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Biochemical and Biophysical Research Communications 333 (2005) 241–246
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Ultraviolet B exposure activates Stat3 signaling via phosphorylation at tyrosine^{705} in skin of SKH1 hairless mouse: A target for the management of skin cancer?

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Received 30 April 2005
Available online 31 May 2005

Abstract

Understanding the molecular determinants of ultraviolet (UV) response may lead to the development of novel targets; and therefore, better approaches for the management of cancers, which mainly arise due to the exposure of skin to UV (particularly its UVB spectrum). Signal transducer and activator of transcription (Stat) proteins have been shown to activate multiple signaling pathways to contribute to oncogenesis. Here, we studied the regulation of Stat3 during UVB exposure-mediated responses in the skin of SKH-1 hairless mouse, a model regarded to possess relevance to human situations. Our data demonstrated that a single UVB (180 mJ/cm^2) exposure to the skin of SKH-1 hairless mice resulted in significant upregulation in (i) protein levels of Stat3 and (ii) phosphorylation of Stat3 at tyrosine^{705}. Further, the activation of Stat3 was found to be associated with a decrease in apoptotic response of UVB and a gradual time-dependent increase in leukocyte infiltration and hyperplasia. In conclusion, we have demonstrated, for the first time, that UVB exposure to skin resulted in an activation of pro-survival protein Stat3. Based on our observation, we suggest that Stat3 could serve as a target for the management of UVB exposure-mediated damages including skin cancer.

Keywords: Stat3; Phospho-(Tyr^{705}) Stat3; UVB; Skin; Ultraviolet; Apoptosis; Skin cancer

Excessive exposure to solar ultraviolet (UV), particularly its UVB component (290–320 nm), to human skin is the major cause for more than a million new cases of non-melanoma skin cancer diagnosed annually in the United States [1]. Exposure to UV also results in the development of precancerous conditions such as actinic keratosis (AK) [2–6] and elicits a variety of other adverse effects including erythema, sunburn cells, inflammation, hyperplasia, hyperpigmentation, and premature skin aging [7–10]. The available options have proven to be inadequate for the management of UV damages including skin cancers. It is, therefore, important to understand the mechanism of UV response that may result in the development of mechanism-based novel approaches for UVB-mediated damages including skin cancer.

Signal transducer and activator of transcription (Stat) proteins belong to a family of latent transcription factors, which are activated by phosphorylation on a single tyrosine residue (near the carboxy terminal) by members of the JAK tyrosine kinases in response to a variety of factors [11,12]. Stats dimerize, through a reciprocal –SH2 phospho-tyrosine interaction, and translocate to the nucleus where it binds to defined DNA elements within the promoter region of target genes and activate their transcription [12]. To date, seven Stats have been identified: Stat -1, -2, -3, -4, -5A, -5B, and -6, whereas Stat1 and Stat3 exhibit two isoforms each, resulting from

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0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.05.106

alternative splicing [13]. Stat1 is important for transducing pro-apoptotic signals whereas Stat3 and 5 have been implicated in promoting cell survival [14].

Studies have shown that constitutive activation of Stat3 exists in several human malignancies [15]. This critical role of Stat3 in the molecular pathogenesis of many tumors provides a validation for targeting this protein for cancer drug discovery [16]. Recent studies have suggested that Stat3 plays a critical role in chemically induced epithelial carcinogenesis [17]. However, chemical carcinogenesis model does not possess much relevance to human skin carcinogenesis and UV radiation is the established cause of 90% of skin cancers and non-cancerous cutaneous disorders. Based on these facts, we designed this study to define the modulation of Stat3 protein in UVB response in vivo in SKH-1 hairless mouse, a model that is regarded to possess relevance to human situations. This study, for the first time, demonstrated an activation of Stat3 during UV exposure-mediated responses in vivo.

Materials and methods

Reagents. The antibodies against mouse monoclonal anti-Stat3 (clone 124H6) and mouse monoclonal anti-phospho-(Tyr^{705}) Stat3 were purchased from Cell Signaling Technology (Beverly, MA) whereas the mouse monoclonal anti-β-actin antibody (clone AC-74) was obtained from Sigma Chemical Company (St. Louis, MO). The BCA protein assay kit and chemiluminescence detection kit were obtained from Pierce (Rockford, IL). All the other chemicals were obtained from Sigma Chemical Company (St. Louis, MO) and were analytical grade reagents.

UVB source. For UVB irradiation, we employed 'Daavlin Research Irradiator' (Daavlin Company, Bryan, OH). This equipment consists of a fixture mounted on fixed legs and contains four UVA and four UVB lamps. The exposure system is controlled using two Daavlin Flex Control Integrating Dosimeters. The dose units, in this equipment, could be entered as mJ/cm^2 (for UVB) or Joules (for UVA). For accuracy, the machine is periodically calibrated using International Light IL 1400, digital light meter (Daavlin, Bryan, OH).

Animals and treatment. Female SKH-1 hairless mice (6 weeks old) obtained from Charles River Laboratories (Wilmington, MA) were used in this study. After their arrival in the animal facility, the animals were allowed to acclimatize for a week prior to the start of experiment. The animals were fed purina chow diet and water ad libitum. Throughout the experimental protocol, the mice were maintained at standard conditions; temperature 24 ± 2 °C, relative humidity $50 \pm 10\%$, and 12 h room light/12 h dark cycle. In total, 40 mice were used for this study. The first group of eight mice did not receive any treatment and served as control (Group 1). The remaining 32 animals were subjected to single exposure of UVB ($180\ mJ/cm^2$) radiation. From this treated group, eight mice (per group) were taken out at specific time intervals; 3 h post-UVB (Group 2), 6 h post-UVB (Group 3), 12 h post-UVB (Group 4), and 24 h post-UVB (Group 5). At the end of specified times post-UVB, the animals were sacrificed and the UV-exposed skin (or similar untreated skin from the control group) was surgically removed and used for further studies.

Preparation of epidermal skin lysate. From the whole skin, epidermis was obtained as described elsewhere [18]. The epidermis was then homogenized in ice-cold lysis buffer [50 mM Tris–HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP-40, 1% Triton X-100, and 1 mM PMSF (pH 7.40)] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III; Calbiochem, La Jolla, CA). The homogenate was centrifuged at 13,000 rpm for 25 min at 4 °C, and the supernatant (total cell lysate) was collected, aliquoted, and stored at −80 °C. The protein content in the lysates was measured by BCA protein assay kit (Pierce, Rockford, IL) as per the manufacturer's protocol.

Immunoblot analysis. For immunoblot analysis, protein (25 μg) was resolved over 12% polyacrylamide gels and transferred onto a nitrocellulose membrane. The non-specific sites were blocked by incubating the membrane with 5% non-fat dried milk in buffer (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20, pH 7.6) for 1 h at room temperature, followed by incubation with the appropriate primary antibody in the blocking buffer for overnight at 4 °C. The antibody was used at the dilutions specified by the manufacturers. The blot was washed twice (5 min each) and then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody (Upstate, Lake Placid, NY) at 1:10,000 dilution for 1 h at room temperature. The blot was washed three times (10 min each) followed by a detection of protein using enhanced chemiluminescence (Pierce, Rockford, IL). Immunoblots were sequentially stripped of antibodies by incubation for 15–20 min at 37 °C with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL), and re-probed with β-actin antibody to confirm protein loading. The quantification of protein levels was performed by a digital analysis of the protein band (as a TIFF image) using *UN-SCAN-IT* software (Silk Scientific, Orem, UT).

Histopathology. To assess the effect of UVB exposure on skin histology including skin hyperplasia and infiltration of leukocytes, the skin samples obtained from control and treated mice were fixed in 10% formalin and embedded in paraffin. Vertical section (6 μm) was cut, mounted on a glass slide, and stained with hematoxylin and eosin followed by microscopic evaluation of the slides.

Immunohistochemical analysis. For immunohistochemical analysis, paraffin-embedded sections (6 μm thickness) were used. The sections were placed on silane-coated (Sigma, St. Louis, MO) glass slides, air-dried overnight, and rehydrated with xylene and graded alcohol. The tissue sections were then subjected to incubation with 3% H_2O_2 in methanol for the quenching of endogenous peroxidase. Antigen retrieval was achieved by boiling the sections in citric acid buffer (pH 6.0) in a microwave oven (at 750 W) until solution boils. At this time, the power of the oven was lowered to 100 W for 15 min. The samples were allowed to cool to room temperature followed by rinsing (three times; 2 min each) with PBS. The non-specific binding sites were blocked by incubating with PBS containing 1% BSA and 0.1% Tween 20 for 10 min, followed by incubation with an appropriate primary antibody for 1 h at 25 °C in a humid chamber. The slides were rinsed in washing buffer three times (5 min each) and then incubated with streptavidin-POD for 30 min at 25 °C in a humid chamber. The sections were washed with washing buffer followed by an incubation (at 25 °C for about 2–3 min) with a freshly prepared substrate solution (diaminobenzidine tetrahydrochloride with hydrogen peroxide and copper sulfate), for color enhancement. The sections were then washed with de-ionized water and counterstained with methyl green for 5 min followed by microscopic evaluation and digital photography.

Statistical analysis. Statistical analysis of all the data was performed by Student's t test. A value of $p < 0.05$ was considered statistically significant. The results are expressed as means ± SD.

Results and discussion

Studies have demonstrated UVB as the major etiological factor for non-melanoma skin cancers [19–21], which is derived from skin epithelial cells and can be further categorized into squamous cell carcinoma and basal

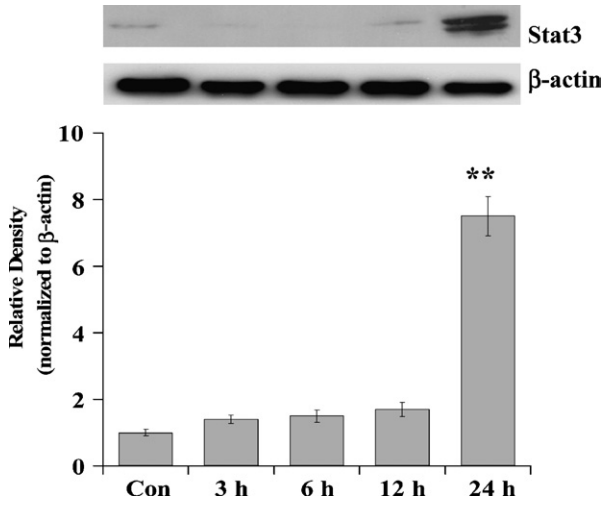

Fig. 1. UVB exposure resulted in a significant upregulation in the levels of Stat3 protein in SKH-1 hairless mouse skin. The SKH-1 hairless mice (8 mice per group) were treated with UVB (180 mJ/cm^2); and at 3, 6, 12, and 24 h post-UVB exposure, skin samples were obtained, and processed for immunoblot analysis as described under Materials and methods. The effect of UVB on modulations in Stat3 protein levels was determined by immunoblot analysis using appropriate antibodies. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The quantification of protein was performed by densitometric analysis using Scion Image Software (Scion, Frederick, MD) and the data (relative density normalized to β-actin) are expressed as means ± SE of three independent experiments (**$p < 0.01$).

cell carcinomas [22]. More than a million cases of non-melanoma skin cancers and more than 40,000 new cases of in situ melanoma are diagnosed each year in the United States [1,23]. The mechanism(s) of UVB-induced skin cancer have not been fully understood. UVB has been shown to cause dysregulation in several transcriptional factors, the normal cell cycle, and apoptosis machinery in the skin; and defects in these important physiological processes may lead to the development of a variety of cutaneous disorders including cancer [24]. In order to design better strategies for the management of skin cancer and other skin-related disorders, it is of utmost importance to completely understand the mechanisms of UVB response. This study was designed to examine the modulation of Stat proteins in skin as a result of UVB response under in vivo situation, employing SKH-1 hairless mouse.

Constitutive activation of Stat3 signaling has been shown to abrogate apoptosis in squamous cell carcinogenesis in vivo [25]. Several studies have shown that tumor cell lines and samples derived from human cancers, including breast, hematopoietic, head and neck, lung, kidney, prostate, and ovarian cancers, frequently express activated or phosphorylated Stat3, suggesting that it plays a critical role in regulating fundamental processes associated with malignant transformation and cell survival [26–29].

In this study, we investigated the involvement of Stat3 in UVB exposure-mediated damages in SKH-1 hairless mouse skin. To achieve our objective, we employed a protocol of single exposure of UVB radiation (180 mJ/cm^2) to the skin of SKH-1 hairless mice and the animals were sacrificed at 3, 6, 12, and 24 h post-UVB exposure. We first assessed the effect of UVB exposure on Stat3 protein levels in the skin of SKH-1 hairless mice. As shown by immunoblot analysis (Fig. 1), single UVB irradiation resulted in a significant upregulation of Stat3 protein expression at 24 h following UVB exposure. This observation was further verified by immunohistochemical analysis of Stat3 in UVB-exposed skin biopsies (Fig. 2). This is an important observation because the activation of Stat3 signaling has been shown to act on several downstream targets such as cdk2, cyclin D1, c-myc, and Bcl-x that has been associated with malignant progression [30,31].

Stat3 activation requires the phosphorylation of both Tyr^{705} and Ser^{727} in response to stimulation by cytokines and growth factors. Therefore, we next investigated whether or not UVB exposure to the skin of SKH-1 hairless mouse causes a phosphorylative activation of Stat3. As shown by immunoblot (Fig. 3) and

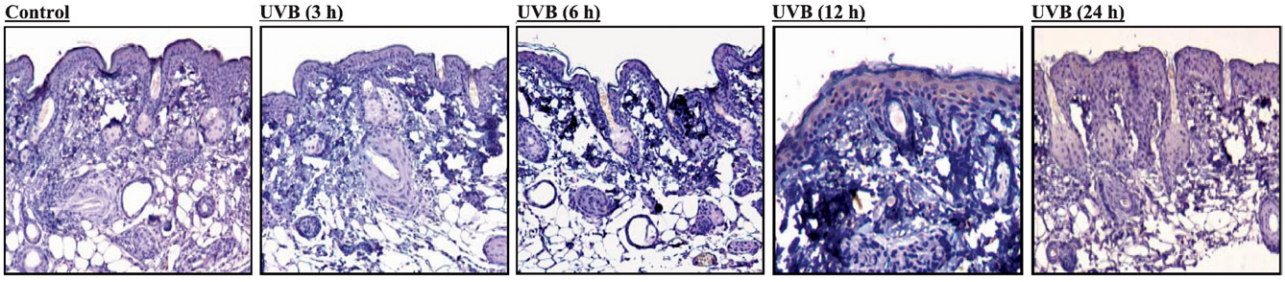

Fig. 2. UVB exposure resulted in a significant upregulation in Stat3 immunostaining in SKH-1 hairless mouse skin. The SKH-1 hairless mice (8 mice per group) were treated with UVB (180 mJ/cm^2); and the skin biopsies were obtained at 3, 6, 12, and 24 h post-UVB exposure, and processed for immunohistochemical analysis as described under Materials and methods. In addition, 6 μm thick paraffin-embedded skin sections were cut and immunohistochemical analysis was performed for Stat3 using appropriate antibodies. The images from immunostaining experiments were captured using a Spot Insight QE Camera (Diagnostics Instruments, USA) attached to a Nikon Optiphot Microscope (Nikon, Japan). The representative pictures are from 3 to 4 skin biopsies.

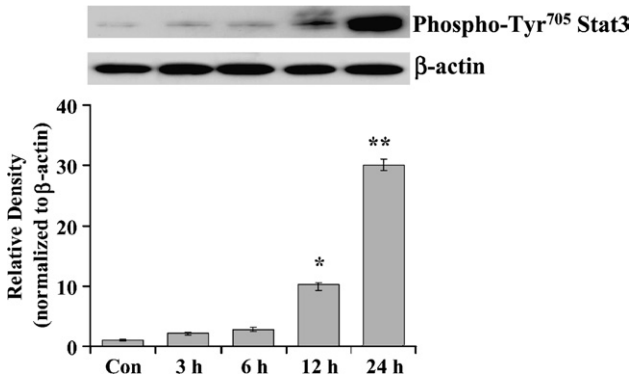

Fig. 3. UVB exposure resulted in a significant upregulation in the protein levels of phospho-(Tyr^{705}) Stat-3, in SKH-1 hairless mouse skin. The SKH-1 hairless mice (8 mice per group) were treated with UVB (180 mJ/cm^2); and at 3, 6, 12, and 24 h post-UVB exposure, skin samples were obtained and processed for immunoblot analysis as described under Materials and methods. The effect of UVB on modulations in phospho-(Tyr^{705}) Stat3 levels was determined by immunoblot analysis using appropriate antibodies. Equal loading was confirmed by stripping the immunoblot and reprobing it for β-actin. The quantification of protein was performed by densitometric analysis using Scion Image Software (Scion, Frederick, MD) and the data (relative density normalized to β-actin) are expressed as means ± SE of three independent experiments (**$p < 0.01$; *$p < 0.05$).

immunohistochemical (Fig. 4) analyses, we found that UVB exposure resulted in an appreciable upregulation of phospho-(Tyr^{705}) Stat3 protein at 12 and 24 h post-UVB-exposed SKH-1 hairless mouse skin. The data suggest that UVB-caused upregulation of Stat3 was associated with an appreciable activation of phospho-(Tyr^{705}) Stat3 protein.

Previous study from our laboratory and by others has shown that UVB exposure to mouse skin results in the infiltration of leukocytes that is probably initiated by locally generated chemotactic stimuli, often accompanied by simultaneous increases in vascular permeability and blood flow [7,32,33]. We therefore, evaluated the effect of single UVB-mediated induction of epidermal hyperplasia and infiltration of leukocytes. As shown in Fig. 3, single exposure of UVB irradiation to the SKH-1 hairless mouse skin resulted in significant increase in the epidermal thickness and vertical thickness of epidermal cell layers at 12 and 24 h, when compared to untreated animals. We also found that single UVB exposure resulted in a marked increase in the infiltration of leukocytes at 12 and 24 h that comprised of mostly neutrophils with some mononuclear cells admixed (Fig. 5).

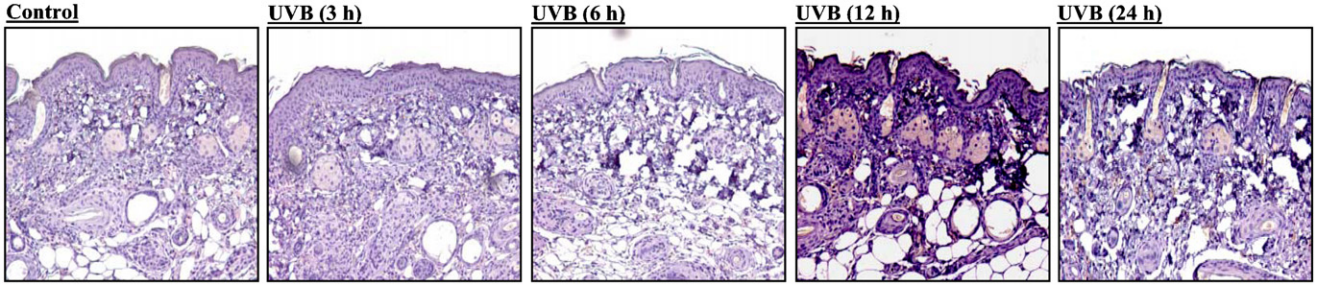

Fig. 4. UVB exposure resulted in a significant upregulation in phospho-(Tyr^{705}) Stat3 immunostaining in SKH-1 hairless mouse skin. The SKH-1 hairless mice (8 mice per group) were treated with UVB (180 mJ/cm^2); and the skin biopsies were obtained at 3, 6, 12, and 24 h post-UVB exposure, and processed for immunohistochemical analysis as described under Materials and methods. In addition, 6 μm thick paraffin-embedded skin sections were cut and immunohistochemical analysis was performed for phospho-(Tyr^{705}) Stat3, using appropriate antibodies. The images from immunostaining experiments were captured using a Spot Insight QE Camera (Diagnostics Instruments, USA) attached to a Nikon Optiphot Microscope (Nikon, Japan). The representative pictures are from 3 to 4 skin biopsies.

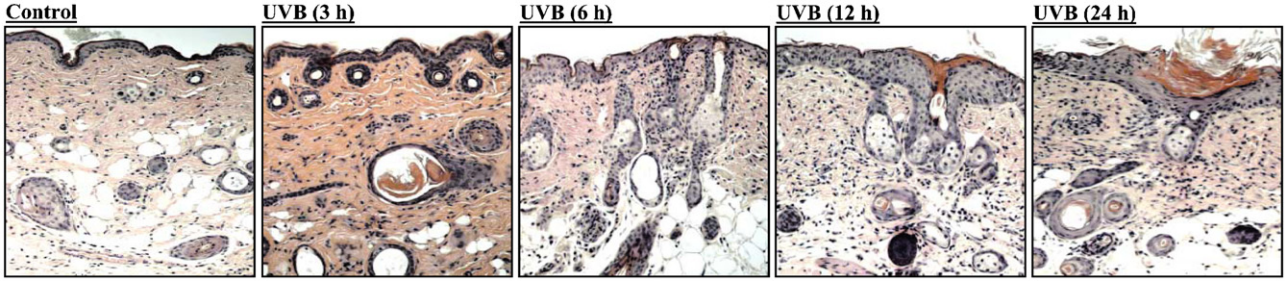

Fig. 5. UVB exposure-mediated increases in hyperplasia and infiltration of leukocytes in SKH-1 hairless mouse skin. The SKH-1 hairless mice (8 mice each group) were subjected to UVB (180 mJ/cm^2) treatments as detailed under Materials and methods. Animals were euthanized and skin biopsies were collected at 3, 6, 12, and 24 h post-UVB exposure. Histopathological analysis was done by using hematoxylin and eosin staining and examination under a microscope. The images from immunostaining experiments were captured using a Spot Insight QE Camera (Diagnostics Instruments, USA) attached to a Nikon Optiphot Microscope (Nikon, Japan). The representative pictures are from 3 to 4 skin biopsies.

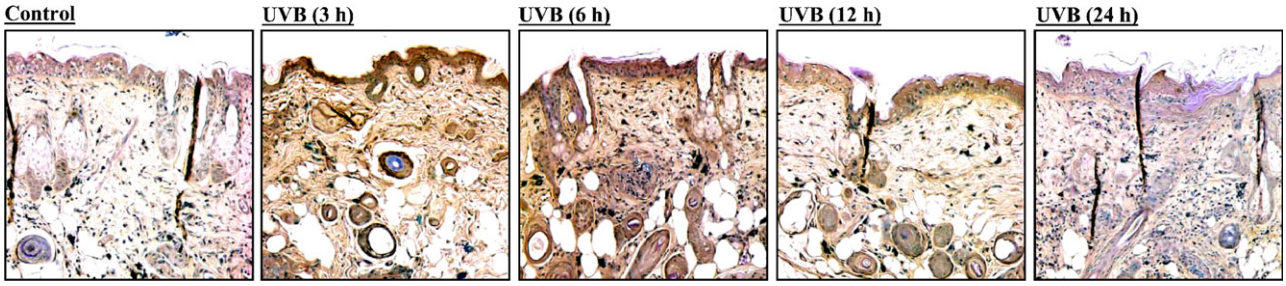

Fig. 6. UVB exposure-mediated modulation in M30 CytoDEATH expression in SKH-1 hairless mouse skin. The SKH-1 hairless mice (8 mice each group) were subjected to UVB (180 mJ/cm^2) treatments as detailed under Materials and methods. Animals were euthanized and skin biopsies were collected at 3, 6, 12, and 24 h post-UVB exposure. In addition, 6 μm thick paraffin-embedded skin sections were cut and immunohistochemical analysis was performed for M30 CytoDEATH using the monoclonal M30 CytoDEATH primary antibody to determine early apoptotic tissues. The images from immunostaining experiments were captured using a Spot Insight QE Camera (Diagnostics Instruments, USA) attached to a Nikon Optiphot Microscope (Nikon, Japan). The representative pictures are from 3 to 4 skin biopsies.

To study the association of UVB exposure-mediated activation of Stat3 with UVB-mediated apoptosis, which is a normal defense mechanism of skin, we assessed apoptosis using M30 CytoDEATH, which is specific for neo-epitope in cytokeratin 18 that becomes available at an early caspase cleavage during apoptosis [34,35]. Our data demonstrated that UVB exposure resulted in a marked increase in M30 CytoDEATH staining in the skin of SKH-1 hairless mouse, when compared to control (Fig. 6). Maximum expression of M30 CytoDEATH was observed at 3 and 6 h post-UVB exposure that gradually diminished at 12 and 24 h post-UVB exposure. These results suggested that UVB exposure-mediated early apoptosis induction in the skin was inhibited by the activation of Stat3. This is not unexpected because studies by Shen et al. [36] have shown that Stat3 could confer protection from apoptosis induced by UV irradiation in fibroblasts.

In conclusion, we have demonstrated, for the first time, that UVB exposure to skin resulted in an activation of pro-survival protein Stat3 (and the associated events) that leads to the inhibition of apoptosis signaling (self-defense), which may ultimately contribute to the development of cancer and possibly other hyperproliferative conditions caused by UVB. Therefore, based on our data, we suggest that Stat3 could serve as a target for the management of UVB exposure-mediated damages including skin cancer.

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