Delayed DNA damage associated with mitotic catastrophe following X-irradiation of HeLa S3 cells*

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Partial loss of the radiation G2/M checkpoint is thought to be an early event in cell immortalization. One of the attributes of immortalized cell lines is an increase in susceptibility to induction of genomic instability by clastogenic agents. Recently we have shown that in irradiated HeLa cells cell cycle delays in late S and G2 lead to overaccumulation of cyclin B1 and that enhanced intracellular levels of this positive regulator of the cell cycle is correlated with cyclin-dependent kinase activation, spontaneous premature chromosome condensation and subsequent mitotic catastrophe occurring following irradiation. Previous studies have shown that spontaneous premature chromosome condensation and mitotic catastrophe are independent of apoptosis. This report shows that 40 h following X-irradiation of HeLa S3 cells, and subsequent to mitotic catastrophe, DNA strand breaks appear which are chemically distinct from those initially produced by ionizing radiation. This delayed damage is recognized by terminal transferase and thus involves generation of free 3'-OH ends. Pulse field gel electrophoresis analysis of DNA size distributions shows that DNA fragments of ~40 kbp and smaller are produced. As strand breaks produced as a direct result of irradiation are generally repaired within a few hours after exposure to X-rays at the doses used, these results describe a novel mechanism for generation of DNA damage occurring a day or more following irradiation. These results may be pertinent to the understanding of mechanisms underlying the delayed lethal effects of irradiation and may provide an initiating mechanism for radiation-induced genomic instability.

Introduction

Exposure of an asynchronous cell population to cytotoxic stress results in a complex pattern of delays in various cell cycle phases. The biochemical bases for some of these phenomena have been elucidated in recent years and a general consensus is that key regulatory mechanisms exist which serve to induce such delays in cell cycle progression. Nuclear envelope breakdown, chromosome condensation and mitotic spindle assembly are all events associated with mitosis and are mediated by multi-protein complexes containing cyclin B and cdc2. Kinase activity associated with these complexes is regulated by post-transcriptional modification of cdc2 and suppressed by specific inhibitory kinases which serve to prevent premature entry into mitosis in the presence of incompletely replicated or damaged DNA (Hartwell and Kastan, 1994; Murnane, 1995; for a review see Nurse, 1990). In spite of these checkpoint pathways, asynchronous HeLa or CHO cells delayed in late S at 41.5°C undergo spontaneous premature chromosome condensation (SPCC) (Mackey et al., 1988, 1992) due to loss of G2/M checkpoint regulation associated with abnormal accumulation of cyclin B1 (Mackey et al., 1996). Further, recent studies (Ianzini and Mackey, 1997) have demonstrated that over-accumulation of cyclin B1 occurs following X-irradiation of asynchronous HeLa cells, with subsequent induction of SPCC and nuclear fragmentation, both hallmarks of mitotic catastrophe (Heald et al., 1993). Previous studies (Swanson et al., 1995) have shown that SPCC and mitotic catastrophe mediated by cyclin B1 over-accumulation (Mackey et al., 1996) are independent of apoptosis in HeLa S3 cells; rather, alterations in nuclear morphology occurring following premature mitotic entry showed unusual reformation of the nuclear membrane within chromatin, suggesting that DNA damage might be produced during mitotic catastrophe.

This paper presents data showing that DNA strand breaks are generated many hours following irradiation of HeLa S3 cells. In particular, kinetics experiments using terminal transferase (TdT) to label residual DNA strand breaks demonstrate that TdT-positive cells are found following nuclear fragmentation starting at 32 h post-irradiation. As strand breaks produced as a direct result of irradiation are generally repaired within a few hours after exposure to X-rays at the doses used and since post-irradiation treatment with inhibitors of kinase activation are known to suppress both SPCC and mitotic catastrophe (Mackey, 1993), these experiments describe a novel mechanism for generation of DNA damage which occurs a day or more following irradiation. These results may lead to an improved understanding of the delayed effects of irradiation and may provide an initiating mechanism for radiation-induced genomic instability. In fact, amongst the many studies regarding genomic instability, some (Cheng and Loeb, 1993; Marder and Morgan, 1993; Morgan and Murnane, 1995) have shown that over-accumulation of cyclin B1 occurs following X-irradiation of asynchronous HeLa cells; delayed in late S at 41.5°C undergo spontaneous premature chromosome condensation (SPCC) (Mackey et al., 1988, 1992) due to loss of G2/M checkpoint regulation associated with abnormal accumulation of cyclin B1 (Mackey et al., 1996). Further, recent studies (Ianzini and Mackey, 1997) have demonstrated that over-accumulation of cyclin B1 occurs following X-irradiation of asynchronous HeLa cells, with subsequent induction of SPCC and nuclear fragmentation, both hallmarks of mitotic catastrophe (Heald et al., 1993). Previous studies (Swanson et al., 1995) have shown that SPCC and mitotic catastrophe mediated by cyclin B1 over-accumulation (Mackey et al., 1996) are independent of apoptosis in HeLa S3 cells; rather, alterations in nuclear morphology occurring following premature mitotic entry showed unusual reformation of the nuclear membrane within chromatin, suggesting that DNA damage might be produced during mitotic catastrophe.

Materials and methods

Cell culture

HeLa S3 cells were grown in suspension culture in Joklik's minimal essential medium (Gibco, Grand Island, NY) containing 10% heat-inactivated calf serum (HyClone Laboratories Inc., Logan, UT) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). Under these growth conditions the cell doubling time was 24 h.

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2Preliminary results of this study were presented at the 5th International Workshop on Radiation Damage to DNA, Bowness-on-Windermere, UK, April 1997.
X-irradiation

X-irradiation was performed at 37°C using a General Electric Maxitron irradiator operating at 220 kV, 11 mA current. Irradiation was delivered without filtration. Dosimetry was performed for each experiment with a Victoreen electroscope and the dose rate was in the range 1.4–1.5 Gy/min.

Sample collection

Samples were collected from the cell suspension culture every 8 h for up to 64 h post-irradiation. All samples needed for each measurement, namely bivariate bromodeoxyuridine (BrdUrd)/proliferation index (PI) and cyclin B1–PI flow cytometry, cell preparations for cytology (nuclear fragmentation and TdT labeling) and agarose-embedded samples for pulse field gel electrophoresis (PFGE) plug preparation, were collected at the same time from both the sham-irradiated control and the X-irradiated cells.

Cytology

Nuclear fragmentation cell preparation. Microscopic determination of nuclear fragmentation was performed on air-dried cell slide preparations. Hypotonically swollen cells, incubated in 75 mM KCl for 8 min, were fixed in 3% glutaraldehyde, 0.1 M NaCl, pH 7.4, and air dried on clean moistened microscope slides. Dried slides were stored at room temperature until ready for staining. Staining was performed using 1 µg/ml Hoechst 33342. The percentage of cells exhibiting nuclear fragmentation was evaluated using an Olympus BX-40 microscope equipped with a mercury lamp and UV filter.

In situ TdT cell labeling. Microscopic determination of TdT-positive cells was performed on forced air-dried cell slide preparations. Detection of DNA strand breaks was carried out by employing a direct in situ free 3'-OH end-labeling technique based on incorporation of fluoroscein thiochocyanate (FITC)-conjugated dUTP at the sites of DNA breaks using the enzyme TdT (Boehringer Mannheim, Indianapolis, IN). Cells washed in phosphate-buffered saline (PBS) were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, applied to clean dry microscope slides and allowed to dry under a hood. Dried slides were stored at -20°C until ready for labeling. TdT labeling was performed following permeabilization with 0.1% Trition X-100, 0.1% sodium citrate (2 min on ice). Fifty microliters of a mixture of TdT enzyme and label solution containing the nucleotide mixture were added to each slide under a plastic coverslip and incubated in a humidified chamber for 60 min at 37°C in the dark. Negative controls were processed without the TdT enzyme, while positive controls were pre-treated for 10 min at room temperature with 100 µg/ml DNase I. After incubation slides were rinsed three times with PBS and air dried.

Flow cytometry

Cell cycle analysis was carried out with 10 µM BrdU employing the pulse labeling technique. Anti-BrdU–PI flow cytometry was performed using a monoclonal antibody to BrdU-substituted chromatin (Immunocytometry System, San Jose, CA) according to the protocol described in Mackey et al. (1992). Flow cytometric analysis was performed using a FACSS IV (Becton Dickinson). Excitation was accomplished using an argon laser emitting at 488 nm with 300 mW power, red fluorescence was detected using a 640 nm low pass filter and green fluorescence using a 525 nm bandpass filter. Data acquisition and analysis were performed using the Cicer data analysis system, interfaced to an IBM-compatible personal computer.

Cell cycle phase distribution was determined using box analysis as described in Ianzini and Mackey (1997). In a previous study (Ianzini and Mackey, 1997) we demonstrated that this radiation-induced delay in the late S and G2 phases is accompanied by premature activation of cyclin B1-dependent kinase and cyclin B1 accumulation and that occurrence of these events results in inappropriate entry of the cells into mitosis. Thus measurement of the levels of cyclin B1 was also performed in these experiments. Figure 2 shows the relative amount of cyclin B1 per cell as a function of time after exposure of HeLa cells to 10 Gy X-rays.

Results

As a result of 10 Gy X-irradiation asynchronous HeLa S3 cells undergo transient delays in progression through the S and G2 phases (Figure 1). An increased fraction of cells in S phase is observed at 8 h post-irradiation, followed by peak G2 accumulation at 16 h. After the cell cycle perturbations a brief period of parasynchrony is observed (24–40 h), followed by a partial return of an asynchronous cell cycle distribution, where the S phase fraction is somewhat depressed and the G2 fraction is enhanced. It is possible that these later features of the cell cycle distribution are attributable to induction of aneuploidy in the cell population, as has been previously shown to occur following mitotic catastrophe (Mackey et al., 1988).

In a previous study (Ianzini and Mackey, 1997) we demonstrated that this radiation-induced delay in the late S and G2 phases is accompanied by premature activation of cyclin B1-dependent kinase and cyclin B1 accumulation and that occurrence of these events results in inappropriate entry of the cells into mitosis. Thus measurement of the levels of cyclin B1 was also performed in these experiments. Figure 2 shows the relative amount of cyclin B1 per cell as a function of time after exposure of HeLa cells to 10 Gy X-rays. Flow cytometric determinations of cyclin B1 levels were obtained using immunofluorescence staining with an anti-cyclin B1 antibody and counterstaining with PI. In this way bivariate analysis of the data provides determination of relative cyclin B1 levels as a function of DNA content. Cyclin B1 levels per cell rise immediately following irradiation, peak at 32 h post-irradiation and return to near control levels by 64 h. Note that in all cases cells containing detectable cyclin B1 levels are those having a late S/G2 DNA content, in accord with the cell cycle dependence of cyclin B1 protein levels in human cells (Gong et al., 1995), and mRNA content (Pines and Hunter, 1989). Thus at the time of onset of S phase delay (Figure 1, 8 h) cyclin B1 begins to accumulate and its amount remains higher than the control level throughout S6 h post-irradiation.

In accordance with data previously published (Ianzini and Mackey, 1997), following exit of cells from the block in S phase, DNA synthesis is resumed and cyclin B1 accumulates much faster, reaching peak levels by 16 h post-irradiation.
Delayed DNA damage

phase and during cyclin accumulation alterations in interphase nuclear and mitotic cell morphology were found. These alterations are the result of premature entry into mitosis, which gives rise to aberrantly condensed chromosomes and nuclear fragmentation. The kinetics of nuclear fragmentation, reported in Figure 3, show that fragmentation begins to rise 24 h post-irradiation. Only those nuclei having three or more fragments per cell were scored in the cell preparations and while scoring those slides cells exhibiting SPCC were noted, prior to accumulation of nuclear fragmentation, with morphologies identical to those described in a previous work (Ianzini and Mackey, 1997).

To assay the possible role played by DNA damage in the process of nuclear fragmentation we performed PFGE. Many different runs, conditions and standards were employed to assay the feasibility of the PFGE technique to address this issue. Figure 4 shows a PFGE time course (0–64 h) of DNA migration following X-irradiation of HeLa S3 cells at 37°C. Dose–response characteristics for DNA damage produced after 5, 10 and 20 Gy X-rays are also presented for comparison with the amount of damage produced at zero time when the cells are kept on ice. Following 10 Gy X-irradiation it appears that DNA is cleaved into fragments of ~40 kbp beginning at ~32–40 h post-irradiation. Under the running conditions of this gel (48 h, 3 V/cm, initial switch time 50 s, final switch time 90 s; Figure 4), and in accordance with what was found in other studies (Ager and Dewey, 1990; Ane et al., 1991; Nevaldine et al., 1993, 1994), two compression zones were evident. Immediately following irradiation the majority of the DNA migrating out of the plug is confined to the upper compression zone, between 720 kb and higher molecular weight values; between 720 and 40 kb DNA is resolved in distinct bands; the second compression zone is from ~40 kb to lower molecular weight values. The size of the fragments revealed by PFGE are in the same range as fragmented DNA obtained upon chromatin digestion with DNase I and approximate the average size of DNA loop domains, ~40 kb (Paulson and Laemmli, 1977; Hancock and Hughens, 1982). However, to rule out any possible misinterpretation of the results due to the lower compression zone that, under these running conditions, is close to 40 kb, we performed PFGE measurements using different running parameters, namely 11 h, 6 V/cm, initial switch time 1 s, final switch time 6 s, and a different molecular weight standard (Figure 5). Under these
Fig. 4. Pseudocolored images of the PFGE time course of DNA damage in HeLa S3 cells following X-irradiation. Lane 1, λ ladder molecular weight standard; lanes 2–4, dose–response curves for the initial DNA damage following 5, 10 and 20 Gy X-rays (cells were irradiated on ice and kept on ice), lane 5, sham-irradiated cells; lanes 6–14, DNA damage 0, 8, 16, 24, 32, 40, 48, 56 and 64 h post-irradiation (the cells were irradiated at 37°C and kept at 37°C). PFGE running parameters: 48 h, 3 V/cm, 50 s initial switch time, 90 s final switch time, 120°, 14°C. The gel was stained with 1:5000 SYBER Green I as described in Materials and methods.

Running conditions the upper compression zone in the gel is evident between 75 kb and higher molecular weights and the lower compression zone is present in the region of molecular weights lower than 5 kb, with linearity of DNA migration present between 75 and 5 kb. Under these conditions the DNA fragment sizes described earlier were verified, with DNA fragments of ~40 kb (and smaller) appearing at 32–40 h and persisting to 63 h following 10 Gy X-irradiation.

To determine if there was a correlation between the onset of nuclear fragmentation and the onset of delayed DNA damage following irradiation of HeLa cells another set of experiments was carried out in which both nuclear fragmentation and in situ DNA damage labeling were monitored. DNA damage was assayed, as described in Materials and methods, by labeling of free 3'-OH ends in cellular DNA using TdT with FITC-conjugated 5'-dUTP as substrate. Figure 6 shows the kinetics of fragmented nuclei in HeLa cells irradiated with 10 Gy X-rays and followed up to 50 h post-irradiation. As for Figure 3, nuclear fragmentation starts to arise 24 h post-irradiation and at 32 h the percentage of fragmented cells peaks at a value of ~65%.

After the increase in nuclear fragmentation (Figure 6, 24 h) an increase in TdT-positive cells was found, as shown in Figure 7, which presents the kinetics of TdT-positive cells following 10 Gy X-rays versus time. At 32 h post-irradiation the percentage of TdT-positive cells begins to rise slowly and peaks at 40 h post-irradiation; note that since this assay uses a direct labeling technique the results are not complicated by non-specific staining of secondary reagents. As expected, positive controls generated using DNase I digestion showed bright fluorescence, while negative controls, incubated without TdT, were unstained (data not shown).

Figure 8 shows photographic images of cells from an earlier experiment presenting TdT-positive labeling. At 32 h post-
irradiation TdT-positive cells were found which contained condensed chromosomes, consistent with the hypothesis that cytoplasmic nucleases are cleaving chromatin in SPCC. Although in this particular experiment the features of TdT-positive cells arose earlier than in that shown in Figure 7, a consistent pattern of cell cycle delay—nuclear fragmentation TdT-positive cells was always observed in these studies. TdT-positive interphase cells exhibit a punctate staining pattern, indicating that the recognized damage is inhomogeneously distributed. It is important to note that these cells are still viable 48 h post-irradiation. Cell viability was assayed in sham-irradiated controls and irradiated cells 48 h post-irradiation. Trypan blue staining showed that both the sham-irradiated control and the irradiated cells are able to extrude the drug from their cytoplasm, 92 and 91% for controls and irradiated cells respectively. Thus while mitotic catastrophe has occurred in the irradiated populations, cells retained physiological viability.

Discussion

Genomic instability has been defined as an increased rate of acquisition of alterations in the mammalian genome (Morgan and Murnane, 1995). Often observed as a result of exposure of cells to clastogenic agents (Murnane, 1996), genomic instability is also considered to be a hallmark of oncogenic transformation (Cheng and Loeb, 1993; Cox, 1994). The mechanistic basis for genomic instability, either as a result of carcinogen exposure or as an intrinsic property of transformed cells, is unknown. While numerous end-points are considered to be reflective of genomic instability, including but not limited to microsatellite instability or polymorphisms (Thibodeau et al., 1993), gene amplification (Ma et al., 1993), persistent production of dicentric chromosomes (Holmberg et al., 1993) or micronuclei (Jamali and Tropp, 1996; Manti et al., 1997), loss of allelic heterozygosity (Cheng and Loeb, 1993), radiation-induced mutagenesis (Chang and Little, 1992; Paquette and Little, 1994), oncogenic transformation (Mendonca et al., 1993; Coleman and Tsongalis, 1995), persistent oxy radical activity (Clutton et al., 1996) and radiation-induced damage attributed to breakage–fusion–bridge cycles (Murtif Park et al., 1992; Alvarez et al., 1993; Day et al., 1993; Pommier et al., 1995), a mechanistic link between genomic instability and DNA damage has not yet been proposed.

Evolutionarily conserved mechanisms have been found which modulate cell cycle progression and there are at least two points in the cell cycle where progression is halted following irradiation exposure. The p53-dependent block in G1 phase (Kastan et al., 1991; Dulic et al., 1994) is thought to facilitate repair of damaged DNA prior to S phase entry, while the radiation-induced G2 block (Whitmore et al., 1961), also described by Painter and Hughes (1961) as a slowing in progression through S phase ("S retention"), is thought to contribute to the repair of potentially lethal radiation damage (Phillips and Tolmach, 1966). The G2 block is currently associated with at least two distinct checkpoint pathways in mammalian cells: inhibition of post-transcriptional modification of cdc2, thus preventing premature entry into mitosis in the presence of incompletely replicated or damaged DNA (Jin et al., 1996); an earlier topoisomerase II-dependent checkpoint which regulates decatenation of replicated DNA necessary for entry of cells into mitosis (Downes et al., 1994). Although it is tempting to suggest that radiation-induced damage to a particular cell cycle control gene acts as a promoter for genomic instability, target theory analyses have indicated that the incidence of this effect is probably too high to be due to such specific genetic alterations (Kadhim et al., 1992).

In the present paper we show that abrogation of a G2/M radiation cell cycle checkpoint which leads to mitotic catastrophe is associated with DNA damage. In particular, PFGE measurements show the presence of damaged DNA migrating as small fragments of ~40 kb (and smaller) in irradiated cells beginning 32–40 h post-irradiation, following the onset of mitotic catastrophe. TdT DNA labeling experiments showed that this DNA damage appears at 32 h, after nuclear fragmentation has occurred, peaks 40 h post-irradiation and is characterized by a punctate staining pattern which indicates that such damage is inhomogeneously distributed within the cell nucleus. Moreover, TdT-positive cells are also found which contain condensed chromosomes, consistent with the hypothesis that cytoplasmic nucleases are cleaving chromatin during or immediately following SPCC. It is important to note that DNA
Fig. 8. Photographic images of TdT-positive HeLa cells. Photographs were taken from the cell preparations labeled with TdT according to Materials and methods (A) Sham-irradiated control; (B) 32 h following 10 Gy X-irradiation; (C) 72 h following 10 Gy X-irradiation.
strand breaks produced by ionizing radiation give rise to ‘sticky ended’ DNA strand breaks and that the TdT assay is not able to recognize these ‘sticky ends’; rather, it labels only ‘clean ends’ (Gorczyca et al., 1993). Also, note that immediately following irradiation with the doses used cells do not present small DNA fragments such as those found in the PFGE time course experiments. Thus these results suggest that the delayed DNA strand breaks reported herein are generated via processes related to SPCC and nuclear fragmentation. In fact, because DNA strand breaks are promptly rejoined after these doses of X-rays and since post-irradiation treatment with inhibitors of cell cycle progression is known to suppress both SPCC and nuclear fragmentation (Mackey, 1993) these results support the hypothesis that SPCC-mediated DNA damage occurs as an outcome of abrogation of G2/M cell cycle checkpoint pathways. SPCC and mitotic catastrophe occurring as a result of over-accumulation of cyclin B1-dependent kinase activity have also been demonstrated in BHK cells by Heald et al. (1993). Thus mitotic catastrophe occurring as a result of over-accumulation of cyclin B1 during the post-irradiation delay in late S and G2 phases may lead to a delayed DNA damage phenotype. In particular, premature entry into mitosis may expose incompletely or improperly condensed chromatin to the DNA degrading action of cytoplasmic nucleases at the time of nuclear membrane breakdown, as the size of the DNA fragments observed are in the same range as those obtained upon chromatin digestion with DNase I and are approximately the same sizes as those found in DNA loop domains (Paulson and Laemmli, 1977; Hancock and Hughens, 1982).

Since the specific DNA damage characterized in this report is found only hours post-irradiation, these results may describe a novel mechanism for generation of DNA damage which occurs a day or more following irradiation and which is chemically distinct from the initial DNA damage caused by ionizing radiation exposure, thus providing a possible initiating mechanism for radiation-induced genomic instability. Further, since not all the cells undergoing radiation-induced SPCC and nuclear fragmentation may be viable, these findings are also particularly pertinent to the understanding of mechanisms underlying the delayed lethal effects of irradiation. In the authors’ laboratory experiments using time-lapse cinemicrography are under way to determine the reproductive fate of those cells undergoing radiation-induced SPCC and subsequent nuclear fragmentation, to determine if these alterations are propagated into subsequent cell generations.

In conclusion, the mechanism for production of this delayed DNA damage may lie in the following sequence of events: X-irradiation causes delays in the late S and G2 phases of the cell cycle, whereupon cyclin B1 accumulates to extraordinary levels; such enhanced levels of cyclin B1 lead to abrogation of the G2/M checkpoint, with premature entry into mitosis, which, in turn, causes nuclear fragmentation and subsequent delayed DNA damage. Further experiments are underway in the authors’ laboratory to test these hypotheses.

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