Melanoma Cells Express Elevated Levels of Phosphorylated Histone H2AX Foci

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When human cells sustain a DNA double-strand break (dsb), histone H2AX in chromatin surrounding the DNA break is phosphorylated, marking repair foci. The number of phosphorylated histone H2AX (γH2AX) foci approximates the number of dsb present in the cell's nuclear DNA. We observed 0.4 γH2AX foci per nucleus in primary human melanocytes. In contrast, in four melanoma cell lines, we detected 7–17 γH2AX foci per nucleus, a 17–42 times increase in the basal level of γH2AX foci in melanoma cells relative to melanocytes (MC). Thus, untreated melanoma cells express significantly greater numbers of γH2AX foci than do untreated MC. Detection and rejoining of ionizing radiation-induced DNA dsb proceeded as rapidly in melanoma cells as in MC. Melanoma cells, however, reduced the number of radiation-induced γH2AX foci down only to pre-irradiation levels. Co-localization of the majority of γH2AX foci with ataxia telangiectasia mutated, BRCA1, 53BP1, and Nbs1 foci in untreated melanoma cells indicated that the additional foci in melanoma cells were associated with a DNA change that the cells interpret as DNA dsb. Co-localization of γH2AX foci with the telomere replication factor 1 protein in untreated melanoma cells indicates that the additional foci in untreated melanoma cells are associated with dysfunctional telomeres that induce a DNA damage stress response.

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The skin is the primary barrier for humans against the external environment. Thus, skin cells are frequently the first cells to be exposed to physical and chemical genotoxic agents such as ultraviolet (UV) and ionizing radiations (IR). Exposure to UV radiation is considered to be the major contributing factor to melanomagenesis (Jhappan *et al*, 2003), although the molecular pathway(s) leading from UV-induced DNA photoproducts to melanoma is as yet unclear. Melanoma develops through well-defined morphological and histological stages that involve the loss of control of cell proliferation, acquisition of invasiveness, and ultimately acquisition of metastatic potential (Clark *et al*, 1984). The normal melanocytic cell, presumably damaged in its genome by sun exposure, develops through primary melanoma *in situ* and ultimately to metastatic melanoma. From its earliest stages, melanomagenesis involves elevated frequencies of mutations and/or loss of tumor suppressors and oncogenes (Albino *et al*, 1997; Hussein and Wood, 2002), microsatellite instability (Hussein and Wood, 2002), aneuploidy detected with increases in the DNA content (Pilch *et al*, 2000; Runger *et al*, 2003), and an increase in the frequency of micronuclei (Runger *et al*, 2003).

The chromosome rearrangements observed in melanoma cells likely require naturally or environmentally induced DNA double-strand breaks (dsb) as intermediates. The presence of DNA dsb in the cell genome induces formation of repair foci (Petrini and Stracker, 2003). So one approach to assess the involvement of DNA dsb in chromosome instability is to evaluate the expression of dsb-induced repair foci in melanoma cells. Nuclear phosphatidylinositol 3–OH serine/threonine protein kinase-like kinases (PIKK) recognize the presence of DNA dsb in the cell DNA, and transduce this fact via phosphorylations to downstream effector proteins that participate in DNA dsb repair processes. One type of DNA dsb repair focus involves phosphorylated H2AX (γH2AX) histone proteins (Sedelnikova *et al*, 2002; Rothkamm and Lobrich, 2003). Production of DNA dsb in nuclear DNA induces phosphorylation of the H2AX histone at serine 139, which is in an evolutionarily conserved PIKK motif (Rogakou *et al*, 1998, 2000; Burma *et al*, 2001). Two nuclear PIKK, the ataxia telangiectasia mutated (ATM) and DNA-dependent protein kinase (DNA PK) protein kinases, have been shown to phosphorylate histone H2AX in response to IR forming nuclear foci (Burma *et al*, 2001; Bakkenist and Kastan, 2003; Park *et al*, 2003; Stiff *et al*, 2004). As the ATM and DNA PK kinases phosphorylate the H2AX protein in chromatin adjacent to a DNA dsb, the locally high concentration of γH2AX protein can be detected by immunofluorescence as foci (Rogakou *et al*, 1999; Rothkamm and Lobrich, 2003). In addition to trauma-induced DNA dsb, phosphorylated H2AX histone foci have been detected in S-phase cells (Mirzoeva and Petrini, 2003) and at uncapped telomeres (Takai *et al*, 2003). There is a close correlation between the number of DNA dsb and the

Abbreviations: ATM, ataxia telangiectasia mutated; DI, DNA index; DNA PK, DNA-dependent protein kinase; dsb, double-strand break; FB, fibroblasts; IR, ionizing radiation; PBS, phosphate-buffered saline; PIKK, protein kinase-like kinases; MC, melanocytes; TRF, telomere replication factor

number of Type II nuclear γH2AX foci induced by IR (Sedelnikova *et al*, 2002; Rothkamm and Lobrich, 2003). Therefore, the number of γH2AX foci has been taken to indicate the number of DNA dsb in the cell's nuclear DNA (Sedelnikova *et al*, 2002; Rothkamm and Lobrich, 2003).

In a recent study of the expression of phosphorylated H2AX histone in cultured primary skin cells and their cancers, we found that untreated cancer cells expressed significantly higher levels of γH2AX foci than did primary skin cells. These phenomenological observations suggested that some characteristics of skin cancer cells result in higher basal levels of DNA repair foci. As some chromosome rearrangements require DNA dsb as an intermediate, and melanoma cells generally express chromosome instability during their development, we speculated that the high basal levels of γH2AX foci might reflect high levels of chromosome instability in these cancer cells. In this report we have examined the relationship between the expression of DNA γH2AX foci and the expression of chromosome instability (i.e., micronuclei) in melanocytes (MC) and melanoma cells.

Results

Phosphorylated H2AX histone foci are detected in untreated MC and melanoma cells We recently examined cultured primary MC and melanoma cells by indirect immunofluorescence for the expression of γH2AX foci. γH2AX foci with a diameter of 0.25–1.0 μm were observed. The number of γH2AX foci observed in untreated MC was low (Fig 1*A*). The distribution of foci counted per nucleus in untreated MC was skewed toward cells containing less than 5 foci per nucleus. Ninety percent of MC contained 1 or no foci (Fig 2*A*). On an average 0.4, 0.8, and 2.0 γH2AX foci were detected per nucleus in untreated MC, fibroblasts (FB), and keratinocytes, respectively (Table I). This is within the range of values, from 0.05 to 1.5 foci per nucleus, reported in the literature for primary human FB (Rogakou *et al*, 1999; Rothkamm and Lobrich, 2003). In contrast, greater numbers of γH2AX foci were detected in the nuclei of untreated melanoma and carcinoma cells (Fig 1*C* and Table I). The distribution of γH2AX foci counted per untreated YUSAC2 nucleus was skewed toward 15–25 foci per nucleus (Fig 2*A*) with an average of 17 foci per cell nucleus (Table I). A shift toward higher numbers of foci/nucleus was also observed in the other melanomas examined (Fig 2*C*). Seventy-five percent of YUSIT1 cells contained 3 or more foci, whereas 76% of YUSAC2 cells contained 6 or more foci per nucleus. Detecting larger numbers of γH2AX foci in melanoma cells was not surprising because cancer cells have been reported to express higher numbers of γH2AX foci. For instance, about 2.5 foci per nucleus were reported to occur in a human astrocytoma and a fibrosarcoma (Rogakou *et al*, 1999; Sedelnikova *et al*, 2002). A range of γH2AX foci yields was also observed in untreated cells from other cancers. For instance, we observe 5.2 foci per HeLa S3 cell nucleus and 13.7 foci per HaCat cell nucleus (Table I). Our results indicated that untreated melanoma and carcinoma cells contain significantly higher numbers of γH2AX foci, and thus presumably DNA dsb, than do untreated primary MC.

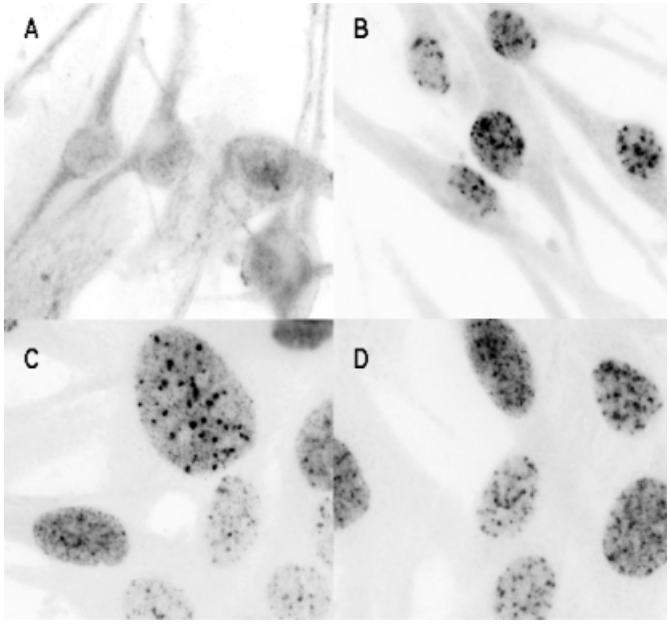

Figure 1
Detection of γH2AX foci in primary melanocytes (MC) and melanoma cells. Primary neonatal MC (*Panels A* and *B*) or YUSAC2 melanoma cells (*Panels C* and *D*) were grown on serum-coated cover slips and either exposed to 1.5 Gy of γ radiation (*Panels B* and *D*) or left unirradiated (*Panels A* and *C*). Cover slips were collected 60 min after treatment, the cells were fixed with formaldehyde, and analyzed for the presence of γH2AX in their nuclei by indirect immunofluorescence, as described above.

DNA dsb detection in MC and melanoma cells A direct relationship between the number of DNA dsb induced by IR and the number of γH2AX foci has been reported (Sedelnikova *et al*, 2002; Rothkamm and Lobrich, 2003), indicating that the number of γH2AX foci detected in cell nuclei reflects the number of DNA dsb. When the length of double-stranded DNA prepared from untreated MC, YUSIT1, or YUSAC2 cells was compared on the type of pulsed field agarose gels that resolve *Schizosaccharomyces pombe* chromosomal DNA (Warters, 1992; Warters and Lyons, 1992), no consistent difference was detected between any of the three cell types (results not shown). But as the number of DNA dsb expected in melanoma cell DNA (i.e., about 17–20) is comparable to a radiation dose of about 0.5 Gy, and as this dose is below the resolution of this agarose gel approach (Warters and Lyons, 1992; Rothkamm and Lobrich, 2003), it is not surprising that we could not detect higher numbers of dsb in the melanoma cells than in MC by physical chemical methods.

To demonstrate that in our hands the γH2AX immunofluorescence approach actually detects DNA dsb, we compared the expression of γH2AX foci at various times after cells were exposed either to H_2O_2 or to γ radiation. H_2O_2 produces few DNA dsb. In contrast, IR is an efficient inducer of DNA dsb. Few additional γH2AX foci were induced over the background level for up to 4 h after cells were exposed to 20 μM H_2O_2 for 15 min at 4°C (results not shown). Significantly greater numbers of foci were detected when MC (Fig 1*B*) or YUSAC2 cells (Fig 1*D*) were collected and analyzed at various times after irradiation. The number of γH2AX foci expressed, as a function of time after