Impaired Processing of DNA Photoproducts and Ultraviolet Hypermutability With Loss of p16INK4a or p19ARF

Papri Sarkar-Agrawal, Irene Vergilis, Norman E. Sharpless, Ronald A. DePinho, Thomas M. Rünger

Reduced DNA repair has been linked to an increased risk of cutaneous malignant melanoma, but insights into the molecular mechanisms of that link are scarce. The INK4a/ARF (CDKN2a) locus, which codes for the p16INK4a and p19ARF proteins, is often mutated in sporadic and familial malignant melanoma, but it has not been directly associated with reduced DNA repair. We transfected unirradiated mouse fibroblast cells with UV-treated DNA to measure DNA repair in normal, p16INK4a mutant, p19ARF mutant, or double mutant mouse host cells. Loss of either p16INK4a or p19ARF reduced the ability of the cells to process UV-induced DNA damage, independent of cell cycle effects incurred by the loss. These results may further explain why INK4a/ARF mutations predispose to malignant melanoma, a UV-induced tumor. [J Natl Cancer Inst 2004;96:1790–93]

Sunlight exposure, fair complexion, moles and atypical moles, and a family history of malignant melanoma are well-established risk factors for cutaneous malignant melanoma. In addition, a reduced capacity to repair UV-induced DNA damage has recently been described as an independent melanoma risk factor in a large case-control study (1). The link between reduced DNA repair and malignant melanoma is further substantiated by a high melanoma risk in patients with the nucleotide excision repair–deficient disease xeroderma pigmentosum (2,3). Baccarelli et al. (4) reported that polymorphisms of the XPD gene are associated with increased melanoma risk in normal subjects over 50 years of age. These XPD variants exhibit a reduced ability to repair UV-induced DNA damage and thus provide the first insights into possible molecular mechanisms that link DNA repair and melanoma risk in the normal population.

In sporadic and familial melanoma, the INK4a/ARF locus at 9p21 often sustains deletions or point mutations (5–8). This locus encodes two tumor suppressor proteins that regulate p53 and Rb (9). p16INK4a inhibits cyclin-dependent kinase activity, leading to Rb hypophosphorylation and therefore impeding S phase entry and subsequent cell division (10). The other product of the INK4a/ARF locus, p14ARF (p19ARF in mice), inhibits MDM2-mediated degradation of p53, and therefore, in addition to other p53-mediated effects, also impedes subsequent cell division (11–14).

Until now, loss of the INK4a/ARF locus has not been directly linked to reduced DNA repair. However such a link is possible, given the association between reduced DNA repair and melanoma. To investigate the possible role of p16INK4a and p19ARF in DNA repair, we measured repair of DNA photoproducts in mouse embryonal fibroblasts lacking p16INK4a and/or p19ARF and in INK4a/ARF-intact littermate control cells (15–17). Because of the lack of cell cycle arrest of p16INK4a−null and p19ARF−null cells after UV irradiation could alter the processing of DNA photoproducts, we used an assay to assess DNA repair without irradiating the cells with UV. This host cell reactivation assay with the pYZ289 plasmid was performed as reported previously (18,19) but modified slightly by using a liposome-mediated transfection procedure (Lipofectamine Plus; Invitrogen, Frederick, MD) and a different type of DNA damage. The plasmid was irradiated with UVB outside the cells, transfected into the mouse host cells for repair, recovered from the cells after 24 hours, and then assayed for survival by transforming Escherichia coli strain MLB100. Plasmid survival is contingent upon removal of the UVB-induced DNA photoproducts and plasmid replication and therefore assesses DNA repair. The mutagenesis marker gene supF in the plasmid was used to screen for mutations (i.e., the result of error-prone repair) by a color reaction in transformed bacteria.

As expected, plasmid survival decreased with increasing plasmid DNA damage (by increasing UVB dose) in all host cell lines (Fig. 1). However, INK4a/ARF−/− cells (lacking both p16INK4a and p19ARF) were found to have a 3.7- to 10.5-fold (P < .001, two-sample t test for difference in means) lower survival of UV-damaged plasmid compared with littermate INK4a/ARF+/+ (wild-type) cells (Fig. 1), indicating a reduced DNA repair capacity. Furthermore, both p16INK4a−/− and p19ARF−/− cells also showed a reduced (1.6- to 2.2-fold, P < .001 and 2.1- to 3-fold, P < .001, respectively; two-sample t test for difference in means) plasmid survival compared with littermate wild-type cells. Plasmid survival was 1.7- to 6-fold (p16INK4a−/−) and 1.2- to 5-fold (p19ARF−/−) higher than survival in the INK4a/ARF−/− cells, indicating that both gene products affect DNA damage processing and that a loss of both functions has an additive effect. Transfection with unirradiated or irradiated plasmid did not change the cell cycle profile, as seen in flow cytometric analysis of propidium iodide–stained cells of all four cell lines (data not shown). Thus, the difference in DNA repair efficiency cannot be explained by a different reaction of the INK4a/ARF−/− cells to the transfection procedure or to DNA damage on the plasmid.

Mutation frequencies also increased with increasing doses of UVB to the plasmids in all cell lines (Fig. 2; the increases were statistically significant only for the highest UVB dose with INK4a/ARF−/−, p16INK4a−/−, and p19ARF−/− and for the intermediate UVB dose, respectively).
Open squares = wild-type; solid diamonds = p16INK4a–/–; solid circles = p19ARF–/–; solid triangles = INK4a/ARF–/–. The plasmid survival of UVB-damaged plasmid reflects DNA repair efficiency of the host cells. A solar simulator (LH153; Kratos Analytical, Ramsey, NJ) was used to irradiate plasmids at a concentration of 30 µg/mL in Tris–EDTA buffer, generating UVB-type DNA photoproducts. The emission spectrum of this source has been published (30). UVB and UVA contained in this source are not expected to generate oxidative DNA damage under these in vitro conditions (i.e., in the absence of cellular photosensitizers) because the only chromophore in the aqueous solution is DNA (31). For dosimetry, an IL-1700 Research Radiometer (International Light, Newburyport, MA) equipped with a UVB-sensor (SED 240, in combination with a 280-nm cutoff filter) was used. Intensity of approximately 0.06 mW/cm² allowed irradiation with, e.g., 100 J/m² UVB in less than 3 minutes. Plasmids were then transfected into host cells using Lipofectamine Plus (Invitrogen, Frederick, MD), according to the manufacturer’s instructions. Twenty-four hours after transfection, replicated plasmids were purified and used to transform Escherichia coli MLB100. To compensate for variations in transfection rates, plasmid replication, and cell viability, triplicate parallel reference samples were used for each data point (mean).

Error bars show upper 95% confidence intervals.

Twenty-four hours after transfection, replicated plasmids were purified and used to transform Escherichia coli MLB100. To compensate for variations in transfection rates, plasmid replication, and cell viability, triplicate parallel reference samples containing unirradiated plasmid were used in every experiment. Plasmid survival was calculated as the percentage of bacterial colonies relative to those obtained from the parallel control samples with unirradiated plasmid. n = 5–8 independent samples for each data point (mean). Error bars show upper 95% confidence intervals. All experiments were performed on mouse embryonal fibroblasts before passage 2 using littermate control cells. The average yield of bacterial colonies after passage of unirradiated control plasmids through the different cell lines was 507 544 ± 66 643.

dose with INK4a/ARF–/–; P < 0.03.

However, after propagation in INK4a/ARF–/– cells, UVB-irradiated plasmids (250 J/m² and 500 J/m²) showed a 7.3- and 9.1-fold higher mutation frequency compared with plasmids propagated in littermate INK4a/ARF+/+ cells (mean with 250 J/m² and INK4a/ARF+/+ cells = 0.48%, mean with INK4a/ARF–/– cells = 3.50%, and difference = 3.02% [95% confidence interval = 1.09% to 4.94%, P = 0.007]; mean with 500 J/m² and INK4a/ARF+/+ cells = 0.98%, mean with INK4a/ARF–/– cells = 8.90%, and difference = 7.92% [95% confidence interval = 3.70% to 12.14%, P = 0.005]), indicating an ultraviolet hypermutability in the INK4a/ARF+/+ cells. In contrast, the repair of DNA photoproducts in either p16INK4a–/– or p19ARF–/– cells was not error-prone (no statistically significant difference), suggesting that both products of the INK4a/ARF locus must be inactivated to confer ultraviolet hypermutability. There were statistically nonsignificant 3.2- and 3.1-fold higher mutation frequencies with the highest UVB dose in p16INK4a–/– and p19ARF–/– cells compared with wild-type cells that may reflect a milder ultraviolet hypermutability than the INK4a/ARF–/– cells. Based on this finding, it appears that when p16INK4a–/– and p19ARF–/– cells are subjected to low levels of DNA damage, their DNA repair capacity is high enough to prevent genetic mutations; however, some mutations will occur when the cells are exposed to high levels of DNA damage. In contrast, the INK4a/ARF–/– cells will incorporate genetic mutations, even when subjected to low levels of DNA damage, and will have higher mutation rates when exposed to higher levels of DNA damage.

Eskandarpour et al. (20) reported a high frequency (95%) of activating Nras mutations in melanomas in members of melanoma-prone families with germline INK4a/ARF mutations as opposed to a low frequency (10%) in sporadic melanomas. This hypermutability in melanomas from INK4a/ARF-deficient backgrounds is consistent with our findings of ultraviolet hypermutability of the pY2289 plasmid propagated in INK4a/ARF+/– cells. Our data are also consistent with findings of a systemic ultraviolet hypermutability in familial melanoma patients, as seen with shuttle vector plasmids propagated in noncancerous cells of these patients (21–23).

Stabilization and activation of p53 have been shown to induce global genome nucleotide excision repair (24,25). Therefore, it is perhaps not surprising that loss of p19ARF, an upstream regulator of p53, results in reduced DNA repair efficiency. More surprisingly, our data also link p16INK4a to the repair of DNA photoproducts. Cells expressing human papillomavirus E7 protein with subsequent low levels of hypophosphorylated Rb demonstrate reduced global genome nucleotide excision repair of DNA photoproducts (26). Loss of p16 can also lead to reduced levels of hypophosphorylated Rb by increasing levels of hyperphosphorylated Rb (9), and it is tempting to speculate that the increased levels of hyperphosphorylated Rb might explain deficient DNA repair in p16INK4a–/– cells.

Consistent with our results, mice lacking p19ARF have been reported to be highly susceptible to UV-induced melanoma, suggesting that this alteration of DNA repair contributes to tumorigenesis in vivo (27). The model in the study by Kannan et al. was characterized by a
single, low-dose UV exposure in RAS-expressing neonatal mice. Although mice lacking p16INK4a only were not tumor-prone, analysis of tumors from these mice demonstrated that Rb pathway inactivation was a required and rate-limiting feature of the model. Therefore, loss of p16INK4a was epistatic to UV treatment in that model. In normal humans, however, many more genetic lesions are no doubt required for melanoma formation, and therefore the decrease in DNA repair induced by p16INK4a deficiency is still likely to be important in the human disease.

Although it is generally thought that p16INK4a and p19ARF suppress malignant proliferation in melanocytes through their acknowledged tumor suppressor roles, our work suggests an additional possibility that reduced repair of UV-induced DNA damage and increased mutation formation with loss of the INK4a/ARF locus might also contribute to melanoma formation. Because the DNA repair defect in xeroderma pigmentosum cells has been shown to affect fibroblasts and melanocytes equally (28), we believe that it is reasonable to assume that the repair deficiency and ultraviolet hypermutability in fibroblasts of INK4a/ARF–/– mice is also present in other cell types of these mice—melanocytes in particular. It remains to be determined whether INK4a/ARF heterozygous cells are already DNA repair–deficient or whether INK4a/ARF heterozygous individuals are more prone to melanoma because their cells are more likely to become DNA repair–deficient because only one hit instead of two hits is necessary to inactivate INK4a/ARF. Cell-specific differences, e.g., in the apoptotic response to UV exposure (29), might explain why loss of the INK4a/ARF locus does not predispose to other types of UV-induced skin cancer.

REFERENCES

T. M. Rünger was supported by a grant from the Skin Cancer Foundation (Henry Shotmeyer Award); N. E. Sharpless, by grants from the Sidney Kimmel Foundation for Cancer Research and from Paul Beeson Scholars in Aging Research; and R. A. DePinho, by Public Health Service grant 5 U01 CA84313-04 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

We thank T. Yagi (University of Kyoto, Japan) for kindly providing the shuttle vector plasmid pYZ289.

Manuscript received April 8, 2004; revised August 31, 2004; accepted September 10, 2004.