Gadd45a Protects against UV Irradiation-induced Skin Tumors, and Promotes Apoptosis and Stress Signaling via MAPK and p53

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ABSTRACT

Skin cancer is the most frequent form of malignancy in the world, and UV radiation is the primary environmental carcinogen responsible for its development. Herein we demonstrate that Gadd45a is a critical factor protecting the epidermis against UV radiation-induced tumorigenesis by promoting damaged keratinocytes to undergo apoptosis and/or cell cycle arrest, two crucial events that prevent the expansion of mutant or deregulated cells. Whereas Gadd45a has been implicated in cell cycle arrest, apoptosis, and DNA repair, to determine the physiological function of endogenous Gadd45a after genotoxic stress, the skin of Gadd45a-null mice was targeted with UV radiation. We report that Gadd45a induces apoptosis and cell cycle arrest by maintaining p38 and c-JNK MAPK activation in keratinocytes. The absence of Gadd45a results in loss of sustained apoptosis, and cell cycle arrest by maintaining p38 and c-JNK MAPK activation in keratinocytes. Consequently, Gadd45a-null mice are more prone to tumors relative to wild-type mice. Therefore, we conclude that Gadd45a, like p53, is a key component protecting skin against UV-induced tumors.

INTRODUCTION

UV radiation is a naturally occurring genotoxic agent and is the primary environmental carcinogen responsible for the development of most skin cancers (1–4). Much of the important damage produced by solar radiation is caused by UVB fluences (290–320 nm), which induce damage to a variety of cellular targets including DNA. In the case of DNA damage, UVB generates oxygen radicals as well as inducing cyclobutane pyrimidine dimers and pyrimidine-pyrimidine (6–4) photoproducts (5–7). These forms of DNA damage will, directly or indirectly, signal the activation of a series of signal transduction cascades comprised of serine/threonine kinases, MAPKs (8–11). In addition, UV radiation rapidly triggers signal transduction pathways involving the MAPKs by activation of growth factor receptors and other non-nuclear targets (12–14). Under normal conditions, MAPKs are known to play critical roles in cellular differentiation, proliferation, and death (10, 15). Whereas the extracellular signal-regulated kinase (ERK) MAPKs are preferentially activated by mitogenic stimuli, p38 and JNK/stress-activated protein kinase MAPKs are activated by growth-suppressive stimuli such as genotoxic stress, transforming growth factor β, and proinflammatory cytokines (16, 17). Activated p38 and JNK in turn will phosphorylate and contribute to the activation of numerous transcription factors, such as p53, c-Myc, c-Jun, c-Fos, and ATF-2, involved in regulating stress-induced genes, which orchestrate events leading to cell cycle arrest, DNA repair, and/or apoptosis (10, 15, 17–24). These targets are not limited to transcription factors only; e.g., p38 has been shown recently to phosphorylate a key inhibitor site in the cell cycle protein Cdc25B (25).

One family of genes that is downstream, and perhaps upstream, of the MAPK cascade is the Gadd45 (growth-arrest and DNA damage-inducible) family. This family is composed of three genes: Gadd45a (Gadd45/Gadd45α), Gadd45b (Myd118/Gadd45β), and Gadd45g (CR6/Gadd45γ/OG137; Refs. 26–30). Whereas all of the family members are stress inducible, Gadd45b and Gadd45g appear to have prominent roles in cellular differentiation events (26, 29, 31), although Gadd45a is a p53-effector and stress-inducible gene (28). Gadd45a is an ubiquitously expressed M, 21,000 acidic protein, which, like p53, has been implicated in many biological processes related to maintenance of genomic stability and apoptosis. Gadd45a (along with Gadd45b and Gadd45g), for instance, has been shown to activate p38 and JNK MAPKs by associating with and activating MEKK4/MTK1 MAPK kinase kinase (32), although this issue remains controversial, and has yet to be shown with a genetic approach for both Gadd45a and Gadd45b (33–35). In the same vein, JNK-mediated apoptosis in vitro has been reported to occur via Brcal-induced Gadd45a transactivation (36). Gadd45a is also able to associate with proteins involved in cell cycle regulation, such as p21 (Cdkn1a), a cyclin-dependent kinase inhibitor (37), and Cdc2/cyclinB, a key kinase for G2/M progression (38); it also associates with proliferating cell nuclear antigen, which is involved in DNA replication and repair (39, 40). Additionally, Gadd45a binds to core histones in damaged DNA (41). The development of Gadd45 a-null mice has provided important insights for the in vivo roles of this gene (42–44). To a great extent, the phenotype of Gadd45a-null mice (42) parallels that of Tp53-null mice (45, 46). Whereas Gadd45a-null mice do not develop spontaneous tumors, these mice have an increased frequency of both ionizing radiation-induced and dimethylbenzanthracene-induced tumors (42, 43). Like p53-deficient cells (47, 48), cells derived from Gadd45a-null mice exhibited genomic instability, single oncogene transformation, loss of normal cellular senescence, increased cellular proliferation, incomplete cytokinesis, centrosome amplification, and reduced DNA repair (42, 44). In the case of apoptosis, Gadd45a-null fibroblasts, thymocytes, and lymphocytes showed proficient apoptosis to a variety of stimuli, such as ionizing (42) and UV radiation (35). Thus, there is substantial but not complete overlap in the cellular roles for Gadd45a and p53. Although Gadd45a is one of numerous downstream targets of p53 (47), the fact that: (a) Gadd45 family members are able to activate the p38/JNK MAPK pathway (32, 49); and (b) Tp53-null and Gadd45a-null mice share many similarities (42), led us to hypothesize that Gadd45a may have some role in the regulation of p53. Because p38 (18), as well as JNK (19), can contribute to p53 activation after
stresses such as UV radiation, and because Gadd45a can be induced by both p53-dependent (50) and independent (28) mechanisms, Gadd45a could conceivably contribute to maintaining p53 activity through a p38/JNK MAPK-mediated feedback loop that in turn leads to additional increased expression of Gadd45a. In this report, we used both in vivo skin and in vitro primary keratinocyte culture systems to additionally elucidate the functions of endogenous Gadd45a pertaining to apoptosis, proliferation, and differentiation, all of which can contribute to tumorigenesis if perturbed. The importance (and relevance) of using UVB and solar radiation (290–320 nm and 290–400 nm, respectively) as the genotoxic agent instead of UVC radiation (240–290 nm) as the genotoxic agent as the preferential site of DNA damage and the contribution of endogenous Gadd45a to the response to UV-induced stresses such as UV radiation, and because Gadd45a could conceivably contribute to maintaining p53 activity through a p38/JNK MAPK-mediated feedback loop that in turn leads to additional increased expression of Gadd45a. 

**MATERIALS AND METHODS**

**Cell Culture.** Primary keratinocytes were derived from multiple litters of 1–2-day-old C57BL/6J129 newborn mice. Mouse trunk skins were treated overnight with Dispase (25 units/ml) at 4°C followed by trypsinization at 37°C. Single cell suspensions were seeded on type IV collagen coated flasks (50 μg/ml; BD PharMingen) and cultured with serum-free keratinocyte medium (Life Technologies, Inc.) supplemented with bovine pituitary extract and bovine serum. Cells were fed every other day.

Dermal fibroblasts were harvested from the dermis of the same mice after Dispase treatment. Dermis was digested with collagenase (3.5 mg/ml; Worthington Biomedical Corp.) in DMEM and subsequently subject to two centrifugation steps to first pellet all dermal cells (fibroblasts and follicular keratinocytes, 3 min at 1200 rpm), and second, to eliminate the heavier follicular keratinocyte pellet (two times 3 min at 400 rpm). Dermal fibroblasts remaining in supernatant are seeded in regular flasks and cultured with DMEM/10% fetal bovine serum. Cells were fed every other day.

**Cell Cycle Analysis.** Primary keratinocytes and dermal fibroblasts were allowed to reach ~70% confluence before they were UV-irradiated with the specified doses. For G1 checkpoint analysis, cells were pulsed for 3 hr with 10 μM BrdUrd 15 h after irradiation. Pulsed cells were in turn harvested by trypsinization followed by centrifugation at 350 × g for 5 min. Cell pellets were fixed in 70% ethanol at –20°C for at least 3 h and hydrolyzed with 2 N HCl/0.5% Triton X-100 for 30 min at room temperature. HCl was in turn neutralized with 0.1 N sodium borate (pH 8.5). After washing the cells with 1× PBS, the pellet was resuspended in blocking solution (PBS containing 0.05% Tween 20% BSA) for 30 min, followed by 1-h incubation with FITC-conjugated anti-BrdUrd antibody (BD Pharmingen). Lastly, cells were washed with 1× PBS and resuspended in FACS solution (PBS with 5 μg propidium iodide/10 μg/ml RNase).

For G2 checkpoint analysis, cells were fixed at indicated time points after UV irradiation in 70% ethanol. After washing the pellet with 1× PBS, cells were blocked with 2% BSA/0.05% Tween 20% in PBS for 20 min at room temperature. Subsequently, cells were incubated for 1 h with blocking solution containing antihistone H3 antibody (1:1000 dilution; Upstate Biotech). Next, cells were incubated with secondary antibody (Cy2-conjugated antirabbit IgG; Amersham Pharmacia Biotech) for 30 min at room temperature, washed with 1× PBS, and resuspended in FACS solution as described above. Mitotic index corresponds to the fraction of cells in mitosis. **Real-Time PCR.** Total RNA was extracted with Trizol from adherent cells as recommended by the manufacturer (Life Technologies, Inc.). cDNA synthesis was performed according to standard protocols (ThermoScript RT PCR System; Life Technologies, Inc.) on 1 μg of DNase-treated (DNA-free; Ambion) total RNA with a combination of random-hexamer and oligo(dT)20 primer. In turn, the single-stranded cDNA was used as template for real-time PCR performed with an ABI PRISM 7700 sequence detection system. Briefly, the total volume/reaction was 25 μl and composed of 50 ng of single-stranded cDNA template, 1× SYBR Green PCR master mix (Applied Biosystems), and 200 nM primer mix (forward
and reverse primers combined). Templates were subject to 40 cycles of: denaturation (94°C, 20 s), annealing (55°C, 20 s), and extension (72°C, 30 s). Primer sets used are as follows: Gadd45α/H9251, 5'-GGTGAGCTGAAGAAGGAAGCT-3' (forward), 5'-H11032-TCCTTGCAGTGCTTTGTAGTTTTG-3' (reverse); Gadd45α/H9252, 5'-TACATATTTGACAGCCCCCTCA-3' (forward), 5'-H11032-CAGAAGGTATCACGGGTAGGGT-3' (reverse); Gadd45α/H9253, 5'-AGCCGACTGCACTGCTCTTT-3' (forward), 5'-H11032-ACGATAGCGTCCTTTAGAA-AATGAA (reverse); and glyceraldehyde-3-phosphate dehydrogenase, 5'-GAAGGTTGGAAGGTCGGAGTC-3' (forward), 5'-H11032-GAAGATTGTGATGGGATTTC-3' (reverse).

**In Vivo Apoptosis Assay.** Either newborn C57BL/6J pups or 6-week-old depilated adult C57BL/6J mice were irradiated with 1000 Jm⁻² of UVB. Twenty-four h after UV radiation, the skin was harvested, fixed in 10% neutral-buffered formalin, and subject to H&E staining. Substantially more dyskeratotic and pyknotic sunburn cells (indicated by arrows) are present in UV-irradiated wt skin (+/+, middle left panel) as compared with Gadd45α-null skin (+/−, middle right panel) or control unirradiated skin (C, top left panel). Quantitation (top right panel) of randomly selected 1-cm segments of irradiated (UVR) and unirradiated controls (C) revealed a 2.5-fold increase in sunburn cells in wt mice (+/+) relative to Gadd45α-null mice (+/−). Moreover, irradiated wt mice frequently developed subcorneal pustules (bottom left panel) and/or complete epidermal erosion (bottom right panel). This degree of inflammation is not observed in Gadd45α-null mice. UV radiation experiments were performed in triplicate, and two 1-cm segments were scored per animal. Representative images are shown. Because hair removal will stimulate keratinocyte proliferation, the relative epidermal thickness of depilated skin is normally increased. Magnification is ×200; bars = ±SD.

Fig. 2. **In vivo** UV radiation-induced sunburn and inflammation in adult mouse skin. Six-week-old adult mice were irradiated on depilated dorsal skin with 1000 Jm⁻² of UVB. Twenty-four h after UV radiation, the skin was harvested, fixed in 10% neutral-buffered formalin, and subject to H&E staining. Substantially more dyskeratotic and pyknotic sunburn cells (indicated by arrows) are present in UV-irradiated wt skin (+/+, middle left panel) as compared with Gadd45α-null skin (+/−, middle right panel) or control unirradiated skin (C, top left panel). Quantitation (top right panel) of randomly selected 1-cm segments of irradiated (UVR) and unirradiated controls (C) revealed a 2.5-fold increase in sunburn cells in wt mice (+/+) relative to Gadd45α-null mice (+/−). Moreover, irradiated wt mice frequently developed subcorneal pustules (bottom left panel) and/or complete epidermal erosion (bottom right panel). This degree of inflammation is not observed in Gadd45α-null mice. UV radiation experiments were performed in triplicate, and two 1-cm segments were scored per animal. Representative images are shown. Because hair removal will stimulate keratinocyte proliferation, the relative epidermal thickness of depilated skin is normally increased. Magnification is ×200; bars = ±SD.
PM2106 UVR detector calibrated to register the energy from 282 to 326 nm. Trunk skin was harvested 24 h after irradiation and immediately fixed in 10% neutral buffered formalin. Five-μm thick sections of paraffin-embedded tissues were deparaffinized and subject to antigen unmasking with 10 mM sodium citrate buffer (pH 6.0). After unmasking, sections were incubated with 1% hydrogen peroxide for 10 min to eliminate endogenous peroxidase activity. After the quenching step, sections were washed several times in water and 1× PBS, and subsequently blocked for 1 h with 5% goat serum in PBS. Primary antibody (1:100 dilution of F-5 anti-p21 monoclonal antibodies; Santa Cruz Biotech.; 1:50 dilution of Phospho-p38 MAPK polyclonal antibody, 1:20 dilution of Phospho-JNK MAPK monoclonal antibody; Cell Signaling Technology, Inc.; and 1:50 dilution of Gadd45a polyclonal antibody; Santa Cruz Biotech.) was then applied in blocking solution, and sections were incubated overnight at 4°C. The following morning, secondary antibody was added (1:200 dilution of biotinylated anti-rabbit or antimouse IgG). For HRP conjugation and substrate detection, the Vectastain ABC kit was used as described by the manufacturer (Vector Laboratories). Four (p21) and 8 (p38, JNK, and Gadd45a) animals per genotype were used (2 irradiated per time point and 2 unirradiated).

UV Carcinogenesis and Histopathology. Hairless mice were generated by crossing SKH1-hairless mice with the established C57BL6/129 Gadd45a-null mouse strain (42). Littermates from hairless Gadd45a−/− crosses were used. Animals were age and gender-matched and subjected to three weekly doses of 1000 J/m2 of UVB (Westinghouse FS40 Sunlamp fluorescent tube) and 7300 J/m2 of UVA (blacklight fluorescent tube; emission spectrum of 310–400 nm with peak at 365 nm) radiation for a total of 52 weeks. For this strain, this exposure corresponds to one minimal erythema dose, and the inclusion of UVA radiation gave a spectrum comparable with solar radiation. Comprehensive histopathology evaluation was performed on skins of 10 mice (5 wt and 5 Gadd45a−/−). Entire skins were fixed in 10% buffered neutral formalin. All of the gross lesions on dorsal skin of trunk, pinnae, and tail were embedded in paraffin, sectioned at 5 μm, and stained with H&E.

Statistical Analysis. Tumor multiplicities between wt and Gadd45a−/− mice were compared using the nonparametric Wilcoxon rank-sum test because of the lack of homogeneity of variance between groups on the specific criterion of interest. Probabilities reported are two-sided.

RESULTS

**Gadd45a−/− Mouse Epidermis Is Resistant to Apoptosis.** Because of the potential role of Gadd45 family proteins in MAPK signaling and stress-induced apoptosis and DNA repair, we investigated whether Gadd45a−/− mice showed altered susceptibility to
sunburn/apoptosis. In the epidermis, the manifestation of apoptotic keratinocytes originating from UV radiation is referred to as sunburn cells (52, 53). UV-induced sunburn cells have been demonstrated previously to require functional p53, the absence of which results in resistance to UV-induced apoptosis (53). Although wt and Gadd45a-null thymocytes, embryonic fibroblasts, and splenic lymphocytes showed comparable levels of apoptosis after ionizing radiation or UV radiation (35, 42), interestingly the epidermis of Gadd45a-null mice was far more resistant to UVB-induced apoptosis (Fig. 1). Both Gadd45a-null and wt newborn mice were irradiated with a dose ranging from 0.8 to 5 minimum erythema doses in different human skin types (54). At the established peak response time of 24 h after irradiation (55), their skin was harvested and analyzed by TUNEL assay. Whereas wt mice had appreciable numbers of TUNEL-positive keratinocytes after irradiation in both the basal and nucleated suprabasal keratinocytes, mice lacking Gadd45a were resistant to apoptosis and only rarely showed sunburn/TUNEL-positive cells. Similar results were obtained from adult mice (Fig. 2, top and middle panels). Histological analysis of the UVB-irradiated adult mouse skin revealed a 2.5-fold greater incidence of sunburn cells in wt mice as compared with Gadd45a-null mice. Interestingly, adult wt mice also demonstrated increased sensitivity to UV irradiation in comparison to Gadd45a-null mice as manifested by more pronounced erythema and the emergence of subcorneal pustules and epidermal erosion (Fig. 2, bottom panels) coincident with elevated inflammatory cytokine responses (Fig. 3). Therefore, we conclude that Gadd45a, like p53, is intimately involved in orchestrating events leading to apoptosis in the skin, which may contribute to the observed inflammation in the dermis.

Gadd45a Is Required for Maintaining p38 and JNK MAPK Activation in Vivo. Because Gadd45 family proteins have been reported to be upstream activators of p38 and JNK MAPK (32, 56), we were interested in determining whether Gadd45a-dependent apoptosis involves activation of these two signaling pathways in epidermal keratinocytes. Although comparable basal levels of p38 and JNK activity were detected by Western blot analysis of unirradiated wt and Gadd45a-null keratinocytes (data not shown), immunohistochemical analysis of irradiated newborn mouse skin revealed that whereas activation of both p38 and JNK MAPK immediately after UVB radiation is independent of Gadd45a, sustained MAPK signaling requires Gadd45a (Fig. 4, A and B). Whereas anti-phospho-specific p38 antibodies (which detect activated p38) clearly stained granular keratinocytes in wt mice 15 min to 1 h after UV radiation, p38 activation was not appreciably maintained and/or detected in the epidermis of irradiated Gadd45a-null mice beyond 15 to 30 min after UV radiation (Fig. 4A).
suprabasal localization of active p38 in murine skin is in accordance to what has been reported previously (57).

The immunohistochemical observations made with anti-phosphospecific JNK antibodies parallel the phosphospecific p38 antibodies (Fig. 4B). Whereas wt mice readily showed evidence for persistent JNK activation within the 15–60 min time frame, this was not observed in Gadd45a-null mice. Only transient and modest JNK activation was detected in Gadd45a-null skin at 30 min after UV radiation. Interestingly, unlike p38, active JNK was detected predominantly in the basal layer of the epidermis. Together, maintenance of p38 and JNK activation correlated well with the accumulation of Gadd45a protein in the epidermis 1 h after UV irradiation (Fig. 4C) and the eventual emergence of TUNEL-positive nuclei of keratinocytes in all of the epidermal layers 24 h after UVR (Fig. 1). These results, along with the observation that Gadd45a is required for UV-induced sunburn cells, are consistent with Gadd45a as an upstream component of the MAPK signaling pathway involved in the normal maintenance of both p38 and JNK MAPK activity (18, 58).

UVB Radiation-induced p53 Activation Is Dependent on Gadd45a. Both p38 and JNK have been implicated in normal p53 activation and p53-mediated apoptosis after UV radiation (18, 19). Furthermore, although it has been estimated that Gadd45a has well over 100 downstream target genes (47), the fact that Gadd45a−/− and Tp53−/− epidermal keratinocytes behave very similarly under genotoxic stress conditions suggests that either: (a) Gadd45a is the primary p53-effector gene in skin; and/or (b) that Gadd45a is not only a downstream p53-effector gene but is also, under some circumstances, necessary for p53 activation. To determine the latter, an UVB radiation-induced p53 transcriptional activation assay was performed with wt and Gadd45a−/− primary keratinocytes. UV irradiation of primary keratinocytes transfected with a chloramphenicol acetyltransferase reporter plasmid driven by a p53 response element clearly demonstrated that in the absence of Gadd45a, keratinocytes had a marked reduction in p53 activation (Fig. 5). Whereas pronounced p53 activation was observed in Gadd45a−/− cells irradiated with UVB (6-fold and 14-fold activation above background for UV doses of 150 and 250 Jm−2, respectively), relatively little or no p53 activation was observed in Gadd45a−/− cells (no activation and 2.5-fold activation above background, respectively, for the two doses; Fig. 5B). To determine whether the same effects occurred in vivo, p53 activity was indirectly determined by performing immunohistochemistry on UV-irradiated newborn mouse skin with anti-p21WAF1/Cip1-specific antibodies (Fig. 5C). Whereas p21 protein levels increase substantially in the nuclei of wt basal keratinocytes 4 h after UV radiation, only modest protein accumulation is detected in Gadd45a-null equivalents, indicative of reduced p53 activity in the absence of Gadd45a protein. The fact that UV radiation-induced p53 activation is dependent on Gadd45a, suggests that whereas Gadd45a is a p53-effector gene, it can also contribute to p53 activation. Consequently, this places Gadd45a both upstream and downstream of p53.

Reduced G1 and G2 Checkpoints after UVB Radiation in Gadd45a−/− Keratinocytes. The fact that we observed Gadd45a is necessary for stress-induced activation of not only p38 and JNK MAPK but also of p53 in epidermal keratinocytes lead us to additionally investigate the importance of Gadd45a in UVB radiation-induced cell cycle arrest. Recent studies demonstrated that JNK and p38 MAPK are not only involved in p53 phosphorylation and activation (18, 19), but that p38 MAPK also mediates activation of a G1/M checkpoint by phosphorylating Cdc25B (25). Consistent with the known role for p53 in G1 checkpoint activation (47, 59) after many stresses including UV radiation (60), FACs analysis of UVB-irradiated primary mouse keratinocytes revealed nearly a complete loss of G1 arrest in p53-null cells and a marked attenuation in Gadd45a−/− cells. For example, BrdUrd labeling of keratinocytes 15 h after UV radiation showed that the G1:S ratio for irradiated wt keratinocytes is ~3-fold greater than the G1:S ratio for irradiated Gadd45a-null cells at 250 Jm−2 (Fig. 6A). Whereas both wt and Gadd45a-null cells have a dose-dependent G1 checkpoint response to UV radiation, Gadd45a-null cells are consistently less responsive. Considering that this checkpoint is nearly completely abrogated in p53-null, our findings in Fig. 5 and Fig. 6A indicate that this checkpoint deficit in Gadd45a-null keratinocytes is because of attenuated p53 signaling. Interestingly, contrary to the observed defects in UV stress-induced G1 checkpoint.
response, Gadd45a-null keratinocytes respond effectively to classic differentiation and antiproliferative stimuli, such as calcium and transforming growth factor β1-induced G1 arrest, which play a role in differentiation of suprabasal epidermal keratinocytes (data not shown; Refs. 61–66).

Both Gadd45a-null and Tp53-null keratinocytes also showed a pronounced reduction in G2 arrest after UV radiation (Fig. 6B). G2 checkpoint activation was measured by a reduction in the mitotic index in the first 4 h, and thus focuses primarily on cells already in G2 when irradiated. Nearly all of the wt cells (>80%) were arrested by 1 h after UV radiation, and this arrest persisted during the following 3 h of the study. In contrast, only a marginal delay is seen for Gadd45a-null cells at 1 h, and the maximum checkpoint at 2 h was <40%. This weak checkpoint was transient, and the mitotic index returned to unirradiated levels by 4 h. A similar response was seen in Tp53-null keratinocytes where only marginal checkpoint activation is seen in the first 2 h with return to unirradiated levels by 4 h. Whereas G1 checkpoint abrogation is more pronounced in Tp53-null cells (Fig. 6A), interestingly the G2 checkpoint deficit is comparable with that of Gadd45a−/− cells. We conclude from these studies that Gadd45a, like p53, plays a pivotal role in cell-cycle regulation in both G1 and G2 after UV radiation-induced stress. What remained to be seen was whether our results with keratinocytes are applicable to other skin cell types.

Abrogation of UVB Radiation-induced G1 and G2 Checkpoints Is Keratinocyte-specific. To determine whether the involvement of Gadd45a in UV radiation-induced cell cycle arrest is cell-type specific, primary dermal fibroblasts were tested under similar conditions. In contrast to keratinocytes, Gadd45a−/− dermal fibroblasts did not manifest any G1 or G2 checkpoint deficits after UV radiation relative to Gadd45a+/− dermal fibroblasts (Fig. 6, C and D, respectively). On the basis of FACS analysis, both cell types responded equally well, in a dose-dependent manner, to various doses of UVB radiation. The observed differences in response to UV radiation for the two cell types could be because of a redundancy within the Gadd45 gene family, and it is conceivable that one or more Gadd45 genes could compensate for the lack of Gadd45a. To test this possibility, a comparison of the relative mRNA expression levels of the three Gadd45 genes in both primary keratinocytes and dermal fibroblasts was performed by quantitative reverse transcription-PCR (Fig. 6, E and F). Interestingly, whereas similar basal levels of Gadd45b expression are detected in keratinocytes and dermal fibroblasts (data not shown), the expression profile for Gadd45a (Fig. 6E) and Gadd45g (Fig. 6F) are significantly different for the two cell types. Primary dermal fibroblasts have a 2.6-fold lower expression of Gadd45a and a 10-fold greater expression of Gadd45g relative to primary keratinocytes. The relative expression levels noted for Gadd45b and Gadd45g in wt cells are maintained in Gadd45a-null cells (data not shown), and indicate that no compensatory mechanism exists in Gadd45a−/− keratinocytes and dermal fibroblasts.

Gadd45a Protects the Epidermis against Solar Radiation-induced Carcinogenesis. Because we demonstrate that: (a) Gadd45a contributes to p53 activation via MAPK signaling; (b) the Gadd45a-null sunburn phenotype mimics that of Tp53-null mice; and (c) it is known that p53 protects against UV radiation-induced squamous cell carcinomas (51, 67, 68), we were interested in determining whether UV-irradiated Gadd45a−/− mice would have increased predisposition to skin tumorigenesis, as do Tp53-null mice (51). Gadd45a-null and wt mice littermates were irradiated three times per week for 1 year with a source that mimics solar radiation and subsequently were processed for comprehensive histopathological examination. Overall, relative to wt mice, UV-irradiated Gadd45a-null mice demonstrated a dramatic increase in the total number of proliferative epidermal lesions including preneoplastic atypical epidermal hyperplasia, squamous cell papillomas, and squamous cell carcinomas (Table 1). The atypical hyperplasias consisted of discrete foci of thickened stratum spinosum with disorganization of nuclei and piling up of the basal epithelial cells, with frequent rete ridge protrusions into the dermis. The squamous cell tumors were typical of those normally described in skin carcinogenesis studies (Fig. 7; Ref. 69).
DISCUSSION

In this report, we provide evidence that endogenous Gadd45a protects epidermal keratinocytes against UV radiation-induced carcinogenesis by contributing to both apoptosis, and G1 and G2 checkpoint regulation via p38 and JNK MAPK signaling, and p53 activation. Interestingly, the epidermal damage induced by UVB radiation also triggers a substantial inflammatory response, which is lacking in Gadd45a-null mice. It remains to be determined whether this response primarily reflects production of inflammatory cytokines by normal skin components or the infiltrating inflammatory cells; IFN-γ, for instance, is produced by inflammatory cells and not keratinocytes. Much of the Gadd45a-dependent regulatory effects we observed in keratinocytes appear to be cell type-specific. Whereas we observed a dramatic difference in p38 and JNK activation in keratinocytes exposed to UV radiation, recent reports demonstrated no changes in p38 and JNK MAPK activation after UV irradiation of embryo fibroblasts derived from the same mouse models (35). Our results with dermal fibroblasts validate this observation, because Gadd45-null dermal fibroblasts behave similarly to the wt counterparts, and effectively block G1/S and G2/M transition after DNA damage. The fact that Gadd45g is expressed at appreciably higher levels in dermal fibroblasts is one possible explanation for why this particular cell type may be able to compensate for the lack of Gadd45a with proficient G1 and G2 checkpoint activation, in contrast to keratinocytes (Fig. 6, A–D).

Additionally, our laboratory has reported previously that unlike Tp53−/− thymocytes, Gadd45a−/− thymocytes are as sensitive to DNA damage-induced apoptosis as are wt thymocytes (42). Once again, these phenotypic differences can be attributed not only to the fact that Gadd45 family proteins have overlapping functions, but also to our observation that the Gadd45 genes are differentially expressed in different cell types (Fig. 6, E and F).

A provocative and controversial reported model implicates Gadd45

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a Mice were UV-irradiated for 52 weeks, and comprehensive histopathology evaluation was performed on skins of 10 age-matched mice (5 wt mice and 5 Gadd45a-null mice). All gross lesions on dorsal skin of trunk were scored.

b The probabilities (two-sided P) are computed for differences in wt versus Gadd45a-null mice with respect to multiplicity.

Table 1 Multiplicity and incidence of solar radiation-induced proliferative lesions for wt and Gadd45a-null mice

Fig. 7. UV-induced hyperproliferative lesions in the skin. Shown are representative H&E-stained skin cross-sections of mice subject to solar irradiation. Wild-type mice (top left panel) typically retained normal skin, whereas Gadd45a-null mice manifested a significant increase in hyperproliferative lesions such as atypical hyperplasia (top right panel), squamous cell papilloma (bottom left panel), and squamous cell carcinoma (bottom right panel). Magnification is ×100.
The manner in which \emph{Gadd45a} \textsuperscript{−/−} keratinocytes mimic the phenotype of \emph{Tp53} \textsuperscript{−/−} keratinocytes is not surprising in the context of \emph{Gadd45a} being both upstream and downstream of p38/JNK. Whereas it is reasonable to assume that the antiproliferative effects of p38 and JNK are achieved via a positive feedback loop with p38 and JNK (Fig. 8). Stress MAPK signaling is rapidly activated by UV radiation with induction of the \emph{Gadd45a} gene by p53 signaling. \emph{Gadd45a}, as well as \emph{Gadd34} (MyD116) and \emph{Gadd153} (CHOP), is also stress-inducible in p53-deficient cells via signaling involving p38 and other MAPKs (28, 47, 73). With increases in the level of \emph{Gadd45a} after UV radiation, “feedback” signaling loops involving \emph{Gadd45a} and upstream MAPK components, such as MTK1, maintain strong signaling of p38 and JNK. p53 activation will result in changes in transcription of p53-effector genes such as p21 (\emph{Cdkn1a}), 14-3-3\textgamma, \emph{Gadd45a}, and others involved in cell cycle control (71). p21 will induce G\textsubscript{1} arrest, whereas multiple factors, such as 14-3-3\textgamma and \emph{Gadd45a}, will contribute to G\textsubscript{2} arrest (74). \emph{Gadd45a} protein will directly contribute to G\textsubscript{2} arrest by disrupting Cdc2/cyclin B1 kinase activity and indirectly by blocking Cdc25B phosphatase activity via p38 (25, 38). p38/JNK activation by \emph{Gadd45a} will also indirectly contribute to G\textsubscript{1} arrest through p38-mediated events. For example, inhibition of p38 activity has been shown previously to markedly attenuate p53 transcriptional activity and p53-mediated apoptosis after UV radiation (18). Whether the cell will undergo either cell cycle arrest or apoptosis may be determined by the severity of the insult as well as cellular context. Whereas transient p38/JNK activation will likely result in cell cycle block, prolonged p38/JNK activation will likely set the apoptotic program in motion. Loss of stress MAPK signaling can lead to inadequate protection against tumorigenesis. Indeed, a recent report clearly demonstrates that p38 MAPK is a critical tumor suppressor protecting against E1A and H-Ras-induced transformation of mouse embryonic fibroblasts (75). Moreover, a relatively high frequency of mutations in the \emph{Gadd45a} gene has been identified recently in pancreatic tumors, additional strengthening its link to cancer (76). Therefore, it is understandable how the absence of \emph{Gadd45a} predisposes irradiated skin to tumors by enabling damaged and deregulated cells to not only survive the genotoxic insult, but also to proliferate after loss of normal checkpoint controls.

In summary, the results presented above not only provide a better understanding of the biological function of \emph{Gadd45a} in a physiologically relevant system but also enable us to clarify and integrate many of the controversies regarding \emph{Gadd45a} and the MAPK signaling pathways. A better understanding of the mechanisms involved in maintaining genomic integrity is essential for the identification of potential targets, such as \emph{Gadd45a}, and development of future therapeutical approaches for the most prevalent form of human neoplasia, the various forms of skin cancer.

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