugate, which was further stained by 3-amo-no-9-ethylcarbazole (AEC) for visualization of the product.

**Fluorescence in situ hybridization (FISH)**

FISH analysis using centromeric probes for chromosomes 1, 3, and 9 was performed essentially according to Rooney and Czepulkowski (1992). Briefly, slides were immersed for 5 min in 2 × SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) at room temperature and then dehydrated in increasing grades of room temperature ethanol (70%, 80%, 96%) for 5 min each. Slides were then treated with RNase (100 µg/ml) in 2 × SSC for 30 min at 37°C and then washed three times in the same solution at 37°C (5 min/wash) and once in PBS for 5 min at room temperature. Subsequently, preparations were treated with pepsin (5 mg/ml 0.1 M HCl) for 10 min at 37°C, washed twice in PBS for 5 min at room temperature and immersed for 10 min in 1% paraformaldehyde in PBS at room temperature. After paraformaldehyde treatment, specimens were washed once in PBS for 5 min and then dehydrated in increasing grades of ethanol (70%, 80%, 96%) at −20°C for 5 min each. Subsequently, specimens were exposed to the DNA probe which was first denatured by boiling for 2 min followed by rapid cooling. Hybridization was performed with 2 ng/ml of the probe in hybridization solution (70% formamide, 10% dextran sulfate, 100 mg/ml salmon sperm DNA) at 37°C for 15−18 h in a humidified chamber. After incubation, specimens were washed three times in 50% formamide/2 × SSC, pH 7.0 at 45°C for 5 min and immersed in 2 × SSC at 37°C for 5 min. For detection of probe hybridization, slides were first washed in 100 ml of 0.05% Tween-20 in 2 × SSC (SSC-T) and then incubated with avidin-FITC in this solution at 70°C for 5 min. Slides were then washed in SSC-T and cells counterstained with 0.3 mg/ml PI in Antifade.

**Separation of giant cells**

On day 5 post-irradiation, Ramos or Namalwa cells were divided by light-scattering (FSC and SSC) criteria into giant and small fractions using a FACS Vantage flow cytometer (Becton Dickinson). The giant cell fraction was demonstrated to contain the polyploid cells by PI DNA content analysis and by light and electron microscopy as described above. A minimum of 100,000 giant cells were separated.

**Re-irradiation of restituted cell lines**

To determine whether the previously irradiated restituted cell line was a resistant sub-clone of the parent cell line, the surviving cell lines were re-irradiated and the kinetics of their response compared with previously untreated cells. Re-irradiation was performed after the primary genotoxic insult, when cells were growing normally in log-phase and appeared morphologically and phenotypically identical to the parent line. The
kinetics of their response was studied with flow cytometry as described below.

**Cell clonogenicity assay**

To measure clonogenic survival, a serial dilution assay was performed, using a method similar to that of Olive *et al.* (1996). Briefly, each sample was treated with a range of doses of irradiation and seeded into individual wells of a 96-well plate over a range of cell densities (10 wells per cell density, eight cell densities per dose).

Cells were plated in 80% fresh medium and 20% conditioned medium. After 10–14 days the fraction

![Graph A](image1)

![Graph B](image2)
RESULTS

Role of giant cells in the response to genotoxic damage in p53-mutated lymphoma cells

The response of p53-mutated cells (Namalwa, Ramos, Daudi, W1-L2-NS) to genotoxic insult was initially investigated using a single 10 Gy dose of irradiation. Figure 1A shows a typical set of DNA histograms generated using flow cytometry over a 3-week period after irradiation in Namalwa cells. Figure 1B represents analysis of the DNA histograms from six independent experiments. There appear to be five distinct components of the response to a single fraction of high dose irradiation and these appear common to all of the human p53 mutant BL cell lines we have studied. The first component of the response is cell cycle arrest at the G2-M checkpoint (4N DNA content) which occurs within the first 24 h (Fig. 1A and B), and is seen up to 5 days after irradiation (Figs 1A and 2). Next, polyploidization is seen with the formation of polyploid cells with >4N DNA content. Following this, an apoptotic population of cells is seen with <2N DNA content (Fig. 1A and B). The fourth component of the response is the depression of the diploid stem line, which becomes suppressed during the first 24 h post-irradiation and remains so throughout the next 14 days until full restitution by day 21 after irradiation. Finally, ‘de-polyploidization’ occurs with the disintegration of the polyploid cells (Fig. 1A and B).

To address whether the lack of wild-type p53 is responsible for this particular response to genotoxic insult, two closely related human lymphoblastoid cell lines derived from the same donor carrying wild-type (TK6) or mutated p53 (WI-L2-NS) were utilized. Figure 2 illustrates the differences in response to genotoxic insult between these lines: WI-L2-NS undergoes profound G2-M arrest, delayed apoptosis and extensive polyploidy whereas the TK6 cells demonstrate a degree of G1 arrest, rapid apoptosis and lack of polyploidy after irradiation. To assess whether other DNA damaging agents produced a similar response, Namalwa cells were treated with Doxorubicin. As shown in the bottom panel of Figure 2, addition of Doxorubicin leads to similar events to those already described after irradiation.

To understand these processes more fully, a comprehensive cytological study was undertaken examining the response of Ramos and Namalwa cell lines to irradiation.

Fig. 1. A: A typical response of BL cells to a single 10 Gy dose of external beam irradiation (one of 10 independent experiments, performed with the Namalwa cell line). Cells were harvested at the time point indicated, washed and stained with PI and the DNA content analysed by flow cytometry (as described in Materials and Methods). The DNA histograms are presented on a logarithmic scale. Profound G2-M arrest (>85% of cells) at day 1 (1d) is followed by the appearance of polyploidy by day 3 (3d). By 3d the number of polyploid cells has significantly increased and the large 8N and 16N peaks are shown gated. At 3d there are still only small amounts of sub-diploid (<2N) apoptotic cells. These increase gradually over the next 96 h to reach a peak or ‘apoptotic crisis’ at around 8 days. Decrease in the total numbers of the polyploid cells subsequently follows until restitution of the stem line is achieved. B: Typical kinetic responses of p53-mutated Ramos BL cells after a single 10 Gy dose of external beam irradiation. Each point represents the mean of six independent experiments and values are calculated from DNA histograms as described in Materials and Methods. The time course of the five responses to a single dose of 10 Gy are demonstrated, including (1) G2-M arrest (2) polyploidization (3) delayed apoptosis (4) depression of the stem-line diploid fraction (5) de-polyploidization. The four major fractions of the cellular population are diploid 2N (●) sub-diploid (apoptotic) <2N (■); tetraploid 4N (▲) and polyploid >4N (▼). Total DNA content is calculated by multiplying the percentage of cells in each gated region by the DNA ploidy content for that region, for example 20% × 2(N)=40, 20% × 4(N)=80. For the polyploidy fraction the values represent a sum of the genomes of all cells having a DNA content equal to or greater than 8N. The hypodiploid apoptotic fraction is expressed as a percentage with a DNA content value of 1.
Mitoses and post-mitotic apoptosis

Mitoses were absent initially at 24 h after irradiation, but reappeared by 48 h and had increased in number by a further 30% or more above the control values at 72 h, before decreasing again by day 5 as determined by cytological counting. After this time and until day 14, no mitoses of normal size were observed. Mitoses reappeared between the second and third week post-irradiation, coincident with the restoration of the diploid stem-line. Although similar, the Namalwa cells restitute some 3–4 days quicker than the Ramos cells.

The first waves of mitoses (in the first 48 h) were markedly aberrant, showing lapses, bridges, uninemic fragments, multipolar spindles and restitution. Subsequent mitoses were either aberrant or pycnotic and the majority (60–70%) of them were TUNEL-positive (data not shown). When pycnotic mitoses (PM) were clearly visible, they always had features of a metaphasic arrangement, strongly suggesting that cells undergoing PM were previously arrested in metaphase. Apoptotic-like degeneration involving segmentation of coalesced chromosomes into highly condensed enveloped bodies in pale cytoplasm was also observed in these cases (Fig. 3A). These data indicate that PM usually follow the first wave of aberrant mitoses and derive primarily from metaphase-arrested cells. This appears to indicate that a spindle checkpoint linked to apoptosis exists for unrepaired cells, which have overridden G1 and G2 checkpoints. An additional interesting observation was that huge PM but no anaphases were seen throughout the whole of the polyploidization–depolyploidization period, representing abortive attempts of polyploid cells to undergo mitosis.

Significant amounts of apoptosis were not seen until 72 h post-irradiation in both cell-lines. This first wave of delayed apoptosis appears to coincide with failure of the mitotic pathway, which is indicated by a peak in the number of PM. By morphology, the apoptosis is mostly of the 'classic' (Fig. 3B) or 'twin' bi-nuclear type (Fig. 3C). The latter have a characteristic zip-contact between two equal-sized nuclei and were abundantly represented between 48–72 h and probably originate after failing mitotic restitution in early anaphase. Some apoptotic polyploid cells also demonstrate these 'zips' and are presumably derived from cells that have failed to restitute multipolar mitoses.

In the later waves of apoptosis, additional mechanisms of cellular disintegration are seen and many polyploid cells ultimately disintegrate as apoptotic polykaryons (Fig. 3D). However, prior to this, these cells may undergo intensive micronucleation and nuclear segmentation (data not shown- see companion article by Erenpreisa et al. in this issue).
Polyploidization and apoptosis

The increase in polyploidy illustrated in Figure 1A and B appears to develop from a step-wise daily doubling in DNA content which can reach up to 64 N in the first week post-irradiation. FISH analysis performed on day 5 for the centromeres of chromosomes 1 and 3 shown in Figure 4A and B (and the centromere of chromosome 9; data not shown) confirm the polygenomic nature of these giant cells. Some of the giant cells do however lack one of the checked chromosomes and this appears to be indicative of their possible origin from restitution of post-aberrant mitoses.

The last peak of serial endoreduplication (on day 5) is followed in the next few days by what we have termed ‘apoptotic crisis’ with a massive disintegration of giant cells seen by TUNEL analysis (Fig. 4C). The level of apoptosis then reaches a plateau embracing more than 80–90% of the cells during the second week post-irradiation and at the end of this period the amount of apoptosis abruptly decreases. As such, apoptosis was found to be strongly positively correlated to polyploidization (r=0.82, \(P<0.002\) for Ramos cells).

The behaviour of the giant cells which survive this ‘apoptotic crisis’ was different from that of the giant cells formed initially and appears to be associated with complex nuclear reorganization, sub-nuclear sorting, and a final disintegration into oligoploid secondary cells (described in detail by Erenpreisa et al. in the companion article in this issue).

Analysis of the surviving stem line

Following irradiation with 10 Gy, the emerging surviving stem line was assessed by various parameters, including cell doubling time, DNA

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**Fig. 3.** Electron micrographs demonstrating four forms of apoptosis observed after irradiation. (A) Clumped pyknotic mitoses were observed beginning from the second mitotic wave after the release of cells from G2-M arrest and in the giant form throughout the whole restitution period. (B) Classical apoptosis, which was in the majority, during the whole of the restitution period; (C) diplo-karyotic ‘twin apoptosis’ with characteristic zip-connection between the nuclear pores of two closely apposed nuclei, was observed in the first week (may represent a failed mitotic restitution); (D) apoptotic polykaryons derived from giant cells. All pictures are of Ramos cells and all forms were TUNEL positive. Bars=1 \(\mu m\).
content profile, karyotype, and phenotype, compared with those of an untreated population of the same cells. All of the B cell antigens analysed by direct immunofluorescence (CD19, CD20, CD22, CD38, sIgM) appeared very similar in their surface levels of expression, as did DNA content (diploid), microscopic appearance and doubling time determined for Ramos and Namalwa cells. Although the karyotype of the rescued line was not identical, it appeared similar, but displayed extra abnormalities consistent with irradiation damage, in 14 of 40 cells examined (data not shown). Forty days after the initial irradiation, the stem-line appeared fully recovered and was re-irradiated at this point. The clonogenic survival of the restituted stem-line was compared by means of a clonogenic assay and found to be very similar to that of the parent line (Fig. 5A). Extremely similar responses and kinetics of apoptosis, polyploidization, and de-polyploidization of giant cells were observed for the surviving stem-lines, which were re-irradiated (Fig. 5B).

Separation and clonogenicity of giant cells

We next sought to establish the origins of the clonogenic survivors by \[^{3}H\]-thymidine labelling of irradiated cell populations. These experiments were carried out initially on unsorted populations and then subsequently on the ‘giant’ and ‘small’ fractions separately, having sorted these populations by size, with cells greater than 4N in the giant cell population. Initial autoradiographic analysis of unsorted labelled cells revealed that: (1) The giant cells demonstrated a very high intensity and frequency of labelling between day 5 and day 6 after 10 Gy, indicating that the vast majority of the giant cells underwent extensive DNA synthesis during this time; (see Fig. 6A for labelling of giant cells). (2) The intensity of labelling on simultaneously exposed autoradiographs suggested that these giant cells appear to undergo only one round of endoreduplication during the period between days 7–14. This data correlates with the DNA content histograms, which show a smaller second wave of DNA replication in the second week. (3) There was no inclusion of the \[^{3}H\]-Thymidine label seen between days 5 to 6 in the population of small (diploid size) cells sampled on day 7 and no normally-sized mitoses were seen from day 5 up to the end of the second week, suggesting the interruption or strong suppression of the mitotic cycle.

When the cells were sorted according to their size and labelled separately, only the giant cell fraction incorporated the label and was found to be
replicating its DNA on day 5 post-irradiation (Fig. 6A). When the giant cell population was \[^{3}H\]-thymidine labelled for 24 h, re-united with an unlabelled small cell fraction and then returned to culture, by day 21 numerous small discreetly labeled diploid cells were found (Fig. 6B). In view of the fact that only the giant cell population was labelled, any cells which possess a \[^{3}H\]-thymidine label when examined on day 21, must have come from the giant cell precursors, which integrated \[^{3}H\]-thymidine into their DNA during exposure on day 5. To confirm that this was indeed the case and that a small population (for example 1 in 1000) of diploid cells had not contaminated the giant cell population and given rise to the labelled diploid cells, the experiment was repeated but with \[^{3}H\]-thymidine label added only to the small cell fraction. On this occasion, the population of cells representing the rescued stem line remained unlabelled (data not shown). The recovery of the diploid stem-line from the separated and cultivated giant cell fraction displayed similar kinetics when compared with the recovery of the unsorted population. In contrast, the small cell fraction did not survive when cultivated alone.

**DISCUSSION**

We have described the responses of radioresistant p53-mutated BL cell lines to genotoxic damage over a 3-week period and described the formation and subsequent breakdown of polyploid ‘giant’ cells as part of this response. We have characterized the post-mitotic events which occur in these cells after irradiation, and have described several different distinct types of delayed apoptosis. Furthermore, we have shown that giant cells appear capable of replenishing the reproductive stem line.

**Reproductive cell death and delayed apoptosis**

There is now considerable evidence supporting p53-independent pathways of apoptosis (Peled et al., 1996; Bracey et al., 1995; Merrit et al., 1997) and the role of p53 in radiosensitivity of lymphoma cell lines (O’Connor et al., 1993). However, the source of this delayed apoptosis and how it differs from rapid apoptosis, also termed ‘interphase death’ (Yamada et al., 1981), have not previously been so extensively characterized.

Delayed apoptosis seen after a single 10 Gy dose of irradiation appears to derive from two sources. Initially, in the first 5 days after irradiation, cells of the mitotic pathway provide much of the delayed apoptosis. These cells fail to complete mitosis, probably due to the presence of unrepaired DNA strand breaks, and ultimately undergo classic or binuclear apoptosis.

Evidence for the continued presence of DNA breaks after release from G2-M arrest is provided by the character of the mitotic aberrations, namely lapses, bridges, fragmentation and uninemic segments. The appearance of micronucleation is also widely accepted as an indicator of chromosome...
breakage (Heddle et al., 1991). After day 5, the majority of delayed apoptosis results from the polyploid cells that exhibit multiple different forms of apoptosis, but which most often die as apoptotic polykaryons.

Cross et al. (1995) demonstrated that cells lacking functional p53 fail to halt the cell cycle in response to microtubule antagonizing drugs, implicating p53 in the spindle checkpoint. Recently Bunz et al. (1998) have demonstrated that p21−/− or p53−/− colorectal cancer cell lines enter mitosis after irradiation but never complete cytokinesis, such that >95% of the cells contain abnormal multilobulated nuclei. Like Bunz et al. (1998) we observed partial arrest in mitosis coupled by restitution into polyploid cells, as well as a transient increase in mitotic index over the control after irradiation, which subsequently decreased to negligible levels. However, the G2 arrest observed in these colorectal tumour cells was much shorter and the delay in the following mitosis and subsequent restitution much more pronounced than in our human p53-mutated BL cells. In addition, we found that the metaphasic arrest in mitosis occurring in the BL cells resulted in apoptosis (PM) more often than in restitution. As a consequence of this difference, the main source of polyploid cells in our BL cells appeared to originate from the G2 arrested cells rather than from the mitotic cells. Here we observed that the switch from the mitotic cycle into the endocycle is metastable and is often linked with delayed apoptosis.

The delayed apoptosis may proceed initially from the compromised mitotic cell cycle and subsequently from the initiation of the endocycle. This switch has recently been shown to be an alternative to apoptosis in human osteosarcoma cells (Erenpreisa et al., in press). Moreover, our more protracted observations also demonstrate that the switch from the mitotic cycle to the endocycle may be reversible.

Fig. 6. Giant cells actively synthesize DNA during the first week post-irradiation and give rise to the diploid stem line. Giant cells were separated from diploid cells by flow cytometry as described in Materials and Methods. The separated Namalwa giant cells were labeled with [3H]-thymidine between day 5 and day 6, washed and recultivated up to day 21. (A) Shows a 7-day sample containing heavily labeled giant cells, not counterstained; (B) Restituted cells containing [3H]-thymidine label on day 21. The magnification is the same in both figures and the Bar=10 μm. The autoradiographs were exposed to emulsion for 3 (A) or 8 weeks (B).
Delayed apoptosis does not predict sensitivity to irradiation

Although suppression of cell death appears to be strongly selected during carcinogenesis, it is interesting that it never appears to involve deletion of the entire apoptotic mechanism. Once malignant, the ability of cancer cells to undergo apoptosis has been argued to predict their sensitivity to treatments (Fisher et al., 1994; Lowe et al., 1993). In BL cell lines, p53 status has been shown to correlate with radiosensitivity and the Namalwa and Ramos cell lines included in our study have been shown to be amongst the most radioresistant (O'Connor et al., 1993). However, we agree with others that the total amount of apoptosis observed is not related to radiosensitivity (Illidge et al., 1998). Despite the vast majority of cells undergoing delayed apoptosis, the population was not eradicated; we would argue that this is not due to the lack of apoptosis, but due instead to the ability of these resistant cells to repair the damage. In arguments pertaining to apoptosis potential and clonogenic survival, we believe that it is critical to consider the potential for repair mechanisms. This argues strongly that not only is the process of polyploid cell formation linked to delayed apoptosis, but also that the entry into the endoreduplication cycle and linked apoptosis occurs as a result of unrepaired DNA.

Giant cells as a repair response for p53-mutated tumours following genotoxic insult

Analysis of the flow cytometry kinetics data reveal a 2-week deep depression of the diploid stem-line, which often appears to be more profound in the second week. This observation appears inconsistent with the suggestion that a small but very resistant, or promptly repairing, subclone of stem cells survives the damage and slowly accumulates during the restitution period. This resistant subclone, which received exactly the same irradiation-induced damage, would be expected to repair the genotoxic damage within the first 48–72 h, whilst in G2-M arrest, and then return to a normal cell cycle. If this was the case, the recovered fraction might be expected to return to logarithmic growth and outnumber the giant cells during the next 14 days. Therefore, it is difficult to understand why this resistant (repaired) subclone would remain quiescent for about a 2-week period.

The possibility that the resistant diploid subclone provides the major repair mechanism is made more unlikely by the fact that when the restituted cell line is re-irradiated the cells respond to the genotoxic insult in a seemingly identical fashion with the reappearance of giant cells. These observations led us to question where the mitotic competent survivors are derived from and how the restitution of the diploid cell line is achieved, whilst it is suppressed for a 2-week period.

Polyploid giant cells have always been regarded as being reproductively dead; however, the observations presented in detail here suggest that this is incorrect, at least for the Ramos and Namalwa BL cell lines. Firstly, only the giant polyploid cells are actively undergoing DNA replication between days 5 and 14 post-irradiation. The diploid population did not take up [3H]-thymidine and was not observed to undergo normal mitoses during this time period. Secondly, data from our separation experiments demonstrate the presence of diploid cells containing [3H]-thymidine in their DNA at the time of restitution of the stem-line (day 21), which were derived from the prelabelled sorted giant cells. The labelled diploid cells were, however, not seen on day 21 in the same experiment with sorted cells carrying 2N or 4N DNA content. For these reasons, with the diploid cells representing such a small proportion of the cellular population and not taking up label on days 5–6, it is difficult to understand how these cells could take up sufficient [3H]-thymidine to account for the observable labeling of the whole population by day 21 and be responsible for restitution of the stem line. However, this data is entirely compatible with the conclusion that the survivors are derived from the giant cell population.

Furthermore, the demonstration that re-irradiation of the surviving stem line produces a seemingly identical response, argues that polyploid cell formation is a necessary component of the survival process.

The question then arises as to how the giant cells might evoke restoration of the stem line and assist in repair of DNA double strand breaks after the
cells have left G2 arrest. If the giant cell fraction
does indeed contain the origin for the survival
of the stem-line, a novel mechanism associated
with polyploidy and depolyploidization must be
involved. The repair mechanism is likely to involve
generic recombination and this has been shown
to be enhanced by irradiation and substantially
increased in p53 mutated tumours (Xia et al.,
1995). It is noteworthy that Xia et al. were able to
render the radiosensitive TK6 cell line radio-
resistant by the successful transfection of TK6 cells
with mutated p53 and that this relative radio-
resistance involved delayed apoptosis, elevated
levels of polyploidy and increased mutational
and recombinational processes (Xia et al., 1997).
Insights into the mechanisms involved in these
processes are discussed in more detail in the com-
panion article by Erenpreisa et al. (in this issue)
and forms the basis of ongoing work.

In conclusion, we believe that giant cells are not
reproductively dead, as previously thought, and
that there can exist an exchange between the endo-
cyclic (polyploid) and mitotic (diploid or tetra-
plloid) populations during the restitution period.
Previously S/M uncoupling responsible for the
formation of giant cells has been suggested to be
one of the factors that can affect sensitivity to
genotoxic agents (Waldman et al., 1996).

Here we provide a mechanism for those observa-
tions and suggest that giant cells are, in principle,
capable of releasing reproductive descendants. We
propose that the endocycle and formation of giant
cells, as well as their subsequent disintegration after
genotoxic damage, may be an important response
and repair mechanism for p53-mutated tumour
cells. In this setting, giant cell formation may
represent an important repair response after cancer
treatments and provide the tumour with an
additional mechanism of resistance and survival
after genotoxic insult. Apoptosis appears in this
context not only to function in bringing about cell
death, but also in selecting for survival. An
enhanced understanding of this response and the
mechanisms of delayed apoptosis is likely to prove
extremely important in discovering potential means
of selectively enhancing the sensitivity of these
resistant tumours to DNA damaging agents.

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