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Role of β -TrCP ubiquitin ligase receptor in UVB mediated responses in skin

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Abstract

Skin cancers are the most common cancers in the United States. Exposure to UVB radiation is a major risk factor for skin cancer induction. SCF ^{β -TrCP} E3 ubiquitin ligase has been found to be involved in cell cycle, cell proliferation and transformation. Aberrant up-regulation of beta-transducin repeats-containing proteins (β -TrCP) is often found in cancer cell lines and primary tumors. We have previously demonstrated that β -TrCP2 is over-expressed in chemically induced mouse skin tumors [1]. Various cellular stress stimuli, including UVB, induce an increase in β -TrCP1 mRNA and protein levels in human cells [2]. We have previously shown that inhibition of β -TrCP function, by induction of dominant negative β -TrCP2 (β -TrCP2 ^{Δ F}), *in vitro* in hTERT immortalized normal keratinocytes, results in increase in UVB induced apoptosis [3]. We have generated transgenic mice with inducible, selective expression of dominant negative β -TrCP2 in epidermis with the Keratin 5 promoter (K5-rTA $\times$ TRE-HA- β -TrCP2 ^{Δ F}). Here we report that inhibition of β -TrCP function in mouse epidermis results in decrease in UVB-induced edema, hyperplasia, and inflammatory response and increment in UVB-induced apoptosis in skin. Our results suggest that β -TrCP may be an essential player in UVB induced responses in skin and can be a potential therapeutic target for skin cancer.

Keywords

Skin Cancer; UVB; β -TrCP; β -TrCP ^{Δ F}transgenic mice; IL-6; Inflammation

Introduction

World Cancer Report 2010 estimates that skin cancer constitutes about one third of all newly diagnosed cancers in the world. It also states that UVB component of the solar radiation is responsible for 90% of non-melanoma skin cancers. Although the mortality rate of non-melanoma skin cancer is relatively low (1900 a year), it represents a major healthcare problem because of high morbidity.

(<http://www.cancer.org/acs/groups/content/@epidemiologysurveillance/documents/document/acspc-026238.pdf>). UV radiation is part of the electromagnetic (light) spectrum

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that reaches the earth from the sun. It consists of wavelengths shorter than visible light which are classified as UVA, UVB and UVC. UVC, shortest wavelength rays, are mostly absorbed by the ozone layer and do not reach the earth. UVA and UVB, however, penetrate the atmosphere and range from 320 to 400 nm and 280 to 320 nm, respectively. UVB, and up to some extent UVA, are essential players in conditions such as premature skin aging, eye damage (including cataracts), and skin cancers.

UVB radiation induces a number of pathologic changes in skin, including erythema, edema, hyperplasia, sunburn cell formation, immune suppression, and skin cancer [4]. Suppression of the immune response is an imperative attribute of exposure to UVB radiation [5]. It has also been known that recruitment of inflammatory cells and neutrophilic infiltration begins after UV exposure and remains high for several days [6]. After exposure to UV radiation, epidermal cells release a variety of cytokines which have been described to act as mediators for immune and inflammatory responses of the skin [7,8].

The ubiquitin proteasome pathway (UPP) is imperative for degradation of proteins involved in complex regulatory mechanisms as cell proliferation and survival. UPP requires concerted play of the three enzymes; E1-ubiquitin-activating enzyme, E2-ubiquitin-conjugating enzyme and E3 ubiquitin ligase to sequentially tag proteins for ubiquitination and subsequent proteasomal degradation. SCF E3 ubiquitin ligases are composed of Skp1, Cul1, Roc1, and F-box proteins, where F-box proteins are substrate recognizing subunits [2]. β -Transducin repeat-containing F-box proteins (β -TrCP) are a key factor in the recruitment of SCF β -TrCP E3 ligases for ubiquitination of specifically phosphorylated substrates and play a pivotal role in the regulation of cell division and various signal transduction pathways, which in turn are essential for many aspects of carcinogenesis [9]. The ubiquitin proteasome pathway controls degradation of the majority of regulatory eukaryotic proteins, including those which play a key role in tumorigenesis [9,10]. Abundance of β -TrCP may serve as a key point of regulation in transducing signals in NF- κ B and β -catenin/Tcf pathways by targeting I κ B and β -catenin proteins respectively [1,11]. Other substrates of β -TrCP include CDK1, CDC25, EMI1, claspin, WEE1, Vpu, ATF4/CREB2, Snail1 and many others [2,3,9,12–14].

We have previously demonstrated that inhibition of β -TrCP function, by induction of dominant negative β -TrCP2 (β -TrCP2 $^{\Delta F}$), in hTERT immortalized normal keratinocytes, results in increase in UVB-induced apoptosis [3]. We also established that this effect was independent of NF- κ B and p53 pathways. In this study, we extended our previous observations to *in vivo* transgenic mouse model with tissue-specific conditional β -TrCP2 $^{\Delta F}$ expression. Here we show that inhibition of β -TrCP function in mouse epidermis results in decrease in UVB-induced inflammatory response and increment in UVB-induced apoptosis in skin. Decline in UVB-induced pathological changes as skin edema and hyperplasia are also pertinent in transgenic mice with induced expression of β -TrCP2 $^{\Delta F}$.

Materials and methods

Animals and treatments

Construct design—The construct containing full-length mouse HA-tagged β -TrCP2 $^{\Delta F}$ were kindly provided by Dr. S. Fuchs (University of Pennsylvania). It was sub-cloned into pBI-G vector which contains a bi-directional tet-responsive promoter (Clontech, Mountain View CA). The resulting construct expresses β -galactosidase on one side and β -TrCP2 $^{\Delta F}$ -HA on the other side.

Generation of transgenic animals—The transgene was excised from the plasmid with AseI restriction enzyme, purified and diluted for microinjections. Transgenic founders were

generated using outbred ICR and inbred FVB mice at the University of Colorado Cancer Center Laboratory Animal and Transgenic Core Facility. Transgenic constructs (approximately 500 copies) were microinjected into the pro-nucleus of a fertilized egg obtained from a super-ovulated female ICR mouse mated with an FVB male. Following the injection, eggs were transferred into the oviduct of a pseudo-pregnant female ICR mouse mated with a vasectomized male. Three to four week old pups were tested for integration of the transgene by PCR analysis of tail DNA using primers for lacZ: 5' primer: 5' GACCCGCATTGACCCTAA 3'; 3' primer: 5' CGCCATTTGACCACTACCA 3'. The presence of transgene was confirmed by southern blot analysis of tail DNA, digested with XbaI and lacZcDNA as a probe. All transgenic lines were initiated by breeding founders with FVB mice and propagated by serially backcrossing with FVB mice and animals were bred into separate lines. The core colonies of transgenic animals were maintained at the AMC Cancer Research Center animal facility (Denver, CO) and University of Wisconsin animal facility (Madison, WI). TRE-HA- β -TrCP2^{ΔF} mice were crossed with K5-rTA mice (kindly provided by Dr. A. Glick) to generate K5-rTA × TRE-HA- β -TrCP2^{ΔF} double transgenic mice, that were used in UVB experiments. K5-rTA mice are a conditional expression model that allows regulated expression of genes in the mouse epidermis. In this model, doxycycline regulated transactivator rTA is expressed in the epidermis with the keratin 5 promoter whereas the regulated target gene is linked to the tetO binding site [15]. In K5-rTA × TRE-HA- β -TrCP2^{ΔF} double transgenic mice, doxycycline is required for transactivation of HA- β -TrCP2^{ΔF} in mouse epidermis.

UVB exposure—For UVB irradiation, a “Daavlin Research Irradiators” obtained from Daavlin Co. (Bryan, OH) was used. This equipment contains four Westinghouse FS-40-T-12 fluorescent sunlamps (National Biological Corp., Twinsburg, OH), and is equipped with UVB Spectra 305 Dosimeter (Daavlin Company, Bryan, OH). This light source emits about 80% radiation in the range of 280–340 nm with peak emission at 314 nm. The sunlamps are covered with Kodacel TA401/407 triacetate filters, which blocks wavelength emission below 280 nm from the FS40 sunlamps.

Male K5-rTA × TRE-HA- β -TrCP2^{ΔF} double transgenic mice, as well as non-transgenic littermates (six weeks old) were divided into four groups of eight mice each; (Group 1) No treatment; (Group 2) Doxycycline treatment only; (Group 3) UVB exposure only and (Group 4) UVB exposure and doxycycline treatment. The third and fourth groups of mice were exposed to 1200 mJ/cm² UVB in three doses of 400 mJ/cm² with 5 min intervals in order to avoid over-heating. The distance between light source to target skin was 9 in. for all UVB irradiation [16]. Mice were treated with 2 mg/ml doxycycline in drinking water just after UVB exposure. Experiment was terminated 24 h following UVB exposure.

Skin edema

The effect of β -TrCP2^{ΔF} expression on UVB-mediated skin edema was studied by evaluating the increase in bi-fold skin thickness and ear-punch weight, measured at 24 h after short-term UVB exposure. The skin thickness was measured with vernier callipers at different sites on the dorsal skin per mouse. For increase in ear-punch weight studies, following short-term UVB exposure, punch skin biopsies from ear (4 mm diameter, two from each ear) were obtained and weighed immediately.

Ornithine decarboxylase enzyme activity

Following the short-term UVB exposure as detailed above, the animals were sacrificed, and skin was surgically removed and made free of connective tissue and fat. Skin was homogenized at 4 °C in a glass-to-glass homogenizer in 10 volumes of ODC buffer [50 mM Tris-HCl buffer (pH 7.5) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM

pyridoxal-5-phosphate, 1 mM 2-mercaptoethanol and 0.1% Tween-80]. The homogenate was centrifuged at $14,000\times g$ at 4°C and the ODC enzyme activity was measured in the supernatant by measuring the release of $^{14}\text{CO}_2$ from DL-[1- ^{14}C] ornithine hydrochloride, as described previously [17].

Skin hyperplasia

The effect of $\beta\text{-TrCP}^{\Delta\text{F}}$ expression on UVB-mediated epidermal hyperplasia was studied by histopathological examination of the tissue sections at 24 h after short-term UVB exposure. The skin from mice was fixed in formalin and embedded in paraffin. 5 μm sections were stained with hematoxylin and eosin (H&E). Epidermal hyperplasia was determined by assessing vertical epidermal thickness and number of vertical epidermal layers.

Western Blotting

Regulated expression of $\text{HOS}^{\Delta\text{F}}\text{-HA}$ was verified by western blot analysis of HEK293T cells, transiently co-transfected with indicated plasmids. Cells were treated with tetracycline (5 $\mu\text{g}/\text{ml}$) where indicated and harvested 24 h later. The lysates were prepared and analyzed by immuno-blotting with anti-HA monoclonal antibody (12CA5, Roche). Skin samples collected from mice were ground with liquid nitrogen, lysed in RIPA buffer, centrifuged and supernatants were collected. These protein samples were exposed to SDS-PAGE and immuno-blotted for HA tag (Cell Signaling Technology, Danvers MA).

Immunohistochemical staining

Formalin fixed and paraffin embedded (FFPE) skin sections were stained for PCNA and IL-6 (SantaCruz Biotechnology, Santa Cruz CA) developed with DAB as described before [18]. PCNA positive cells were counted and calculated as a percent of total cells. Sections were also stained for HA-tag (Cell Signaling Technology, Danvers MA) and developed with secondary antibody labeled with FITC (Jackson Immunoresearch, West Grove PA). These sections were also co-stained with $\beta\text{-tubulin}$ Alexa Fluor 555 conjugate (Cell Signaling Technology, Danvers MA) and mounted with Prolong Gold Antifade Reagent with DAPI (Invitrogen, Carlsbad CA).

TUNEL staining

Formalin fixed and paraffin embedded (FFPE) sections were stained for TUNEL (Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling) using DeadEndTM Fluorometric TUNEL System (Promega Corporation, Madison WI) and mounted with Prolong Gold Antifade Reagent with DAPI (Invitrogen, Carlsbad CA). Cells positive for TUNEL (green) were counted and calculated as a percent to total cells.

Results and discussion

Generation and characterization of K5-rTA $\times$ TRE-HA- $\beta\text{-TrCP}^{\Delta\text{F}}$ transgenic animals

In order to analyze the contribution of $\beta\text{-TrCP}$ ubiquitin ligase receptor to the UVB mediated response in skin, we have generated transgenic mice with conditional expression of dominant negative mutant of $\beta\text{-TrCP}2$ ($\beta\text{-TrCP}^{\Delta\text{F}}$) in mouse epidermis. We have subcloned HA-tagged $\beta\text{-TrCP}^{\Delta\text{F}}$ under the control of tetracycline responsive element (TRE) in pBI-G vector (Fig. 1A). To confirm the inducibility of this construct we co-transfected it with tet-on plasmid in 293T cells. As shown in Fig. 1B, western blotting by antibody against HA-tag confirmed the expression of HA- $\beta\text{-TrCP}^{\Delta\text{F}}$ only in the presence of tetracycline. This construct was used to generate TRE-HA- $\beta\text{-TrCP}^{\Delta\text{F}}$ transgenic mice. In K5-rTA transgenic mice, transactivator rTA is expressed under the control of keratin 5 promoter that targets the

transgene expression to the basal layer of mouse epidermis [15]. TRE-HA- β -TrCP^{ΔF} mice were crossed with K5-rTA mice in order to get progeny expressing both the genes, hence, resulting in conditional expression of β -TrCP^{ΔF} in mouse epidermis. Gene expression was checked by PCR and confirmed by southern blotting for lacZ from tail DNA (data not shown). Double transgenic progeny express HA- β -TrCP^{ΔF} in the epidermis only in presence of doxycycline (synthetic tetracycline derivative) as demonstrated in Fig. 1C. These mice were given 2 mg/ml doxycycline in drinking water for 24–48 h. HA tag antibody was used to check the expression of protein in all experiments. Fig. 1D shows HA-tag expression in skin of double transgenic mice with and without doxycycline treatment. These double transgenic mice provide a coherent model in order to study the role of β -TrCP in epidermal tissue.

β -TrCP^{ΔF} protects mouse skin from UVB induced pathological and biochemical alterations

To assess the effect of inhibition of β -TrCP function on UVB mediated responses, we have treated double-transgenic (TRE-HA β -TrCP^{ΔF}; K5-rTA) mice and non-transgenic FVB mice with UVB (1200 mJ/cm²), and doxycycline in drinking water (2 mg/ml, 24 h). To estimate skin edema, we measured bi-fold skin thickness and weighed equal size skin punches of mice before and after UVB exposure. As shown in Fig. 2A and B, UVB exposure resulted in ~20–40% increase in skin thickness and punch weight of transgenic and non-transgenic counterparts. β -TrCP^{ΔF} transgenic mice exposed to UVB and treated with doxycycline show 30–40% decrease in skin edema, an effect not observed in their non-transgenic counterparts. These results suggest that inhibition of β -TrCP function reduces UVB induced skin edema, and that the observed phenomenon is specific to transgene induction, and not a non-specific effect of treatment with doxycycline.

Cellular proliferation, in response to UVB, is a trademark of photo-carcinogenesis [19]. Hence, we also estimated UVB induced proliferation by staining FFPE skin sections with PCNA antibody (Fig. 2C). Double transgenic mice treated with doxycycline establish 37% decrease in UVB induced proliferation as compared to no doxycycline treatment or non-transgenic counterparts, suggesting that inhibition of β -TrCP function attenuates UVB-induced proliferation in mouse epidermis.

Absorption of UV light by molecules in the cells results in the generation of reactive oxygen species (ROS), which can cause oxidative DNA damage. Ornithine decarboxylase is an essential enzyme for cell growth and DNA double strand repair as it is involved in synthesis of polyamines which are important for stabilizing DNA structure, DNA double strand-break repair pathway and as antioxidants [20]. ODC has been implicated in diverse biological processes as cell growth, differentiation, transformation and apoptosis. The induction of ODC activity is a hallmark of skin exposure to UVB radiation [21,22]. As expected, we observed substantial induction in ODC activity in mouse skin. However, expression of β -TrCP^{ΔF} in mice, with treatment of doxycycline, resulted in 50% decrease in UVB induced ODC enzyme activity as compared to no doxycycline treated or non-transgenic mice (Fig. 2D), suggesting that β -TrCP is involved in the induction of ODC enzyme activity by UVB radiation.

UVB exposure is also known to cause variety of morphological and pathological changes [5,19]. Fig. 3 lays out H&E stained FFPE sections displaying morphological and pathological changes brought about by UVB exposure. Stars, arrows and triangles display skin edema, neutrophil infiltration and apoptotic keratinocytes/sunburn cells respectively. As a consequence of UVB exposure, skin sections show edema, neutrophil infiltration and apoptosis. Neutrophils are considered hallmarks of acute inflammation as they migrate to the site of inflammation in response to secretion of cytokines. Moderate neutrophilic infiltration was observed in mice exposed to UVB, whereas, expression of β -TrCP^{ΔF} resulted in

abatement of the inflammatory response. Together these results suggest that inhibition of β -TrCP function reduces UVB induced edema, inflammatory response, proliferation, and ODC activity in mouse skin.

UVB invoked Interleukin 6 (IL-6) secretion is diminished by β -TrCP^{ΔF} expression

It has been known that some cytokines participate in immune and inflammatory responses. IL-1 β , TNF- α , and IL-6 are important pro-inflammatory cytokines. It has been reported that production of IL-1 β and IL-6 was induced in humans following UV irradiation. IL-1 β is reported to be a potent inducer of IL-6 and to have synergistic effects with IL-6. IL-6 has been reported to be increased in mouse serum, human plasma, human keratinocytes, and pterygium epithelial cells following UVB irradiation [8,23–26]. IL-6, a cytokine with multiple effects, is one of the cytokines most readily inducible in a wide number of cell types and has been reported to be involved in immune responses. IL-6 has been shown to be a crucial player in UV induced immune response in skin and a potent inducer of IL-10 [8,27]. In view of this, we looked for IL-6 expression in FFPE skin sections. As demonstrated in Fig. 4, accumulation of IL-6 was observed after UVB exposure which was conspicuously diminished in mice expressing β -TrCP^{ΔF}, suggesting that β -TrCP contributes to the induction of IL-6 expression by UVB radiation. Higher levels of IL-6 have been detected in cutaneous inflammatory diseases and cancer patients [28]. In multiple myelomas, IL-6 is both produced and required for survival by cancer cells itself [29]. Hence there is an interest in developing anti-IL-6 agents as therapy against many of the hyper-proliferative diseases as cancer [30]. Thus observed regulation of IL-6 by β -TrCP may have broader implications, and may potentially be used as a therapeutic modality in down-regulation of IL-6 expression.

β -TrCP^{ΔF} expression boosts UVB induced apoptosis in mouse skin

As exhibited in Fig. 5, down-regulation of β -TrCP in UVB exposed skin augments apoptosis. Our previous results in mouse squamous cancer cells have demonstrated that ectopic expression of β -TrCP^{ΔF} induces cell death (data not shown). It has been reported that ectopic expression of dominant-negative β -TrCP2 leads to apoptosis in melanoma cells [31]. β -TrCP2 inhibitors have also been reported to induce apoptosis in mammary adenocarcinoma, neuroblastoma, prostate cancer and colorectal cancer cells [2,32]. Induction of apoptosis by down-regulation of β -TrCP function is usually attributed to its effects on NF- κ B signaling via stabilization of I κ B [9]. Although, β -TrCP serves as an essential player in NF- κ B activation, we did not observe down-regulation of UVB-induced NF- κ B activity, measured as nuclear accumulation of p65 (RelA), with ectopic expression of β -TrCP^{ΔF}. These results are in line with our previous study where inhibition of β -TrCP function did not affect NF- κ B activity *in vitro* in UVB exposed keratinocytes [3]. Involvement of NF- κ B in UV-induced skin carcinogenesis has been well reported [33]. Different views have been reported about signaling mechanism/s controlling UV-induced NF- κ B activation, such as, by a distinct pathway from other stimuli such as TNF α or could be I κ B α independent [34,35]. It has been reported that there may be distinct signaling pathways and mechanisms of activation induced by immune and inflammatory stimuli [36–38].

Collectively, these results corroborate that β -TrCP expression is imperative for UVB exposure induced responses such as edema, hyperplasia, sunburn cell formation, immune suppression and inflammatory response. Future studies will evaluate the role of β -TrCP in UVB-induced malignancies, and may establish SCF ^{β -TrCP} ubiquitin ligase as a promising target for prevention and therapy.

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Fig. 1.

Generation and characterization of transgenic animals. (A) Construct preparation. The construct, pBI-G-HA- β -TrCP Δ^F , containing full-length mouse HA-tagged HOS Δ^F was sub-cloned into pBI-G vector (Clontech, Mountain View CA) which contains a bi-directional tet-responsive promoter. The resulting construct expresses β -galactosidase on one side and HOS Δ^F -HA on the other side. (B) Expression of Construct in vitro. 293T human embryo kidney cells were co-transfected with Tet-On and pBI-G vector or pBI-G-HA- β -TrCP Δ^F . Cells were treated with tetracycline (5 μ g/ml) where indicated and harvested 24 h later. Lysates were prepared and analyzed by immunoblotting with anti-HA 12CA5 monoclonal antibody (Roche Applied Science, Germany). (C and D). HA-Tag expression in K5-Tet-On-TRE-HA- β -TrCP Δ^F transgenic mice. (C) Mice were treated with 2 mg/ml doxycycline in drinking water for 24 h. Skin was collected, lysates were prepared and analyzed by immunoblotting as mentioned previously. (D) Skin sections were fixed in formalin and embedded in paraffin. Five micrometer sections were cut and stained for TUNEL (DeadEndTM Fluorometric TUNEL System, Promega Corporation, Madison WI) and β -tubulin Alexa Fluor 555 Conjugate (Cell Signaling Technology, Danvers, MA) and mounted with Prolong Gold Antifade with DAPI (Invitrogen, Carlsbad CA).

Fig. 2.

Effect of inhibition of β -TrCP function on UVB-induced proliferation, skin thickness and Ornithine decarboxylase activity. Non-transgenic (FVB) and transgenic (K5-Tet-On-TRE-HA- β -TrCP ΔF) mice were exposed to UVB and treated with doxycycline as described in materials and methods. (A) Skin thickness. Skin thickness was measured by vernier calipers. Bars demonstrate bi fold skin thickness with and without UVB exposure and doxycycline in FVB and transgenic mice. (B) Skin punch weight. Skin punches of the same size were collected from each ear and weighed. Bars show skin punch weight with and without UVB exposure and doxycycline in FVB and transgenic mice. (C) Immunohistostaining for PCNA. FFPE skin sections were stained with PCNA antibody (Santa-Cruz Biotechnology, SantaCruz CA) using M.O.M kit (Vector Laboratories Inc., Burlingame, CA) by following manufacturers directions and developing with DAB. PCNA positive cells were counted and calculated as a percent of total cells. Bars show PCNA positive cells as a percent of total cells with and without UVB exposure and doxycycline in FVB and transgenic mice. (D) Ornithine decarboxylase Activity. Ornithine decarboxylase activity (ODC) was assayed as described before [17]. Bars demonstrate ODC activity as measured by pmol $^{14}CO_2$ released/hr/mg protein with and without UVB exposure and doxycycline in FVB and transgenic mice. * $p < 0.05$ (as compared to untreated transgenic animals); ** $p < 0.05$ (as compared to UVB only treated transgenic animals).

Fig. 3. Effect of inhibition of β -TrCP function on UVB-induced pathological changes in the skin of transgenic animals. FFPE skin sections of UVB exposed non-transgenic and transgenic mice with and without doxycycline treatment, were stained with hematoxylin and eosin and analyzed for pathological changes. Stars demonstrate edema, arrows demonstrate neutrophilic infiltration, whereas triangles demonstrate apoptotic keratinocytes.

Fig. 4. Effect of ectopic expression of β -TrCP ΔF on UVB-induced Interleukin-6 (IL-6) expression. FFPE skin sections of UVB exposed non-transgenic and transgenic mice, with or without doxycycline treatment, were stained with IL-6 antibody (Santa-Cruz Biotechnology, SantaCruz CA) and developed with DAB.

Fig. 5.

Increase in UVB-induced apoptosis in transgenic animals with ectopic expression of β -TrCP ΔF . (A) FFPE skin sections of UVB exposed non-transgenic and transgenic mice, with or without doxycycline treatment, as mentioned previously in Materials and methods, were processed for Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) using DeadEndTM Fluorometric TUNEL System (Promega Corporation, Madison WI) and mounted with Prolong Gold Antifade Reagent with DAPI (Invitrogen, Carlsbad CA). (B) Cells positive for TUNEL (green) were counted and calculated as a percent to total cells.