

Bystander effects in radiation-induced genomic instability

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Abstract

Exposure of GM10115 hamster–human hybrid cells to X-rays can result in the induction of chromosomal instability in the progeny of surviving cells. This instability manifests as the dynamic production of novel sub-populations of cells with unique cytogenetic rearrangements involving the “marker” human chromosome. We have used the comet assay to investigate whether there was an elevated level of endogenous DNA breaks in chromosomally unstable clones that could provide a source for the chromosomal rearrangements and thus account for the persistent instability observed. Our results indicate no significant difference in comet tail measurement between non-irradiated and radiation-induced chromosomally unstable clones. Using two-color fluorescence in situ hybridization we also investigated whether recombinational events involving the interstitial telomere repeat-like sequences in GM10115 cells were involved at frequencies higher than random processes would otherwise predict. Nine of 11 clones demonstrated a significantly higher than expected involvement of these interstitial telomere repeat-like sequences at the recombination junction between the human and hamster chromosomes. Since elevated levels of endogenous breaks were not detected in unstable clones we propose that epigenetic or bystander effects (BSEs) lead to the activation of recombinational pathways that perpetuate the unstable phenotype. Specifically, we expand upon the hypothesis that radiation induces conditions and/or factors that stimulate the production of reactive oxygen species (ROS). These reactive intermediates then contribute to a chronic pro-oxidant environment that cycles over multiple generations, promoting chromosomal recombination and other phenotypes associated with genomic instability. © 2002 Elsevier Science B.V. All rights reserved.

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1. Introduction

Exposure to ionizing radiation can result in the manifestation of a number of deleterious endpoints in the progeny of irradiated cells, multiple generations af-

ter radiation exposure. These deleterious effects are broadly grouped under the all-embracing phenomena of radiation-induced genomic instability, which we define as the increased rate of acquisition of alterations in the genome. Because these effects can be observed days, weeks, months, even years after exposure of the original progenitor cell to radiation, we believe that radiation initiates a process within a cell that is perpetuated in the progeny of that irradiated cell [1,2]. The

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consequences of the initiation and perpetuation of genomic instability includes such deleterious endpoints as chromosomal rearrangements, delayed mutation, DNA nucleotide repeat instability, cellular transformation, and even cell death (reviewed in [1,3]). Whatever the process(es) that radiation initiates it/these must be capable of inducing gross genomic rearrangements for prolonged periods of time over sequential cell generations.

We have been investigating chromosomal destabilization as one endpoint of radiation-induced genomic instability. Conventional wisdom dictates that the primary DNA lesion leading to chromosomal rearrangements is the DNA double-strand break [4,5]. This suggests that in our chromosomally unstable cell clones there should be increased levels of endogenous DNA breaks that contribute to the novel chromosome rearrangements observed. We have tested this hypothesis using the comet assay, a sensitive means of detecting DNA strand breaks, in clones of irradiated GM10115 cells that were chromosomally stable or that demonstrated significant radiation-induced chromosomal instability. In addition, we have measured chromosomal recombination at interstitial telomere repeat-like sequences (ITBs) to determine the necessity of strand-breakage as a prerequisite to the chromosomal changes observed in unstable clones. Large discrete chromosomal regions containing telomere repeat-like sequences are common in many rodent cells [6], and we have previously demonstrated that these ITBs are involved in many of the chromosomal rearrangements observed in unstable cell clones [7]. Using two-color fluorescence in situ hybridization we have expanded these initial observations to investigate whether the ITBs are disproportionately involved in delayed rearrangements involving the human chromosome within the hamster genome, thus implicating an involvement of recombinational processes in driving chromosomal rearrangements.

2. Materials and methods

GM10115 (from the Human Genetic Mutant Cell Repository, Camden, NJ) contain a single copy of human chromosome 4 in a background of 21–24 hamster chromosomes. These cells are maintained in exponential growth in Dulbecco's modified Eagle's medium

supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 penicillin, 100 mg/ml streptomycin and 0.2 mM L-proline. This hamster–human hybrid cell line is cultured at 34 °C in an atmosphere of 5% CO₂ in air.

The generation of chromosomally unstable clones of cells by exposure to X-rays has been described elsewhere [8–10]. A clone is classified as chromosomally unstable if there are ≥ 3 sub-populations of cells within the clone that show different cytogenetic rearrangements involving the human chromosome. The total number of rearrangements involving the human chromosome must make up at least 5% the total number of metaphase cells analyzed per clone. This is an operational definition and instability is based on analysis of at least 200 metaphase cells per clone following fluorescence in situ hybridization of probes to human chromosome 4. Following exposure to X-rays we have isolated a number of highly unstable clones that were used in the experiments described [9,10].

Analysis of DNA single-strand breaks utilized the alkaline comet assay as described by Tebbs et al. [11]. Briefly, cells were trypsinized and re-suspended in 40 μ l of 0.5% low gelling point agarose and pipetted onto agarose-precoated slides. The agarose suspension was covered with a 25 mm \times 25 mm coverslip and placed at 4 °C. After 5 min the coverslip was gently removed and the slide was submerged into lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 10% DMSO, 1% Triton X-100, pH 10) for at least 1 h. After lysis, the slides were equilibrated for 40 min in a jar containing alkaline buffer (300 mM NaOH, 1 mM EDTA, pH >13), transferred into an electrophoresis unit with alkaline buffer, and subjected to an electric field of 0.86 V/cm for 15 min. Following electrophoresis the microgels were neutralized in 0.4 M Tris (pH 7.5), rinsed, dehydrated in 100% ethanol for 2 min and allowed to dry at room temperature. The DNA was stained with YOYO[®]-1 (Molecular Probes, Oregon, USA) (1:1000 in antifade) and visually examined by fluorescence microscopy. Analysis of DNA strand breakage was performed according to Collins et al. [12]. Based on the extent of strand breakage, each cell was assigned to one of five classes, ranging from 0 (no visible tail) to 4 (maximally damaged). A cumulative score (damage units) was calculated by multiplying the damage class by the percentage of cells per class, giving a cumulative score between 0 (100% of

cells in class 0) and 400 (100% of cells in class 4). Although these units are arbitrary, they are related to the relative tail intensity as a function of break frequency [13]. Data are derived from two independent experiments per clone with at least 100 cells per experiment (from two replicate slides each) analyzed.

Analysis of the potential involvement of ITBs in the recombination between the human and hamster chromosomes was by two-color fluorescence in situ hybridization. The ITBs were identified after hybridization with the fluorescence labeled plasmid pSXNeo1.6, containing 1.6kb of T₂AG₃ repeats kindly provided by Dr. J.P. Murnane at the University of California, San Francisco. The human chromosome was identified after hybridization and binding of a fluorescence antibody against a labeled Bluescript vector based library of human chromosome 4-specific DNA sequences kindly provided by Drs. J. Gray and D. Pinkel, University of California, San Francisco.

The protocols for plasmid expansion, DNA labeling, two-color hybridization and analysis were as previously described [7]. At least 200 metaphase cells were analyzed per clone. From previous measurements we know that the ITB makes up ~5% the total length of all the chromosomes in metaphase cells containing 21–24 chromosomes [7].

3. Results

The mean values from two independent experiments assigned to the tail lengths for non-irradiated GM10115 cells, two clones derived from cells that survived irradiation, but were chromosomally stable, and four chromosomally unstable clones are presented in Fig. 1. The distribution of comet sizes within these clones is summarized in Fig. 2. There was no significant difference in the endogenous level of DNA

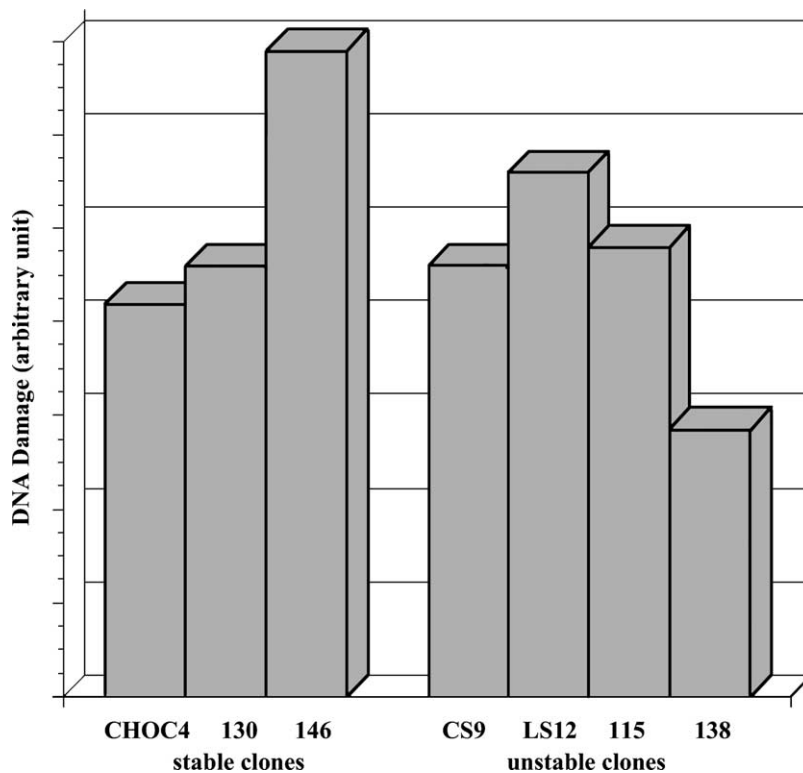


Fig. 1. The mean value assigned to comet length in non-irradiated GM10115 (CHOC4) cells, two irradiated but chromosomally stable clones (146, 130), and four radiation-induced chromosomally unstable clones (CS9, LS12, 138 and 115). The results are the mean of two independent experiments.

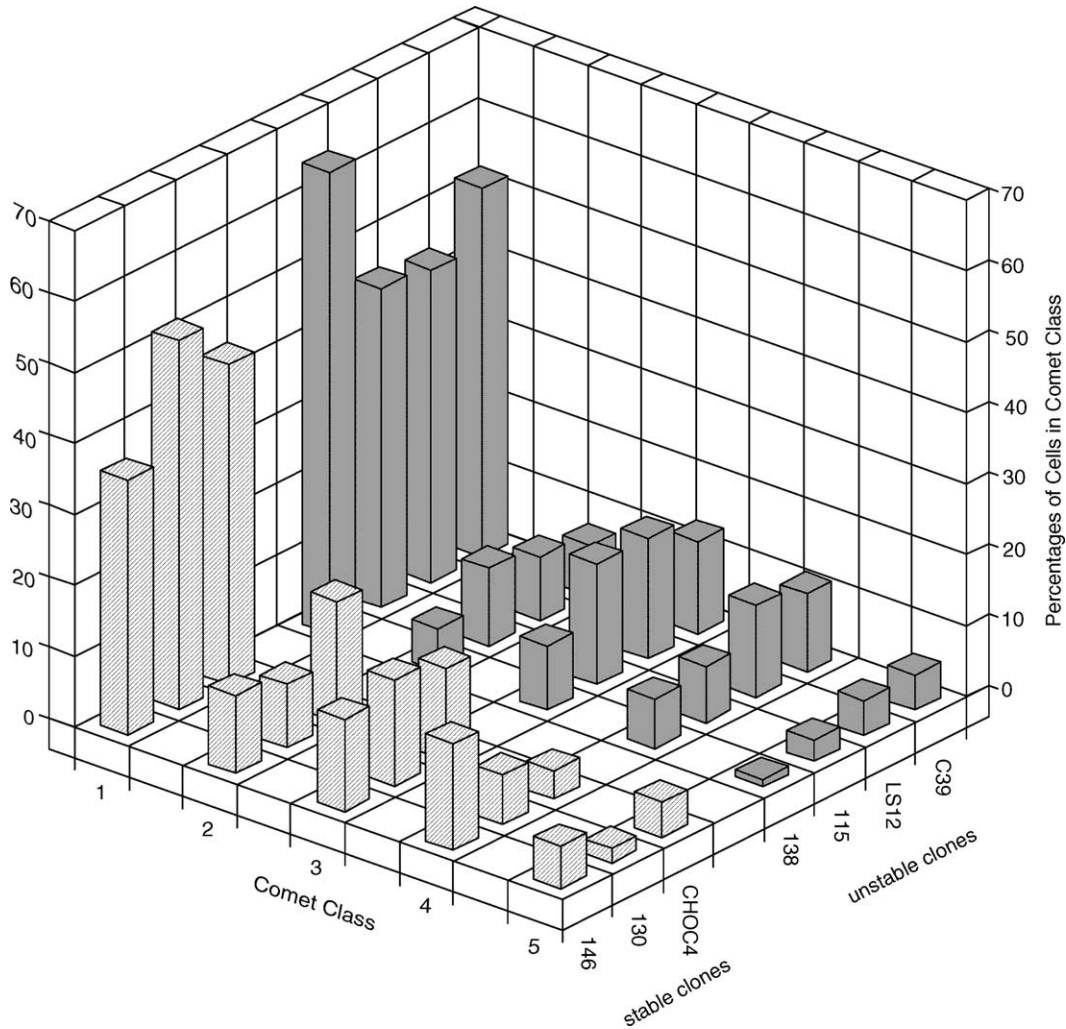


Fig. 2. The distribution of comet sizes within the clones.

breaks observed between chromosomally stable and unstable clones.

Following two-color chromosome painting the number of sub-populations of cells within a clone showing unique rearrangements involving human chromosome 4 and the percentage of tricolor junctions (hamster-ITB-human) was determined (Table 1). All clones examined had between 21 and 23 chromosomes, and between 14 and 16 ITBs (Fig. 3A). With the exception of clones 24 and 115, unstable clones showed significantly higher frequencies of rearrangements that juxtaposed human chromatin and

hamster ITBs (Fig. 3B) than would be expected by chance. Two clones, 138 and CS9 showed $\geq 96\%$ of the rearrangements involved three color junctions. ITBs make up $\sim 5\%$ of the GM10115 genome [7] so if the observed rearrangements involving human chromosome 4 were random, we would expect them to be involved in $\sim 5\%$ of chromosomal rearrangements. Clearly for the majority of our unstable clones, the sub-populations of chromosomally unstable cells showed the ITBs to be involved significantly more often than expected by chance. However, this was not always the case. In two of our unstable clones, 24

Table 1

Two-color fluorescence in situ analysis of chromosomal instability, and the involvement of the ITB in chromosomal recombination between the human and hamster chromosomes

Clone	Cells with chromosome 4 rearranged (%)	No. of sub-populations ^a	Three color junctions (%)	Chromosome no. ^b	ITBs per metaphase cell ^b
GM101115 ^c	0	1	–	22.9 ± 0.3	15.8 ± 0.5
Stables					
102	2	2	0.0	22.8 ± 0.5	15.5 ± 0.6
110	100	2	85.0	22.7 ± 0.6	15.6 ± 0.6
114	100	1	0.0	22.8 ± 0.5	15.8 ± 0.6
118	0	1	–	22.8 ± 0.5	15.7 ± 0.5
130	1	2	0.0	22.3 ± 0.7	14.6 ± 0.9
132	100	1	0.0	22.8 ± 0.5	15.6 ± 0.6
133	100	1	0.0	22.2 ± 0.6	15.2 ± 0.8
141	3	2	33.0	22.6 ± 0.6	15.5 ± 0.6
145	0	1	–	22.8 ± 0.4	15.7 ± 0.6
LS1	1	2	0.0	22.8 ± 0.5	15.7 ± 0.7
Unstables					
126	5.3	3	20.0	22.8 ± 0.7	15.7 ± 0.5
152	5.5	5	27.3	22.8 ± 0.4	15.7 ± 0.6
24	100	8	0.0	22.2 ± 0.7	14.6 ± 0.8
147	10.1	7	26.7	22.6 ± 0.7	14.7 ± 0.8
115	98.5	19	0.5	22.3 ± 0.7	15.8 ± 0.6
CS9	100	17	96.3	21.2 ± 0.7	15.0 ± 0.8
2	96.2	17	72.8	21.5 ± 0.8	14.8 ± 1.0
7	100	>20	14.5	22.5 ± 0.8	15.8 ± 1.0
LS12	95.9	>20	55.1	22.3 ± 0.7	15.3 ± 1.0
23	100	16	36.5	23.0 ± 0.8	15.9 ± 0.8
138	100	16	96.0	22.0 ± 0.6	15.8 ± 0.6

^a Sub-populations = populations of cells within a clone that show unique rearrangements of human chromosome 4.

^b Mean ± S.D.

^c Five individual non-irradiated clones of GM10115 cells were analyzed.

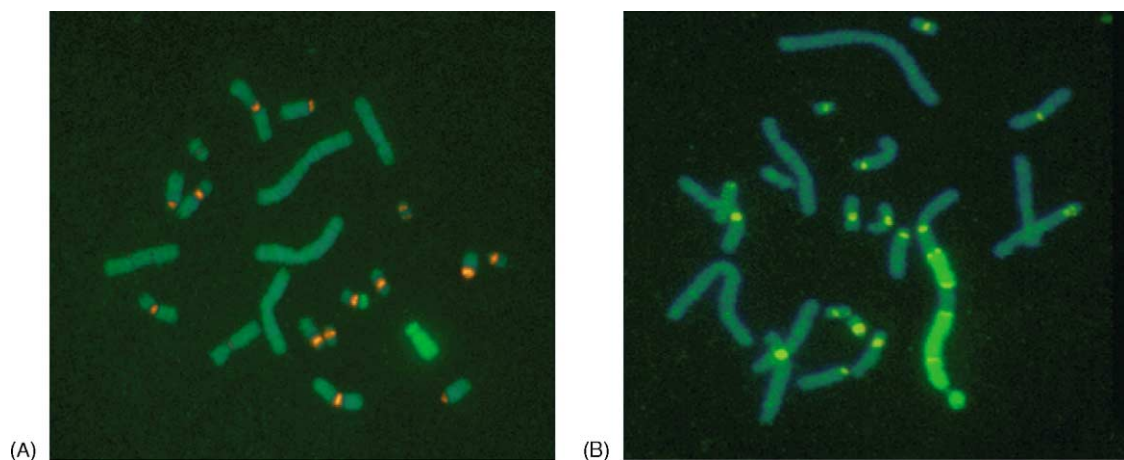


Fig. 3. Two-color fluorescence in situ hybridization using probes against the ITB (red) and human chromosome 4 (green). CHO chromosomes are counterstained with DAPI. (A) Control GM10115 cells. (B) Unstable CS9 cells, multiple hamster–ITB–human chromatin junctions are apparent.

and 115, the ITBs were not involved in any of the rearrangements involving the human chromosome in the multiple sub-populations of rearranged cells.

We also examined the potential involvement of ITBs in chromosomal rearrangements in a number of our clones derived from cells that survived irradiation, but were chromosomally stable. Some of these, e.g. 110 and 141 showed a high frequency of metaphase cells with three-color junctions, but these obviously did not go on and become unstable. Clones 114, 132 and 133 were all homogeneous with respect to a single type of aberration involving the human chromosome. These were probably the result of aberrations directly induced by the radiation. Other clones, e.g. 141, may have initiated the instability process but the unstable sub-population failed to thrive within the clone and consequently did not evolve and did not meet our criteria for instability.

4. Discussion

The alkaline comet assay can measure DNA strand breaks induced by X-ray doses as low as 0.6 cGy [14] indicating it is a very sensitive assay for analysis of DNA breakage. Using this assay we were unable to detect any significant difference in endogenous break frequency between non-irradiated cells, our chromosomally unstable clones, or our stable clones. This does not rule out the possibility that in unstable clones there may be transient increases in DNA breaks, or that the level of breaks may be elevated but still below the level of detection using this assay. Indeed, Suzuki et al. have recently demonstrated that radiation-induced genomic instability causes delayed DNA breakage, and subsequent activation of TP53 function may eliminate cells which would otherwise accumulate genomic alterations (Suzuki et al., manuscript submitted for publication). Despite the importance of DNA damage in the initial responses to radiation exposure, DNA strand breakage appears to play a significantly more minor role in delayed genomic instability [5].

On the other hand, analysis of three-color junctions involving recombination between the human chromosome, an ITB, and hamster chromatin indicates that they are involved at a significantly higher frequency than would be expected by chance. This confirms and expands our previous observations [7] suggesting

that a recombinational process, involving, or perhaps driven by ITBs can perpetuate chromosomal instability in GM10115 cells. If this is indeed the case, as our studies suggest, then what stimulates this recombinational process? We propose that this involves epigenetic effects or bystander effects (BSEs).

BSEs are those effects observed in cells that were themselves not irradiated, but were bystanders at the time of irradiation. These effects include induction of mutation, micronuclei, sister chromatid exchange, transformation, gene expression and even cell death. There is a long literature on clastogenic factors and other “compounds” secreted by cells after exposure to DNA damaging agents that stimulate and/or modify responses in cells that were not damaged [15–20]. By far the most compelling evidence for BSEs come from studies using charged particle microbeams [21,22]. Irradiation of cellular cytoplasm with either a single or an exact number of α -particles results in gene mutation in the nucleus [22] or micronuclei in non-irradiated cells [21,23]. The majority of studies to date on BSEs have used high LET radiation. Low LET X-rays can also elicit this effect. Mothersill and Seymour took medium from irradiated human epithelial cells and demonstrated that this was sufficient to reduce the clonogenic survival in unirradiated cells [24,25]. This implicates a secreted factor that can kill unirradiated cells and we propose that factors similar to, or including these bystander factors described by others play a significant role in the initiation and perpetuation of the multiple endpoints associated with radiation-induced genomic instability.

Cellular irradiation leads to the production of reactive oxygen species (ROS) such as the superoxide anion, hydrogen peroxide, and hydroxy radicals, which can indiscriminately damage a host of cellular components. A number of studies from Lehnert and co-workers have suggested that ROS also contribute to the induction of SCEs in bystander cells [26–28]. The observed increases in SCEs are very small but suggest that a long-lived radical can persist over multiple cell generations to elicit SCEs, or that new ROS are continuously being produced to increase SCEs. There is evidence for long-lived radicals produced by ionizing radiation [29] and there is recent evidence for a role for ROS in modulating the mutagenic responses of cytoplasmic irradiation. Wu et al. [22] pre-treated cells with the antioxidant dimethyl sulfoxide

(DMSO) to reduce ROS, or the thiol-depleting drug buthionine-*S-R*-sulfoximine (BSO) to reduce intracellular glutathione, and observed significant reductions in mutation yields in human hamster A_L cells irradiated with four α -particles through the cytoplasm. Furthermore, these investigators also demonstrated the induction of 8-hydroxy-deoxyguanosine, a marker of oxidative DNA damage in the nucleus of A_L cells irradiated with eight α -particles through the cytoplasm.

While we do not dispute a role for ROS in the BSE it is difficult to envisage a long-lived radical produced by radiation that is capable of such striking levels of cell kill, mutation induction, and chromosome damage as those described for BSE. Instead we propose that some BSE factor (BSEF) is stimulated by cellular irradiation and secreted into the culture medium. This BSEF could itself be responsible for the observed BSEs and/or could generate extra-cellular ROS and act in conjunction with these ROS could elicit the observed BSEs. In turn these ROS could stimulate BSEF creating cycles of BSEF \rightarrow ROS \rightarrow BSEF \rightarrow ROS, thus perpetuating a source of damage over time that could account for the delayed instability observed. A version of this hypothesis was originally proposed by Iyer and Lehnert [30] and there is precedence for this: transforming growth factor- β 1 (TGF- β 1) is rapidly secreted in response to low doses of ionizing radiation [31,32]. TGF- β 1 can also activate cell surface membrane-associated NADH oxidase releasing H₂O₂ and other ROS as a consequence [33–36], which in turn can stimulate TGF- β 1 [36,37]. TGF- β 1 is increased in the supernatants of irradiated cells and addition of recombinant TGF- β 1 to unirradiated cells and its ability to produce an SCE response can be totally inhibited by anti-TGF- β 1 neutralizing antibodies [30]. We do not wish to imply that all BSEs observed are due to TGF- β 1 secretion and subsequent redox activation. Indeed, a number of genes are induced by radiation including tissue-type plasminogen activator, collagenase, cytokines such as tumor necrosis factor- α , α -interferon, and fibroblast growth factor 2 (reviewed in [37]). Instead we are trying to establish precedence for BSEF \rightarrow ROS \rightarrow BSEF cycles to play a role in the observed detrimental effects associated with radiation exposure in surviving cells. A host of compounds may be secreted by cells and function alone, or in concert to elicit BSEs. For example, Narayanan et al. [38] have also shown that low doses of α -particles increase

both the expression and production of interleukin-8 which in turn is linked to induced increases in ROS. These investigators demonstrated that treating cells with interleukin-8 at concentrations commensurate with those occurring after α -particle irradiation can induce increased ROS in otherwise untreated cells.

Our studies to date argue against ROS acting alone to induce instability in our GM10115 cells. Using concentrations of hydrogen peroxide that cause up to three logs of cell kill [9], or concentrations of xanthine/xanthine oxidase that cause up to four logs of cell killing [39], we did not find any chromosomal instability in >80 independently isolated clones [10,39]. These data demonstrate that ROS induced by a single acute chemical exposure are not important in initiating instability in our model system. However, chronic exposure to endogenous metabolic or exogenous environmental oxidative stress can contribute to gene amplification and genomic instability in other model systems [40], and it is more likely that chronic long term exposure is necessary to elicit a delayed response.

Alternatively, normal cells have a “control mechanism” that normally suppresses genomic instability. Stimulating a BSEF may disrupt this control resulting in activation of a destabilizing process that leads to instability (Barcellos-Hoff, personal communication). For example, similar recombination events involving the ITB to those described here have been observed in other hamster cell lines in the absence of radiation [41,42]. This suggests that cells may normally suppress instability but that radiation may stimulate factors that overcome this suppression and promote an endogenous process.

There is direct evidence that genomic instability has an epigenetic component, i.e. that radiation might mediate its delayed effects via extranuclear and even extracellular events. Kadhim et al. [43] analyzed chromosomal instability in murine stem cells following α -particle irradiation. Many of the surviving stem cells analyzed by these investigators were those that, by chance, did not get “hit” during irradiation. In fact, Kadhim et al. [43] calculated from their survival data that the probability of a stem cell surviving a “hit” or passage of a single α -particle was \sim 10%, but cytogenetic analysis of surviving colonies revealed that 40–60% had karyotypic abnormalities. In more recent studies these investigators have unambiguously demonstrated that when a protective grid

shielded cells it was the non-irradiated cells that exhibited delayed chromosomal instability [44]. These data indicate that BSEs can result in chromosomal instability, which has significant implications for the fate of cells surviving high LET irradiation. However, the significance of these studies following exposure to low LET radiation is not clear since Kadhim et al. did not observe instability using the bone marrow assay system after X-irradiation [43].

Interestingly, one consistent characteristic of the large battery of chromosomally unstable clones we have generated in the past is that they almost all show a significantly reduced PE [8,10]. That is, our chromosomally unstable clones also exhibit lethal mutations [45–47] or delayed reproductive cell death [48,49], another endpoint associated with radiation-induced genomic instability. We have recently demonstrated that reproductive failure in chromosomally unstable clones can in large part be rationalized by a fraction of the clonal population predisposed to undergoing apoptosis [39]. Apoptosis involves a complex series of biochemical reactions that result in a programmed elimination of a cell from the proliferating population. Induction of apoptosis by actinomycin-D, etoposide and staurosporine, and induction of necrosis by sodium azide in GM10115 cells was accompanied by an increase in the level of intracellular peroxy radicals and lipid peroxidation products, two independent endpoints typically associated with oxidative stress. We observed similar findings in several of our chromosomally unstable clones which showed delayed apoptosis [39]. These results suggest that the elevated levels of cellular free radical damage detected in chromosomally unstable clones could be secreted by unstable cells or derived from the fraction of cells dying by apoptotic and/or necrotic processes. Our observations support those of Clutton et al. [50] who demonstrated oxidative stress and oxy-radical formation in α -particle-induced chromosomal instability in human and murine bone marrow cells. We propose that the increased oxy-radical levels described by us [39] and by Clutton et al. [50] are a byproduct of genomic instability. These apoptosing cells constitute a risk to the cells in culture by releasing a variety of factors, e.g. inflammatory cytokines, nitric oxide-dependent factors, potential nucleases and signaling molecules in addition to the peroxy radicals that can trigger potentially detrimental cellular responses. We propose that these

contribute to the cycles of BSEFIROSIBSEFIROS, etc. which in turn serve to perpetuate the de novo formation of novel chromosomal rearrangements we observe as radiation-induced chromosomal instability.

There is increasing in vitro and in vivo evidence that BSEs may be major players in the biological consequences of radiation exposure [51,52]. This paradigm shift away from the dogma that radiation-induced deposition of energy in the nucleus is paramount for the deleterious biological effects of radiation will impact on how we interpret and evaluate the risk(s) associated with radiation exposure. Consequently it is important we understand what the BSE is, its mechanism(s) of action, the molecular and cellular events associated with its induction, and ultimately its biological and clinical significance.

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