Available online at www.sciencedirect.com

ScienceDirect

Biochimie xx (2008) 1–9

BIOCHIMIE

www.elsevier.com/locate/biochi

Research paper

Ultraviolet B and A irradiation induces fibromodulin expression in human fibroblasts *in vitro*

Barbara Iovine [a], Massimiliano Nino [b], Carlo Irace [c], Maria Assunta Bevilacqua [a,*], Giuseppe Monfrecola [b]

[a] *Department of Biochemistry, Faculty of Biotechnology, University of Naples ''Federico II'', via S. Pansini 5, 80131 Naples, Italy*
[b] *Department of Systematic Pathology, Section of Dermatology, Faculty of Medicine, University of Naples ''Federico II'', via S. Pansini 5, 80131 Naples, Italy*
[c] *Department of Experimental Pharmacology, Faculty of Pharmacy, University of Naples ''Federico II'', via D. Montesano 49, 80131 Naples, Italy*

Received 30 July 2008; accepted 10 October 2008

Abstract

Ultraviolet (UV) radiation affects the extracellular matrix (ECM) of the human skin. The small leucine-rich repeat protein fibromodulin interacts with type I and II collagen fibrils, thereby affecting ECM assembly. The aim of this study was to evaluate whether short wave UV (UVB) or long wave UV (UVA) irradiation influences fibromodulin expression. Exponentially growing human fibroblasts (IMR-90 cells) were exposed to increasing doses of UVB (2.5–60 mJ/cm^2) or UVA (0.5–10 J/cm^2). After UV irradiation fibromodulin, p21 and GADD45 levels were evaluated as well as cell viability, reactive oxygen species formation (ROS) and DNA damage. We found that fibromodulin expression: (i) increased after UVB and UVA irradiation; (ii) was 10-fold higher after UVA (10 J/cm^2) versus 5-fold with UVB (10 mJ/cm^2); (iii) correlated with reactive oxygen species formation, particularly after UVA; and (iv) was linked to the DNA damage binding protein (DDB1) translocation in the nucleus, particularly after UVB. These results further suggest that the UV-induced fibromodulin increase could counteract the UV-induced connective tissue damage, promoting the assembly of new collagen fibrils.

Keywords: Fibromodulin (Fmod); Extracellular matrix (ECM); Ultraviolet B; Ultraviolet A; DNA damage binding protein

1. Introduction

The ultraviolet (UV) radiation in sunlight is the major environmental cause of skin damage [1]. Interactions between UV and molecular components of the epidermis and dermis cause such acute and chronic cutaneous changes as erythema, immunosuppression, skin cancers and photoaging [2]. Different ranges of UV wavelengths induce different effects through different mechanisms: UVC (200–290 nm) and UVB (290–320 nm) rays act mainly by exciting the DNA molecule that directly absorbs these wavebands, whereas UVA (320–400 nm) rays influence macromolecules, such as nucleic acids, proteins and lipids, chiefly through the generation of reactive oxygen species [3]. UVC rays have scarce clinical interest; being absorbed by the terrestrial gaseous atmosphere, they do not reach the earth's surface. Thus, studies on the effect of

Abbreviations: ECM, extracellular matrix; UVR, ultraviolet radiation; UVB and UVA, ultraviolet B and A; SLRPs, small leucine-rich repeat protein family; DBB1, DNA damage binding protein 1; PDL, population doubling level; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; ROS, reactive oxygen species; NAC, *N*-acetylcysteine; H_2DCF-DA, 2′,7′-dichlorofluorescin-diacetate; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; SA β–gal, senescence associated-β-galactosidase activity; CDKI, cyclin-dependent kinase inhibitor; Gadd45, growth arrest and DNA damage-inducible; GSH, glutathione; CSA, Cockayne syndrome protein; NER, nucleotide excision repair; AP-1, nuclear transcription factor activator protein 1; MMPs, matrix-degrading metalloproteinases.

* Corresponding author. Tel.: +39 81 746 3642.
E-mail address: bevilacqua@dbbm.unina.it (M.A. Bevilacqua).

0300-9084/$ - see front matter © 2008 Elsevier Masson SAS. All rights reserved.
doi:10.1016/j.biochi.2008.10.017

sunlight on cutaneous collagen metabolism are carried out using UVB or UVA irradiation. UVB radiation is the most energetic and DNA-damaging; it penetrates the epidermis and, to a lesser extent, the upper part of the dermis, whereas UVA radiation penetrates more deeply into the dermis.

UV-induced changes to the molecular components of the extracellular matrix (ECM) have been investigated *in vivo* and *in vitro* [4,5]. The ECM is constituted by a network of collagen fibers to which other molecules are associated in order to provide stability and interaction with surrounding structures. These molecules include negatively charged proteoglycans and members of the small leucine-rich repeat protein family (SLRPs) [6]. SLRPs, which are secreted in the extracellular matrix, are usually bound to the collagen fibril surface by means of their protein core and project their side chains into the interfibrillar space thereby forming a three-dimensional network that extends across the whole ECM. The interaction of the core protein with the collagen fibril surface is one of the factors that limit lateral growth during fibrillogenesis. Any alteration to this fraction affects the three-dimensional layout of the ECM. Fibromodulin, a 59-kDa cytosolic-secreted protein belonging to SLRP family, is involved in the assembly of the ECM by virtue of its ability to interact with type I and type II collagen fibrils [7].

Fibromodulin is normally produced by collagen-rich tissues, and reaches its highest levels in articular cartilage, tendon, bone and connective tissue [8]. Mice knocked-out for fibromodulin have malformation of collagen fibrils, resulting from an increase in the amount of small diameter fibrils, and symptoms resembling osteoarthritis [9,10]. In addition, the fibromodulin (−/−) tendons contain irregular collagen fibrils leading to decreased tendon stiffness [11].

We recently studied the regulation of fibromodulin gene expression, and demonstrated that: (i) fibromodulin expression is lower in senescent cells than in normal exponentially growing human fibroblasts; (ii) UV exposure increases fibromodulin levels by about 50-fold in exponentially growing human fibroblasts but not in senescent cells; and (iii) DNA damage binding protein 1 (DDB1) is a key factor in UV-dependent activation of the fibromodulin gene [12]. These results were obtained in cells irradiated with UVC (15 J/m^2), which, as indicated above, does not reach the earth's surface. The aim of the present study was to investigate the expression of fibromodulin in human proliferating fibroblasts (IMR-90 cells) exposed to increasing doses of UVB (290−320 nm) or UVA (320−400 nm), both of which are involved in acute and chronic skin damage.

2. Materials and methods

2.1. Cell culture

IMR-90, human primary fibroblasts, were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen). Cultures were maintained at 37 °C in a 5% CO_2-humidified atmosphere. The population doubling level (PDL) of IMR-90 cells was calculated by using the following equation: log(*nh*/*ni*)/log 2, where *ni* is the initial number of cells and *nh* is the final number of cells at each passage (23). The cells used were at 28 PDL (HF-28 cells).

2.2. UV irradiation

Before UV irradiation, cells were washed and covered with phosphate-buffered saline (PBS). As a source of UVB, six Philips TL12/60W fluorescent lamps (Philips, Eindhoven, Netherlands) emitting UV light mainly between 290 and 320 nm with a peak at 300 nm, were used. A bank of six fluorescent tubes Philips TL09/60 W, whose emission ranged from 320 to 400 nm peaking at 365 nm, served as UVA source. During the irradiation sessions with UVA, wavelengths below 320 nm were removed using a 5 mm glass window. The intensity of irradiation, measured with a UV meter. (Spectrolyne mod., Spectronics Corp., Westbury, NY, USA) was 0.8 mW/cm^2 for UVB and 5 mW/cm^2 for UVA. For each irradiation session, cells received the following doses of UVB 2.5, 5, 10, 20 and 40 mJ/cm^2 or of UVA 0.5, 1.25, 2.5, 5, 10 J/cm^2.

2.3. Analysis of cell vitality

Cell vitality was determined with the trypan blue method. Twenty-four hours after irradiation, cells were washed twice with PBS and incubated with trypsin/EDTA. Cells were then harvested and centrifuged at 1000 × *g* for 10 min. The cell pellet was resuspended in an appropriate volume of PBS, and 0.5 ml of the cell suspension were combined with 0.5 ml of trypan blue solution. The mix was incubated for 15 min at room temperature and the number of unstained cells (vitality cells) and the total number of cells (vital and not) were determined on the hemacytometer under a microscope (dead cells will take up the trypan blue stain). The percentage of viable cells was determined dividing the number of unstained cells by the total number of cells.

2.4. RNA extraction and real time PCR

A total of 1.0 × 10^6 fibroblasts were seeded onto 100 mm culture plates in 10 ml of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After incubation for 1 day at 37 °C in 5% CO_2, the medium was replaced with 1 ml of cold phosphate-buffered saline, and the monolayer of subconfluent cells was irradiated with UVA (320−400 nm) and UVB (290−320 nm). The phosphate-buffered saline was then replaced with 10 ml of fresh culture medium, and the cells were allowed to recover for 24 h. Total RNA was prepared from IMR-90 cells by using the RNeasy mini kit (Qiagen) and subjected to cDNA synthesis with random hexanucleotide primers and MultiScribe reverse transcriptase (Invitrogen) at 48 °C for 1 h. The cDNA was then amplified in an iCycler iQ real time PCR detection system (Bio-Rad) using iQTM SYBR Green Supermix in triplicate in 25 µl reaction volumes. Relative quantification of gene expression was performed using the $2_T^{-\Delta\Delta c}$ method referente

Please cite this article in press as: B. Iovine et al., Ultraviolet B and A irradiation induces fibromodulin expression in human fibroblasts *in vitro*, Biochimie (2008), doi:10.1016/j.biochi.2008.10.017

mRNA was that of c-Abl [12]. The ratios between $2_T^{-\Delta\Delta c}$ before the UV treatment and those calculated for the samples at various times/doses after the exposure to UVA and UVB light are expressed as fold changes. The primer sequences were as follows:

c-Abl forward, 5′-TGGAGATAACACTCTAAGCATAACT AAAGGT-3′
c-Abl reverse, 5′-GATGTAGTTGCTTGGGACCCA-3′
fibromodulin forward, 5′-CAACCAAGTGCAGAAGATCC-3′
fibromodulin reverse, 5′-CACCTGCAGCTTGGAGAGT-3′
$p21^{waf1}$ forward, 5′-CTGGAGACTCTCAGGGTCGAA-3′
$p21^{waf1}$ reverse, 5′-CGGCGTTTGGAGTGGTAGAA-3′
GADD45 forward, 5′-AGACCCCGGACCTGCACT-3′
GADD45 reverse, 5′-CCGGCAAAAACAAATAAGTTGA CT-3′

2.5. Assay for β-galactosidase activity in situ

In situ β-gal staining with X-gal was performed as previously described with a slight modification [13]. Briefly, cells were fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde, washed twice with PBS, and then incubated for 48 h at 37 °C with 1 mg/ml X-gal dissolved in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 150 mM NaCl and 40 mM sodium citrate.

2.6. Comet assay

DNA damage was evaluated with the Comet assay [14] with slight modifications of the manufacturer's instructions: briefly, the cells underwent UV treatment, as described, washed with PBS, trypsinized, re-suspended in PBS, and combined with LM-agarose (supplied in the Trevigen kit assay) at a ratio of 1:8 (cells:agarose). Electrophoretic run and qualitative and/or quantitative analyses were carried out according to the Trevigen protocol. Quantitative analyses of the results were done by using the Image software (National Institutes of Health, Bethesda, MD, USA), as suggested by the manufacturer. Data are reported as the ratio between tail and nucleus areas.

2.7. Measurement of reactive oxygen species and NAC treatment

The formation of ROS was evaluated by means of the probe 2′,7′-dichlorofluorescin-diacetate (H_2DCF-DA) [15]. Briefly, IMR-90 cells were grown in DMEM containing 10% (v/v) fetal bovine serum, and then plated at a density of 5000 cells/well in 96-well dishes. Cells were allowed to grow for 24 h, incubated in the growth medium containing 5 μM of H_2DCF-DA (Sigma) for 2 h at 37 °C and then irradiated with UVA and UVB at different times/doses of exposure. After treatment, cells were washed twice with PBS buffer and plates were placed in a fluorescent microplate reader (Perkin Elmer LS 55 Luminescence Spectrometer, Perkin-Elmer Ltd., Beaconsfield, England).

Fluorescence was monitored using an excitation wavelength of 485 nm and an emission wavelength of 538 nm. In each experiment, fluorescence increase was measured in three replicate cultures for each UV treatment. *N*-acetylcysteine (NAC), were obtained from Sigma. These were dissolved in the designated solution buffer at 100 mM as a stock solution and kept at 4 or −20 °C.

2.8. Western blot analysis

Cytosolic and nuclear extracts of IMR-90 were prepared 24 h after UVB irradiation. Briefly, harvested cells (3×10^6) were washed twice with ice-cold PBS and centrifuged at 180 × *g* for 10 min at 4 °C. The cell pellet was resuspended in 100 μl of ice-cold hypotonic lysis buffer (10 mM HEPES, 10 mM KCl, 0.5 mM phenylmethylsulphonyfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated on ice for 15 min. The cells were lysed by rapid passage through a syringe needle for five or six times, and the cytoplasmic fraction was then obtained by centrifugation for 1 min at 13,000 × *g*. The supernatant containing the cytosolic fraction was removed and stored at −80 °C. The nuclear pellet was resuspended in high salt extraction buffer (20 mM HEPES pH 7.9, 10 mM NaCl, 0.2 mM EDTA, 25% v/v glycerol, 0.5 mM phenylmethylsulphonyfluoride, 1.5 μg/ml soybean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0.1 mM benzamidine, 0.5 mM dithiothreitol) and incubated with shaking at 4 °C for 30 min. The nuclear extract was then centrifuged for 15 min at 13,000 × *g*, and the supernatant was aliquoted and stored at −80 °C. Protein concentration was determined by a Bio-Rad (Italy) protein assay kit. For Western blot analyses, nucleus and cytosolic proteins from IMR-90 cells were challenged with antibodies against DDB1 (Zymed) and tubulin (Santa Cruz Biotechnology), and the signals were detected by using the ECL kit (Amersham Biosciences).

2.9. Fluorescence microscopy

Fibroblasts were grown on slides for 24 h and irradiated with UVA and UVB. After 24 h of irradiation, cells were washed with PBS and fixed with cold 3.7% formaldehyde in PBS at 4 °C for 30 min and then were permeabilized with 0.2% Triton in PBS. After 15 min, cells were washed twice with PBS buffer and incubated with 0.1% Triton, 10% FBS in PBS for 1 h at room temperature. The cells, washed twice with PBS buffer, were first treated with polyclonal rabbit anti-DDB1 antibody (1:500 Santa Cruz Biotechnology) in PBS 10% FBS and 0.1% Triton for 3 h at room temperature and subsequently with goat anti-rabbit IgG-FITC (1:100, Santa Cruz Biotechnology) in PBS 10% FBS and 0.1% Triton for 1 h at room temperature in the dark. After washing with PBS, cells were stained with 0.1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma) at room temperature for 30 min. Slides were mounted in 50% glycerol in PBS and cells were then examined by fluorescence microscopy on a Zeiss Axiophot at 40× resolution with appropriate filters for DDB1 and DAPI.

Please cite this article in press as: B. Iovine et al., Ultraviolet B and A irradiation induces fibromodulin expression in human fibroblasts *in vitro*, Biochimie (2008), doi:10.1016/j.biochi.2008.10.017

2.10. Statistics

Results were expressed as the mean ± SE of *n* experiments. Statistical significance was calculated by one-way analysis of variance (ANOVA) and *P* value for a multiple comparison test [16]. The level of statistical significance was defined as $P < 0.05$.

3. Results

3.1. Effects of ultraviolet B and A irradiation on fibromodulin expression and cell viability

To examine the effects of UVB and UVA irradiation on the expression of the fibromodulin gene, we irradiated exponentially growing human fibroblasts (IMR-90 at 28 PDL) with increasing doses of UVB (from 2.5 to 60 mJ/cm^2) or UVA (from 0.50 to 10 J/cm^2). The expression of fibromodulin was examined by real time PCR (see Section 2). As shown in Fig. 1, fibromodulin levels were significantly increased in both UVB and UVA-irradiated IMR90 cells compared with un-irradiated control cells. UVB irradiation dose-dependently activated fibromodulin expression starting from 2.5 mJ/cm^2 with maximal induction at 10 mJ/cm^2 (Fig. 1A). At increasing UVB doses up to 60 mJ/cm^2 there was a gradual decrease of fibromodulin expression. UVA irradiation caused a rapid jump in fibromodulin expression from 0.5 to 10 J/cm^2 then a plateau (Fig. 1B). The effects of UVB and UVA irradiation on cell viability are reported in Fig. 1C,D. UVB irradiation (between 2.5 and 60 mJ/cm^2) provoked a linear decrease of cell viability compared with control cells, whereas UVA irradiation (between 0.5 and 5 J/cm^2) did not affect cell viability.

3.2. Analysis of UV-induced damage using the Comet assay

We used the Comet assay to examine the DNA damage induced in proliferating IMR-90 fibroblasts irradiated with increasing doses of UVB or UVA. As shown in Fig. 2A, in UVB-irradiated cells the tail/nucleus ratio of comets is consistent with dose-dependent DNA damage from 2.5 to 60 mJ/cm^2. Differently, cells were more resistant to UVA irradiation (Fig. 2B). These results are in agreement with the viability curves (Fig. 1C,D). As a control, we measured the levels of the GADD45 (growth arrest and DNA damage-inducible) gene in both UVB- and UVA-irradiated IMR-90 cells by real time PCR (Fig. 2C,D): transcription of this gene is rapidly induced upon DNA damage [17,18].

3.3. UV-irradiation promotes senescence in IMR-90 cells

To identify cells in replicative senescence, we determined the levels of p21^{waf1} mRNA and the number of SA-βgal (senescence associated-β-galactosidase activity)-positive cells. p21^{waf1} plays an important role in the regulation of cellular senescence because this cyclin-dependent kinase inhibitor (CDKI) binds to "cyclin-CDK" complexes and causes cell cycle arrest in the G1/S phase [19]. As shown in Fig. 3A,C, UVB-irradiation at doses between 10 and 60 mJ/cm^2 provoked a gradual increase of p21^{waf1} mRNA and a concomitant

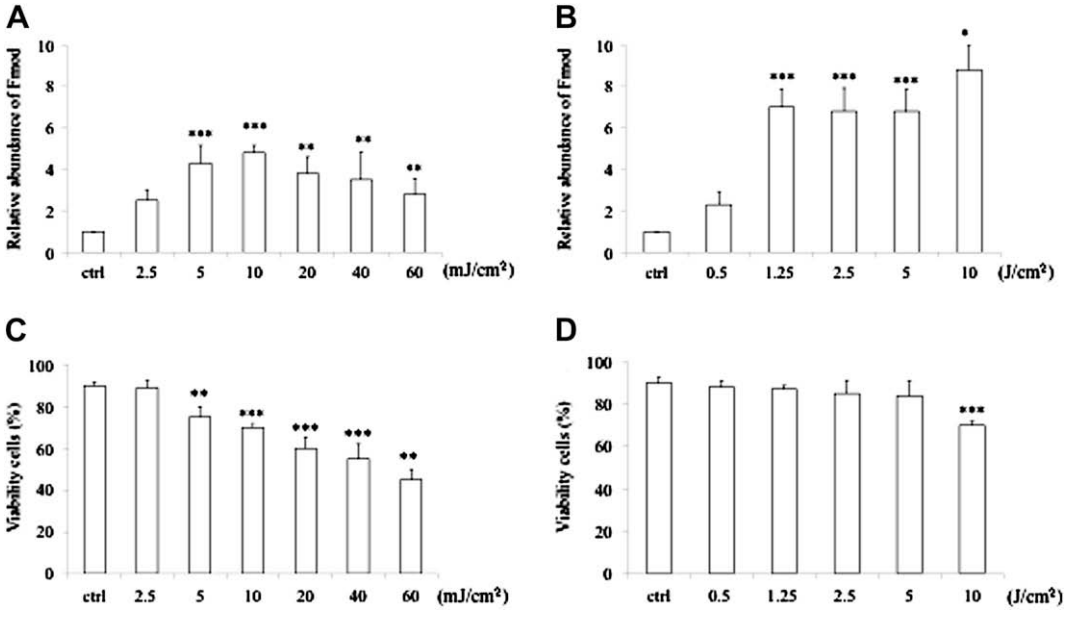

Fig. 1. Effects of UVB and UVA irradiation on fibromodulin expression and on cell viability in exponentially growing human fibroblasts. (A, B) Fibromodulin mRNA was measured by real time PCR in a total RNA preparation from exponentially growing human fibroblasts (IMR-90 cells 28 PDL) 24 h after UVB (from 2.5 to 60 mJ/cm^2) or UVA (from 0.5 to 10 J/cm^2) irradiation. The bars indicate the relative abundance of the mRNA; +1 is the abundance of fibromodulin mRNA in un-irradiated cells. All values represent the mean ± SD of triplicate experiments on two distinct total RNA preparations. $^{**}p < 0.001$, $^{***}p < 0.0001$. (C, D) The viability of IMR-90 was measured by trypan blue 24 h after UVB or UVA irradiation at increasing doses. The results are indicated as percentage of cell viability and the values represent the mean values ± SD of three independent experiments. $^{**}p < 0.001$, $^{***}p < 0.0001$. (Ctrl: un-irradiated control cells).

Please cite this article in press as: B. Iovine et al., Ultraviolet B and A irradiation induces fibromodulin expression in human fibroblasts *in vitro*, Biochimie (2008), doi:10.1016/j.biochi.2008.10.017

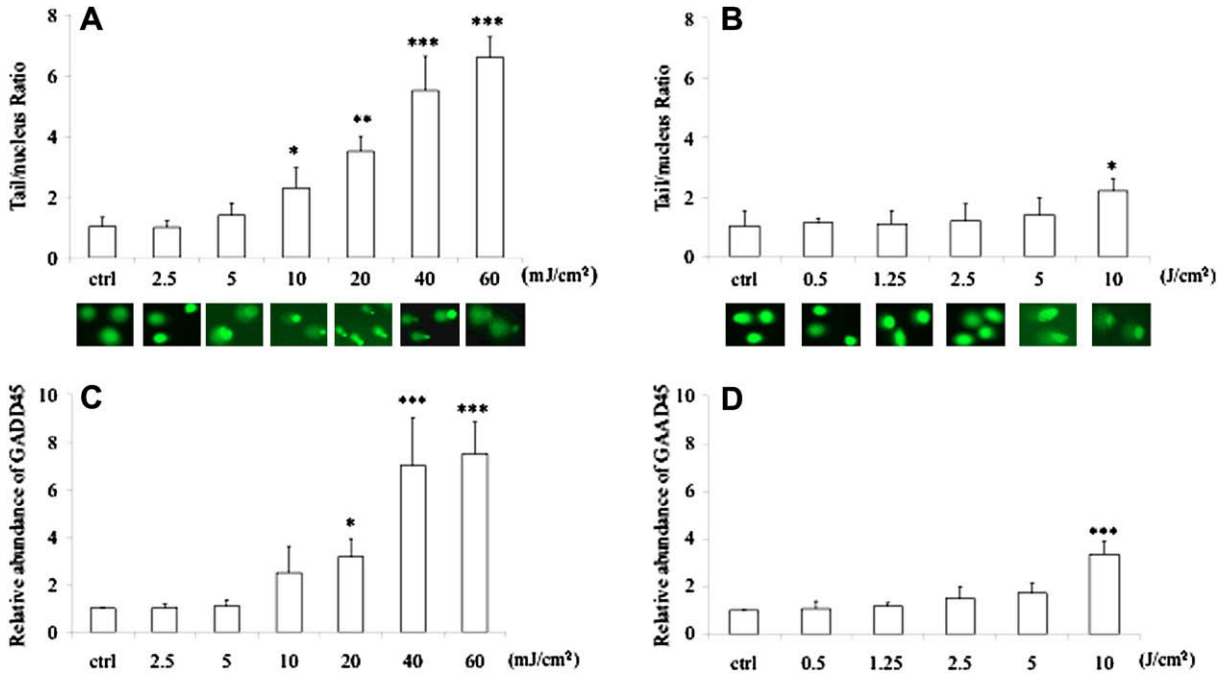

Fig. 2. Effects of UVB and UVA irradiation on the damage DNA. (A, B) IMR-90 cells were grown as described under Section 2, and treated with increasing doses of UVB (from 2.5 to 60 mJ/cm^2) or UVA (from 0.5 to 10 J/cm^2) irradiation. The Comet assay procedure (see Section 2) was performed according to the manufacturer's instructions. The results were quantified using the NIH Image Software. The data are reported as tail/nucleus ratio and represent the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$. (C, D) The total RNAs, from the same cells used in the experiments of Fig. 1A,B, were used to determine the levels of GADD45 mRNA, 24 h after UVB or UVA exposure. The bars indicate the relative abundance of the mRNA; +1 is the abundance in IMR-90 un-irradiated cells. All values represent the mean ± SD of triplicate experiments on two distinct total RNA preparations. *$p < 0.05$, ***$p < 0.0001$.

increase of SA β-gal-positive cells (about 50% at 60 mJ/cm^2 UVB irradiation). On the contrary, UVA (from 0.5 to 10 J/cm^2) induce a minor but statistically significant increase in p21^{waf1} expression (Fig. 3B) at 5 and 10 J/cm^2 nor the appearance of senescent cells (SA β-gal) (data not shown).

3.4. Intracellular formation of reactive oxygen species and the N-acetylcysteine effect on fibromodulin expression

To investigate if irradiation with UVB and UVA provokes an intracellular increase of reactive oxygen species (ROS) we measured their formation by means of the fluorescent dye H_2DCF-DA [15]. As shown in Figs. 4A and 5A, both types of irradiation significantly increased ROS formation. We also investigated the effects of the antioxidant *N*-acetylcysteine (NAC) on fibromodulin expression. Pre-treatment with NAC (1 mM) did not significantly affect UVB-induced fibromodulin expression, whereas it impaired the UVA-induced increase in fibromodulin expression (Figs. 4b and 5b)

3.5. Cellular distribution of DDB1 after UV irradiations

We recently reported that DDB1 is a key factor in the UVC-dependent activation of the Fmod gene [12]. In un-irradiated cells, DDB1 is mostly a cytosolic protein, whereas it moved into the nucleus after UV irradiation. Here we have examined the effect of UVB and UVA on DDB1 protein levels. As shown in Fig. 6A,B, after UVB-irradiation DDB1 moves in the nucleus.

The levels of DDB1 in UVA-irradiated cells were similar to those in control cells (data not shown). We also analyzed the sub-cellular distribution of DDB1 after UVB and UVA irradiation by immunostaining for DDB1. To this aim, UVB- and UVA-irradiated human fibroblasts were cultured in slide chambers, and the slides were analyzed by fluorescence microscopy. Fig. 6C,D show the stains of the nucleus (DAPI signal), as well as the overlay (DDB1 and DAPI signals). The intense nuclear staining in the UVB-irradiated cells (Fig. 6D) demonstrates that 10 mJ/cm^2 UVB irradiation promotes the translocation of DDB1 in the nucleus.

In un-irradiated cells, the staining for DDB1 is mainly visible in the cytoplasm (Fig. 6C); the same finding was obtained with UVA-irradiated cells (data not shown).

4. Discussion

We evaluated the effects of ultraviolet rays on the expression of the fibromodulin gene in normal exponentially growing human fibroblasts (IMR-90 cells). At least with the doses we used, both UVB and UVA increased fibromodulin levels versus sham-irradiated cells. UVB irradiation dose-dependently increased fibromodulin expression, which increased 5-fold at 10 mJ/cm^2 and gradually decreased thereafter. This decrease could be related to the decrease in cell viability (about 40%) after irradiation with 60 mJ/cm^2 UVB. UVA-induced fibromodulin expression was more pronounced; it started at the minimum dose (1.25 J/cm^2) and reached

Please cite this article in press as: B. Iovine et al., Ultraviolet B and A irradiation induces fibromodulin expression in human fibroblasts *in vitro*, Biochimie (2008), doi:10.1016/j.biochi.2008.10.017

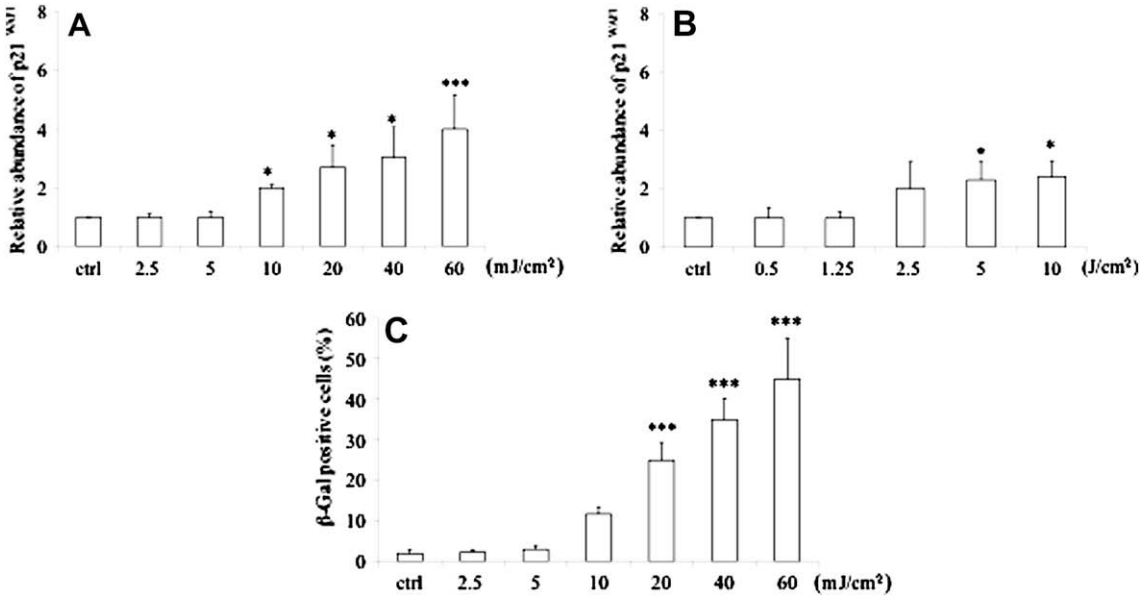

Fig. 3. The appearance of β-galactosidase (SA β-gal) positive cells and $p21^{waf1}$ mRNA expression in IMR-90 UVB–UVA irradiated cells. (A, B) $p21^{Waf1}$ mRNA was measured by real time PCR in total RNA preparations from exponentially growing human fibroblasts (IMR-90 cells 28 PDL) 24 h after UVB (from 2.5 to 60 mJ/cm^2) or UVA (from 0.5 to 10 J/cm^2) irradiation. The bars indicate the relative abundance of $p21^{Waf1}$ mRNA; +1 is the abundance of the mRNA in IMR-90 cells not exposed to UV irradiation. All values represent the mean ± SD of triplicate experiments on two distinct total RNA preparations. $*p < 0.05$, $***p < 0.0001$. (C) The β-galactosidase activity of IMR-90 cells was measured at 48 h after UVB irradiation (from 2.5 to 60 mJ/cm^2). SA β-gal activity was determined as described by Dimri et al. (1995). The population of positive cells was determined by counting 400 cells per dish. The proportions of cells positive for SA β-gal activity are given as percentage of the total number of cells counted in each dish. The results are expressed as mean ± SD of three different experiments. $***p < 0.0001$.

a maximum (10-fold) at the maximum dose (10 J/cm^2). UVA had little effect on cell viability. The results of the Comet assay, showing the extent of DNA damage induced by UVB and UVA irradiation, are in accordance with the increased expression of the GADD45 gene after UVB irradiation and its decreased expression after UVA irradiation. In a previous study, UVB irradiation induced a typical senescent morphology in exponentially growing human fibroblasts, with the appearance of SA β-gal-positive cells and growth arrest in G1 and a several fold increase of $p21^{waf1}$ mRNA [20]. Interestingly, we show that the $p21^{waf1}$ increase and SA β-gal-positive cells triggered by UVB parallels the gradual decrease of fibromodulin levels. Differently, in UVA-irradiated cells, $p21^{waf1}$ mRNA levels were only slightly increased and there were no SA β-gal-positive cells (data not shown). These results are hardly surprising because the appearance of replicative senescence is closely related with a state of irreversible growth rest [21]. Moreover, we previously found that the regulation of fibromodulin gene expression is impaired in senescent human fibroblasts [12].

In an attempt to elucidate the mechanisms involved in UV-induced fibromodulin, we measured ROS production after UV irradiation. As expected, both UV wavebands significantly increased ROS formation. To verify the effect of ROS on UV-induced fibromodulin, we investigated the effects of NAC on fibromodulin expression. NAC is a potent antioxidant and a glutathione (GSH) precursor. It directly scavenges hydrogen peroxide, hydroxyl free radicals, and hypochloric acid *in vitro* [22], and indirectly decreases free radical levels by increasing GSH synthesis [23]. Pre-treatment with NAC did not influence UVB-induced fibromodulin levels, suggesting that the UVB-induced increase of fibromodulin was only partially due to ROS generation. On the contrary, NAC significantly reduced the UVA induction of fibromodulin. In addition, another point concerns the finding that Fmod gene expression, apparently link the UV-induced gene regulation to the machinery involved in the repair of UV-induced DNA lesions. In fact, recently we have demonstrated that the accumulation of the fibromodulin is dependent on DNA damage binding protein named DDB1, a protein involved in the repair of UV-damaged DNA. Suppressing the expression of DDB1 by RNA interference results in an almost complete abolishment of the Fmod gene activation upon UVC exposure [12]. In un-irradiated cells, DDB1 is mostly a cytosolic protein, which moves into the nucleus only after UV irradiation, where it binds with DDB2 or CSA (the Cockayne syndrome protein), both of which act as repair machineries for nucleotide excision (NER) [24,25]. Western blot analysis demonstrated that DDB1 is present in both the nucleus and the cytoplasm in normal fibroblasts. Following UVB irradiation, cellular distribution of DDB1 by western blot and immunostaining revealed that the UVB exposure, promotes the translocation of DDB1 in the nucleus. Especially at 10 mJ/cm^2 UVB irradiation when the Fmod levels increased, the 80% of cells are still viable, only the 8% are senescent. Before UVA irradiation, DDB1 was primarily cytoplasmic, with some cells (20% of all cells examined) showing a uniform distribution (data not shown). In conclusion, we here demonstrated that UVB and UVA

Please cite this article in press as: B. Iovine et al., Ultraviolet B and A irradiation induces fibromodulin expression in human fibroblasts *in vitro*, Biochimie (2008), doi:10.1016/j.biochi.2008.10.017

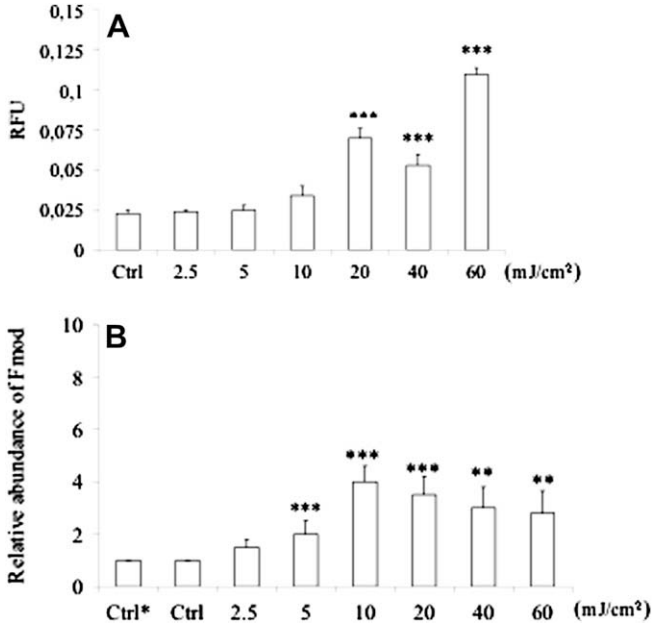

Fig. 4. Intracellular ROS formation and effects of the antioxidant *N*-acetylcysteine (NAC) on the fibromodulin expression after UVB irradiation. (A) 24 h after UVB irradiation (from 2.5 to 60 mJ/cm^2), the formation of ROS was evaluated by means of the probe 2′,7′-dich-lorofluorescin-diacetate (H_2DCF-DA), as described under Section 2. Fluorescence increase was measured in three replicate cultures for each test, using a fluorescent micro-plate reader. The results are expressed as mean of relative fluorescence units (RFU) ± SD of three independent experiments. ***$p < 0.0001$. (B) Fibromodulin mRNA was measured by real time PCR in total RNA preparations from IMR-90 cells 28 PDL incubated for 2 h with 1 mM NAC and harvested after UVB (from 2.5 to 10 mJ/cm^2) irradiation. The bars indicate the relative abundance of the mRNA; +1 is the abundance of the fibromodulin mRNA in un-irradiated cells. All values represent the mean ± SD of triplicate experiments on two distinct total RNA preparations. **$p < 0.001$, ***$p < 0.0001$. (Ctrl: un-irradiated control cells). (Ctrl*: untreated control cells).

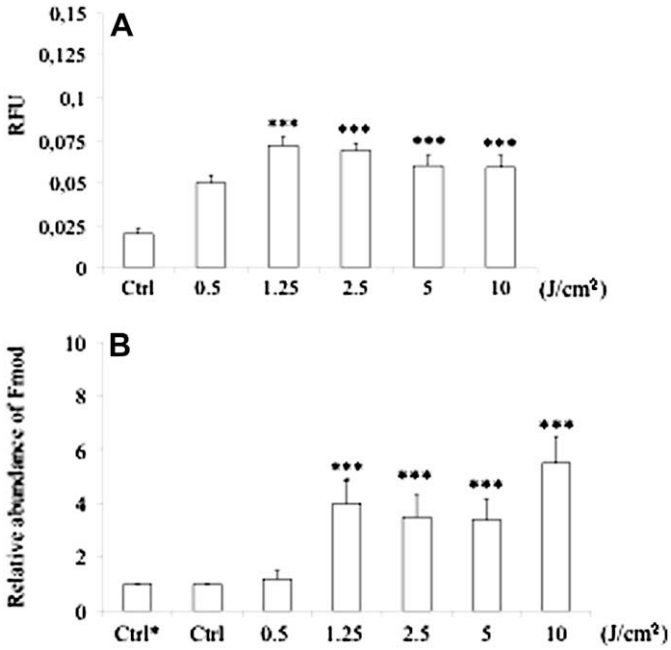

Fig. 5. Intracellular ROS formation and effects of antioxidant *N*-acetylcysteine (NAC) on the fibromodulin expression after UVA session. (A) 24 h after UVA irradiation (from 0.5 to 10 J/cm^2), the formation of ROS was evaluated by means of the probe 2′,7′-dichlorofluorescin-diacetate (H_2DCF-DA), as described under Section 2. Fluorescence increase was measured in three replicate cultures for each test, using a fluorescent micro-plate reader. The results are expressed as mean of relative fluorescence units (RFU) ± SD of three independent experiments. ***$p < 0.0001$. (B) Fibromodulin mRNA was measured by real time PCR in total RNA preparations from IMR-90 cells 28 PDL incubated for 2 h with 1 mM NAC and harvested after UVA (from 0.5 to 10 J/cm^2) irradiation. The bars indicate the relative abundance of the mRNA; +1 is the abundance of the fibromodulin mRNA in un-irradiated cells. Values represent the mean ± SD of triplicate experiments on two distinct total RNA preparations. **$p < 0.001$, ***$p < 0.0001$. (Ctrl: un-irradiated control cells). (Ctrl*: untreated control cells).

radiations both induce the Fmod with a different mechanism. Obviously, our study was carried out *in vitro* and it remains to be established if UV affects fibromodulin *in vivo*. Nevertheless, we provide data about the acute effects of UVB and UVA on the expression of a molecule important in the assembly of collagen fibrils and their three-dimensional stability.

The structure and function of the dermis are the result of a dynamic balance between synthesis and degradation of its extracellular components. The metabolism of collagen fibers, elastic fibers and the ECM is influenced by intrinsic (i.e. genetic pattern, senescence) and extrinsic (environmental) factors. At dermal level, UV rays, through ROS production, induce the expression of the nuclear transcription factor activator protein 1 (AP-1), which controls the transcription of matrix-degrading metalloproteinases (MMPs), a family of proteolytic enzymes, responsible for degradation of the ECM [26–28]. The basal expression of these enzymes in normal un-irradiated skin is relatively low; however, MMPs are up-regulated by UV irradiation *in vivo* and *in vitro* [26,29,30]. The balance between collagen synthesis and degradation leading to collagen deficiency is different in photo- aged and chrono-aged skin. In fact, *in vivo* the natural aging process decreases collagen synthesis and increases the expression of matrix MMPs, whereas photoaging results in an increase of collagen synthesis and a greater matrix MMP expression [31]. During photoaging, UV rays continuously degrade and promote the assembly of new collagen fibrils – a process that involves fibromodulin. Therefore, the induction of fibromodulin by UVB or UVA irradiation, could be either a mechanism to correct the connective tissue damaged resulting from UV insult, or a remodeling process involving the synthesis of new matrix protein.

Subcytotoxic doses of UVB-induced premature senescence of human diploid skin or lung fibroblasts [32,33]. Interestingly in this context, we demonstrate that in exponentially growing human fibroblasts (IMR-90 cells), one UVB dose stressed the senescence markers ($p21^{waf1}$ and SA β-gal), activated the repair mechanism by DDB1 translocation and affected the fibromodulin levels. Recently it has been proved that the number of senescent cells in skin increased exponentially with donor age, made up of about 4% of the connective tissue cell population in 5 year olds, while in 30 year olds the number rose to as high as 20% [34]. Therefore, the lower fibromodulin expression could alter the extracellular matrix assembly in these individuals, leading to a condition similar to that observed in fibromodulin

Please cite this article in press as: B. Iovine et al., Ultraviolet B and A irradiation induces fibromodulin expression in human fibroblasts *in vitro*, Biochimie (2008), doi:10.1016/j.biochi.2008.10.017

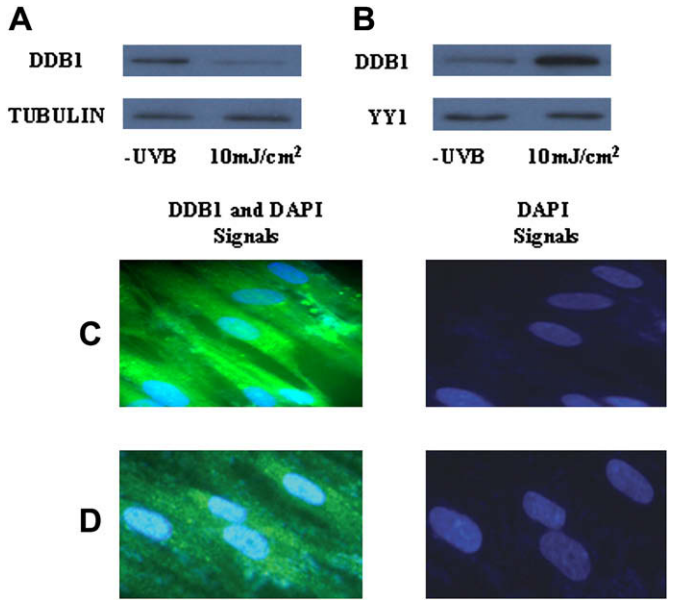

Fig. 6. Western blot analysis and cellular distribution of DDB1 after UV radiations. (A, B) Cytosolic and nuclear extracts of IMR-90 were tested with antibodies against DDB1, tubulin and YY1 in a Western blot, 24 h after exposure to different doses of UVB irradiation. Tubulin and YY1 were used as a control of loading. (C) Overlay DDB1 and DAPI signals and DAPI signal in control cells not exposed to UVB. (D) The nucleus, DAPI signal, and the overlay, DDB1 and DAPI signals, in IMR-90 cells exposed to UVB irradiation at 10 mJ/cm^2. Sham-irradiated cells control and irradiated with UVB were fixed as described under Section 2. Cells were examined with a fluorescence microscope at 40× magnification using filters for secondary antibody of DDB1 protein and for 4′,6-diamidino-2-phenylindole (DAPI).

knock-out mice that is characterized by skin fragility, reduced corneal transparency, and age-dependent osteoarthritis. The lack of response of the fibromodulin gene to UV irradiation could have deleterious effects on the sensitivity of aged tissue exposed to sun rays. In addition, the efficiency of repair of DNA double strand breaks is decreased in senescent and an accumulation of unrepairable double strand breaks is suggested to have a causative role in aging [35]. Studies of senescent cells or skin fibroblast cultures from skin biopsies are required to establish whether the analysis of fibromodulin levels could be a relevant experimental model to evaluate the correlation between cellular aging and UV-induced DNA damage.

Acknowledgments

We are grateful to Professors F. Cimino and T. Russo for discussion and suggestions.

We thank Jean Ann Gilder for editing the text.

References

[1] G. Jenkins, Molecular mechanisms of skin ageing, Mech. Ageing Dev. 123 (2002) 801–810.
[2] Y. Matsumura, H.N. Ananthaswamy, Short-term and long-term cellular and molecular events following UV irradiation of skin: implications for molecular medicine, Expert Rev. Mol. Med 4 (2002) 1–22.
[3] J. Campisi, Replicative senescence: an old lives' tale? Cell 84 (1996) 497–500.
[4] K.C. Choi, S. Lee, S.Y. Kwak, M.S. Kim, H.K. Choi, K.H. Kim, Increased expression of 1433 varepsilon protein in intrinsically aged and photoaged human skin in vivo, Mech. Ageing Dev. 126 (2005) 629–936.
[5] N. Philips, T. Keller, C. Hendrix, S. Hamilton, R. Arena, M. Tuason, S. Gonzalez, Regulation of the extracellular matrix remodeling by lutein in dermal fibroblasts, melanoma cells, and ultraviolet radiation exposed fibroblasts, Arch. Dermatol. Res. 299 (2007) 373–379.
[6] D. Heinegård, A. Aspberg, A. Franzén, P. Lorenzo, in: P. Royce, B. Steinmann (Eds.), Connective Tissue and Its Heritable Disorders: Molecular, Genetic, and Medical Aspects, Wiley-Liss, Inc, Press, New York, 2002, pp. 271–291.
[7] A.M. Hocking, T. Shinomura, D.J. Mcquillan, Leucine-rich repeat glycoproteins of the extracellular matrix, Matrix Biol. 17 (1998) 1–19.
[8] P. Onnerfjord, T.F. Heathfield, D. Heinegård, Identification of tyrosine sulfation in extracellular leucine-rich repeat proteins using mass spectrometry, J. Biol. Chem. 279 (2004) 26–33.
[9] L. Svensson, A. Aszódi, F.P. Reinholt, R. Fässler, D. Heinegård, A. Oldberg, Fibromodulin-null mice have abnormal collagen fibrils, tissue organization, and altered lumican deposition in tendon, J. Biol. Chem. 274 (1999) 9636–9647.
[10] L. Ameye, D. Aria, K. Jepsen, A. Oldberg, T. Xu, M.F. Young, Abnormal collagen fibrils in tendons of biglycan/fibromodulin-deficient mice lead to gait impairment, ectopic ossification, and osteoarthritis, FASEB J 16 (2002) 673–680.
[11] K.J. Jepsen, F. Wu, J.H. Peragallo, J. Paul, L. Roberts, Y. Ezura, A. Olbrrg, D.E. Birk, S. Chakravarti, A syndrome of joint laxity and impaired tendon integrity in lumican and fibromodulin deficient mice, J. Biol. Chem. 277 (2002) 35532–35540.
[12] M.A. Bevilacqua, B. Iovine, N. Zambrano, C. D'Ambrosio, A. Scaloni, T. Russo, F. Cimino, Fibromodulin gene transcription is induced by ultraviolet irradiation, and its regulation is impaired in senescent human fibroblasts, J. Biol. Chem. 280 (2005) 31809–31817.
[13] G.P. Dimri, X. Lee, G. Basile, M. Acosta, G. Scott, C. Roskelley, E.E. Medrano, M. Linskens, I. Rubelj, O. Pereira-Smith, et al., A biomarker that identifies senescent human cells in culture and in aging skin in vivo, Proc. Natl. Acad. Sci. U.S.A. 92 (1995) 9363–9367.
[14] N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp. Cell Res. 175 (1988) 184–189.
[15] R. Santamaria, C. Irace, M. Festa, C. Maffettone, A. Colonna, Induction of ferritin expression by oxalomalate, Biochim. Biophys. Acta 1691 (2004) 151–159.
[16] W.J. Ferguson, The Palmer notation system and its use with personal computer applications, Br. Dent 198 (2005) 551–553.
[17] M.A. Papathanasiou, N.C. Kerr, J.H. Robbins, O.W. McBride, I. Alamo, S.F. Barrett, I.D. Hickson, A.J. Fornace Jr., Induction by ionizing radiation of the GADD45 gene in cultured human cells: lack of mediation by protein kinase C, Mol. Cell Biol. 11 (1991) 1009–1016.
[18] R. Thyss, V. Virolle, V. Imbert, J.F. Peyron, D. Aberdam, T. Virolle, NF-kappaB/Egr-1/GADD45 are sequentially activated upon UVB irradiation to mediate epidermal cell death, EMBO J. 24 (2005) 128–137.
[19] H. Mattock, D.P. Lane, E. Warbrick, Inhibition of cell proliferation by the PCNA binding region of p21 expressed as a GFP miniprotein, Exp. Cell Res. 265 (2001) 234–241.
[20] J.P. de Magalhàes, F. Chainiaux, J. Remacle, O. Toussaint, Stress-induced premature senescence in BJ and hTERT-BJ human foreskin fibroblasts, FEBS Lett. 523 (2002) 157–162.
[21] M. Collado, M.A. Blasco, M. Serrano, Cellular senescence in cancer and aging, Review Cell 27 (2007) 223–233.
[22] O. Aruoma, H. Kaur, B. Halliwell, Oxygen free radicals and human diseases, J.R. Soc. Health 111 (1991) 172–177.
[23] M. Song, J.A. Kellum, H. Kaldas, M.P. Fink, Evidence that glutathione depletion is a mechanism responsible for the anti-inflammatory effects of ethyl pyruvate in cultured lipopolysaccharide-stimulated RAW 264.7 cells, J. Pharmacol. Exp. Ther. 308 (2004) 307–316.

Please cite this article in press as: B. Iovine et al., Ultraviolet B and A irradiation induces fibromodulin expression in human fibroblasts *in vitro*, Biochimie (2008), doi:10.1016/j.biochi.2008.10.017

[24] R. Groisman, J. Polanowska, I. Kuraoka, J. Sawada, M. Saijo, R. Drapkin, A.F. Kisselev, K. Tanaka, J. Nakatani, The ubiquitin ligase activity in the DDB2 and CSA complexes is differentially regulated by the COP9 signalosome in response to DNA damage, Cell 113 (2003) 357−367.
[25] W. Liu, A.F. Nichols, J.A. Graham, R. Dualan, A. Abbas, S. Linn, Nuclear transport of human DDB protein induced by ultraviolet light, J. Biol. Chem. 275 (2000) 21429−21434.
[26] G.J. Fisher, Z.Q. Wang, S.C. Datta, J. Varani, S. Kang, J.J. Voorhees, Pathophysiology of premature skin aging induced by ultraviolet light, New Engl. J. Med. 337 (1997) 1419−1428.
[27] G.J. Fisher, S. Kang, J. Varani, Z. Bata-Csorgo, Y. Wan, S. Datta, J.J. Voorhees, Mechanisms of photoaging and chronological skin aging, Arch. Dermatol. 138 (2002) 1462−1470.
[28] D.E. Heck, D.R. Gerecke, A.M. Vetrano, J.D. Laskin, Solar ultraviolet radiation as a trigger of cell signal transduction, Toxicol. Appl. Pharmacol. 195 (2004) 288−297.
[29] B. Stein, H.J. Rahmsdorf, A. Steffen, M. Litfin, P. Herrlich, UV-induced DNA damage is an intermediate step in UV-induced expression of human immunodeficiency virus type 1, collagenase, c-fos and metallothionein, Mol. Cell. Biol. 9 (1989) 5169−5181.
[30] M.J. Peterson, C. Hansen, S. Craig, Ultraviolet A irradiation stimulates collagenase production in cultured human fibroblasts, J. Invest. Dermatol. 99 (1992) 440−444.
[31] J.H. Chung, J.Y. Seo, H.R. Choi, M.K. Lee, C.S. Youn, G. Rhie, K.H. Cho, K.C. Park, H.C. Eun, Modulation of skin collagen metabolism in aged and photoaged human skin in vivo, J. Invest. Dermatol. 117 (2001) 1218−1224.
[32] F. Chainiaux, J.P. Magalhaes, F. Eliaers, J. Remacle, O. Toussaint, UVB-induced premature senescence of human diploid skin fibroblasts, Int. J. Biochem. Cell Biol. 34 (2002) 1331−1339.
[33] E. Straface, R. Vona, B. Ascione, P. Matarrese, T. Strudthoff, F. Franconi, W. Maloni, Single exposure of human fibroblasts (WI-38) to a sub-cytotoxic dose of UVB induces premature senescence, FEBS Lett. 581 (2007) 4342−4348.
[34] U. Herbig, M. Ferreira, L. Condel, D. Carey, J.M. Sedivy, Cellular senescence in aging primates, Science 311 (2006) 1257−1259.
[35] O.A. Sedelnikova, I. Horikawa, D.B. Zimonjic, N.C. Popescu, W.M. Bonner, J.C. Barrett, Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks, Nat. Cell Biol. 6 (2004) 168−170.

Please cite this article in press as: B. Iovine et al., Ultraviolet B and A irradiation induces fibromodulin expression in human fibroblasts *in vitro*, Biochimie (2008), doi:10.1016/j.biochi.2008.10.017