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UVA exposure of human skin reconstructed *in vitro* induces apoptosis of dermal fibroblasts: subsequent connective tissue repair and implications in photoaging

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Abstract

The skin reconstructed *in vitro* has been previously shown to be a useful model to investigate the effects of UVB exposure (Bernerd and Asselineau, 1997). The present study describes the response to UVA irradiation. Major alterations were observed within the dermal compartment. Apoptosis of fibroblasts located in the superficial area of the dermal equivalent was observed as soon as 6 h after irradiation, leading to their disappearance after 48 h. This effect was obtained without major alterations of epidermal keratinocytes suggesting a differential cell type sensitivity to UVA radiations. In addition, collagenase I was secreted by dermal fibroblasts. The UVA dermal effects could be observed even after removal of the epidermis during the post irradiation period, demonstrating that they were independent of the keratinocyte response. The analysis of the tissue regeneration during the following 2 weeks revealed a connective tissue repair via fibroblasts proliferation, migration and active synthesis of extracellular matrix proteins such as fibronectin and procollagen I. This cellular recolonization of the superficial part of the dermal equivalent was due to activation of surviving fibroblasts located deeply in the dermal equivalent. The direct damage in the dermis and the subsequent connective tissue repair may contribute to the formation of UVA-induced dermal alterations.

Keywords: skin equivalent; UVA radiation; dermal alterations; skin morphogenesis; photoaging

Abbreviations: UV, ultraviolet; UVB, 280 – 320 nm; UVA, 320 – 400 nm; UVA1, 340 – 400 nm; FITC, fluorescein isothiocyanate; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling; SBC, sunburn cell; BED, biologically efficient dose; MED, minimal erythemal dose; MMP1, matrix metalloproteinase I or interstitial collagenase; BrdU, bromodeoxyuridine

Introduction

UV exposure of skin leads to short term responses (erythema, sunburn and suntan) as well as long term effects, such as skin cancers and photoaging. Solar UV light comprises UVB wavelengths (290 – 320 nm) and UVA wavelengths (320 – 400 nm), which represent the majority of UV radiations reaching earth. Although UVA wavelengths are less energetic than UVB wavelengths, they seem to be implicated in UV-carcinogenesis (Freeman *et al*, 1987; Drobetsky *et al*, 1995; Robert *et al*, 1996). The role of UVA in the photoaging process has been suspected for several years because these radiations have high penetration properties (Gilchrest, 1989).

Analysis of photoaged skin revealed major changes in the deep compartment of the skin, i.e., the dermis, including a degradation of the connective tissue, a decrease in collagen content and the accumulation of degenerative elastic fibers corresponding to the so-called 'solar elastosis' (Gilchrest, 1989; Lavker, 1995; Lavker and Kligman, 1988; Oikarinen *et al*, 1985). This elastotic material not only comprises impaired elastin but also associated proteins, such as fibrillin, desmosin or fibronectin (Chen *et al*, 1986; Yaar and Gilchrest, 1995). Studies of sun exposed *versus* sun protected skin revealed dermal alterations (Berstein *et al*, 1996; Warren *et al*, 1991), but do not allow determination of the respective roles of UVB and UVA radiations. Induction of dermal actinic damages after chronic UVA exposures has been obtained using hairless mice (Kligman *et al*, 1985). Studies on fibroblast or keratinocyte cultures revealed that through the generation of oxidative species (Tyrell and Keyse, 1990), UVA induces a decrease in Epidermal Growth Factor (EGF) binding (Djavaheri-Mergny *et al*, 1994), a modification of phospholipid metabolism (Hanson and deLeo, 1990) and lipid peroxidation (Morlière *et al*, 1991; Moysan *et al*, 1995). In addition, UVA also leads to the production of extracellular degrading enzymes, stromelysin 1 and interstitial collagenase (Petersen *et al*, 1995; Sawamura *et al*, 1996; Scharffetter *et al*, 1991, 1993; Wlaschek *et al*, 1994, 1995).

The identification of early cellular markers of UVA effects on skin may provide key insights into the long term process of photoaging. Recent studies with human volunteers revealed that several days of UVA exposure induced slight dermal changes (Lavker *et al*, 1995a,b; Lowe *et al*, 1995). However, experimental chronic UV exposure are difficult to perform in humans for ethical reasons. On the other hand, conventional cell cultures do not reproduce accurate physiological conditions. Human skin reconstructed *in vitro* can be an alternative means. This three dimensional skin system includes a dermal equivalent, in which human fibroblasts have physiological properties closer to *in vivo* conditions compared to classical cultures

systems (Coulomb *et al*, 1983; Nusgens *et al*, 1984). On this dermal support a full thickness epidermis can be reconstructed, that displays normal keratinocyte differentiation markers and horny layers (Asselineau *et al*, 1985; 1989). This skin model is suitable not only for evaluating the effects of pharmacological agents, such as retinoids (Asselineau *et al*, 1989; Asselineau and Darmon, 1995; Magnaldo *et al*, 1992) but also the response to environmental stimuli. This system was recently used to study the typical early DNA damages induced in epidermis by UVB as well as the subsequent recovery phase (Bernerd and Asselineau, 1997).

The present work shows the effect of UVA exposure on skin reconstructed *in vitro*. A complete time course of events was recorded. In contrast with the epidermal UVB response, UVA irradiation induced major damages within the dermal compartment, including apoptosis of superficial fibroblasts. A progressive recolonization of the upper dermis occurred afterwards through proliferation and migration of surviving fibroblasts located in deeper dermis. These cells also displayed a considerable increase in procollagen I and fibronectin synthesis.

Results

UVA sources

The absence of an UVB emission by the UVA1 and UVA sources was carefully checked as shown in Figure 1.

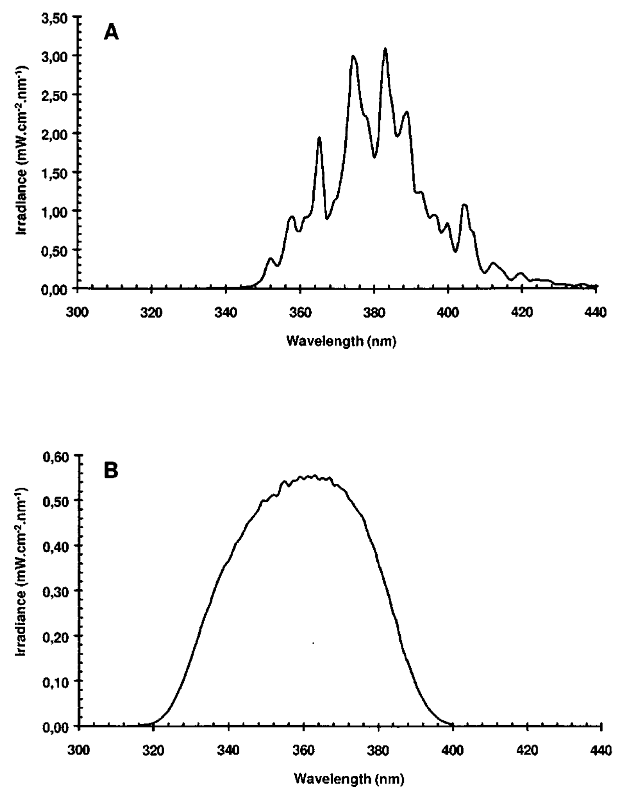

Figure 1 Spectra of UVA sources. (**A**) UVASUN spectrum: 340–440 nm (UVA1). (**B**) Solar simulator IDEM 300 spectrum, filtered with UG11 1 mm and WG335 3 mm: 320–400 nm (UVA)

Determination of the UVA Biologically Efficient Dose (BED)

The BED is the minimal dose necessary to induce morphological or structural changes of the skin without leading to death of the tissue (Bernerd and Asselineau, 1997). Sham irradiated skin reconstructed *in vitro* displays a fully differentiated epidermis (Figure 2A) characterized by the formation of well structured horny layers. In histological sections, the dermal equivalent showed fibroblasts embedded in the collagen matrix. Total UVA BED was found to be 25 J/cm^2 (Figure 2B). The alterations were mostly observed in the dermal compartment 48 h post irradiation. As shown in Figure 2B the fibroblasts located in the superficial part of dermal equivalent disappeared after UVA treatment. When a higher UVA dose was delivered (30 J/cm^2), the upper layers of epidermis were also affected (Figure 2C). However the basal and suprabasal keratinocytes do not show histological modifications. Immunostaining of vimentin, a cytoskeleton protein constitutively expressed in fibroblasts (Lazarides, 1982), revealed the absence of vimentin positive cells in the upper part of the dermal equivalent after UVA irradiation (Figure 2D–F), confirming the deletion of fibroblasts in that area. Basal keratinocytes of the reconstructed epidermis also synthesize vimentin protein. As shown in Figure 2, UVA irradiation does not alter this immunostaining, which corresponds to unmodified basal epidermal layer on histological sections. UVA 1 irradiation obtained using UVASUN 3000, induced similar effects as UVA, but the minimal BED was determined to be 30 J/cm^2 (data not shown).

Apoptotic process

TUNEL reaction performed on samples obtained 6 h after BED-UVA exposure revealed clearly positive fibroblasts in the superficial zone of the dermal equivalent compared to a sham-irradiated sample (Figure 3). Only a few granular keratinocytes had a higher signal after UVA exposure (Figure 3B). Even 24 to 48 h after UVA exposure, TUNEL reaction remains negative in basal and suprabasal epidermal keratinocytes (data not shown).

Epidermal alterations

The global architecture of the epidermis was not significantly altered 24 to 48 h after UVA exposure. Histologically, the uppermost epidermal cells displayed some morphological alterations at 30 J/cm^2 total UVA exposure (Figure 2C). Immunostainings using specific antibodies directed against keratinocyte differentiation proteins, such as keratin 10, involucrin, filaggrin, loricrin and keratinocyte transglutaminase were performed. The only modification observed at the UVA-BED concerned the subcellular localization of loricrin reactivity. Normally detected at the cell periphery (Figure 4A), UVA irradiation induced a wider distribution within the cytoplasm of keratinocyte (Figure 4B).

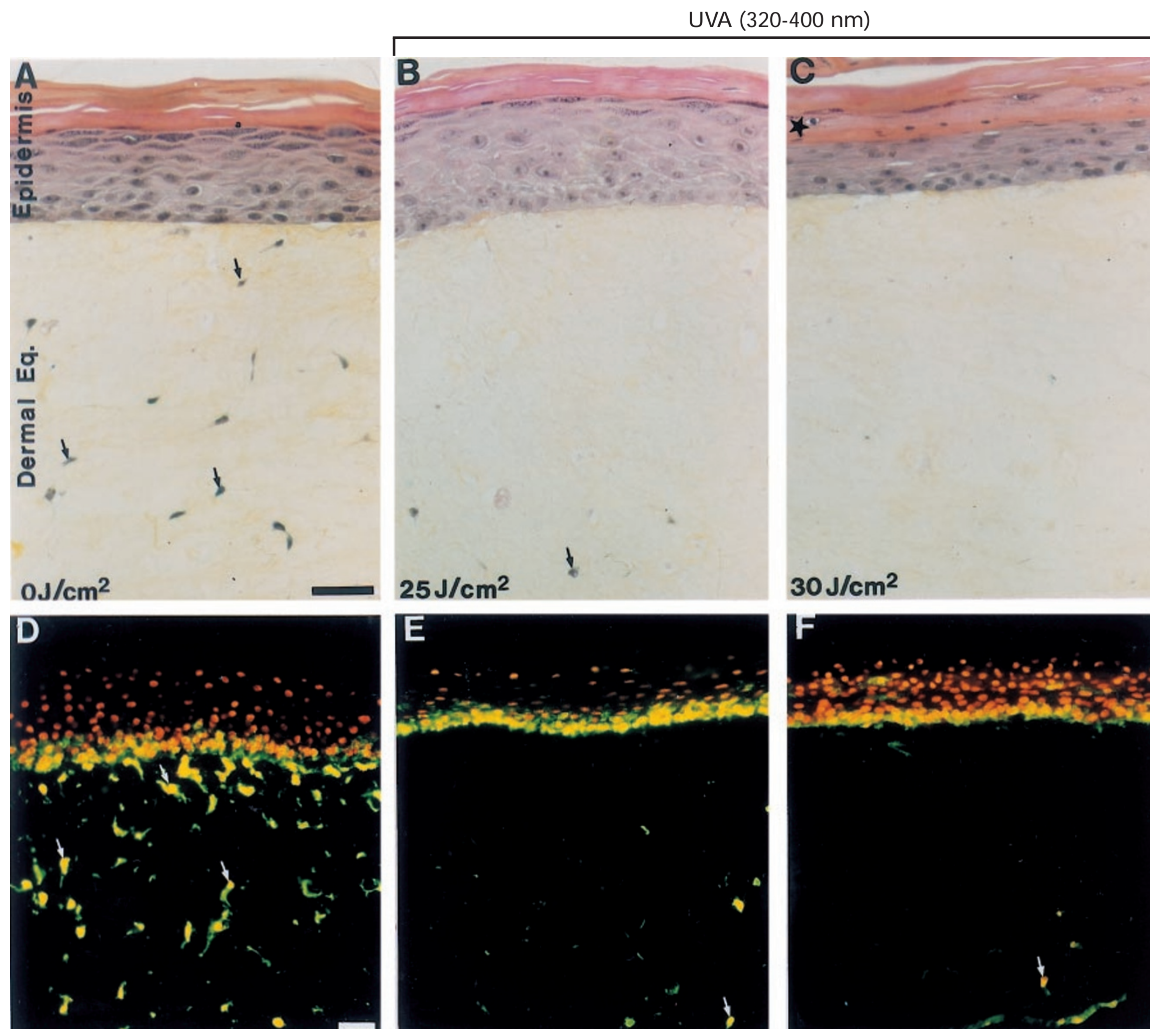

Figure 2 UVA-BED effect on skin reconstructed *in vitro* 48 h after exposure. (Solar simulator source, UVA 320–400 nm). (**A–C**) Histology; (**D–F**) Immunolabelling using anti vimentin antibody. (**A,D**) Sham-irradiated sample. (**B,E**) UVA 25 J/cm^2. (**C,F**) UVA 30 J/cm^2. Note that the UVA-BED is 25 J/cm^2. This dose induces the disappearance of superficial fibroblasts in the dermal equivalent (arrows), without major alterations in the epidermis. At 30 J/cm^2 UVA, the epidermis is slightly affected in the upper layers (star), and parakeratosis is observed. Scale is 50 μm

Typical UVB damages were not significantly induced by UVA irradiation

A classical UVB effect is the sunburn cell (SBC) formation in the epidermis 24 h after exposure (Young, 1987). UVA BED failed to induce SBC formation (data not shown). The induction of pyrimidine dimers was also analyzed by immunostaining using H3 monoclonal antibody. After 30 J/cm^2 total UVA, a weak positive signal was found (Figure 5C). However, the intensity of the fluorescence was far weaker compared to a control sample irradiated with 50 mJ/cm^2 UVB (Figure 5B). No fluorescence was observed after UVA1 irradiation (data not shown).

The effect of UVA exposure is due to the physical penetration properties of UVA radiation

UVA irradiation preferentially affected the superficial fibroblasts and spared the deeper ones. These observations raised these questions: (i) what is the role of the penetration properties of UVA? and (ii) what is the influence of medium proximity for deep fibroblasts? (the culture is on a grid fed by capillary action). To address these questions, dermal equivalents of different thickness were prepared by increasing the volume of initial collagen-fibroblasts mixture (see Materials and Methods). The proportion of collagen and cells was kept constant, which allowed similar contraction kinetics and cell density. The reconstructed skins obtained were then UVA irradiated and analyzed by histology and vimentin immunochemistry. Irrespectively of the dermal equivalent thickness, the disappearance of superficial fibroblasts, its depth in the dermis, and the presence of surviving cells in the lower part of the dermis were found to be similar (Figure 6A and B). The response seemed to be related to UVA penetration into the dermal compartment, the upper part of the dermis being more impaired than the deeper portion.

Epidermis does not play an essential role in the UVA dermal response

Because the skin system is comprised of two living compartments and therefore allows signal exchanges between them, we wondered whether the dermal alterations after UVA exposure could be indirectly mediated by an epidermal response (i.e. soluble factors). Normal reconstructed skins *in vitro* were irradiated with UVA-BED and the epidermis removed immediately after irradiation. The samples were then kept for 48 h to allow the effects of UVA to develop. This procedure ensured that the dermis received the same dose of UVA. Figure 7 shows that UVA alterations on dermal fibroblasts were still observed in the absence of epidermis during the post irradiation period, suggesting that UVA dermal effects do not depend on an epidermal response.

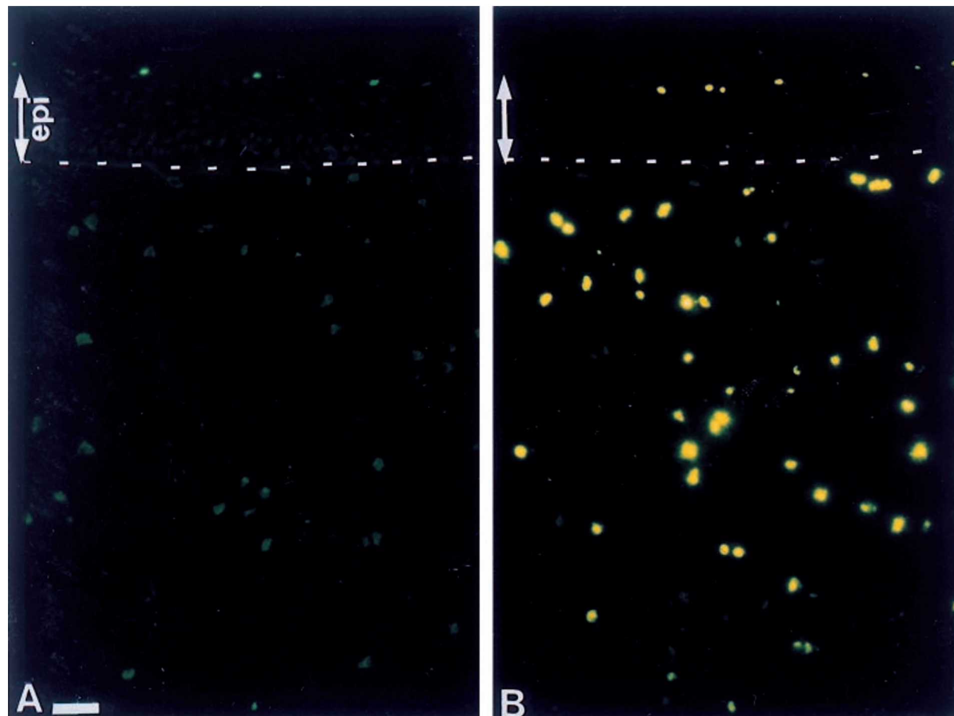

Figure 3 TUNEL assay on UVA irradiated reconstructed skin 6 h after exposure. (**A**) Sham irradiated skin; (**B**) 25 J/cm^2 UVA-irradiated skin. Note the presence of positive fibroblasts within the dermal equivalent after UVA irradiation (320–400 nm). Epi=epidermis. Scale is 50 μm

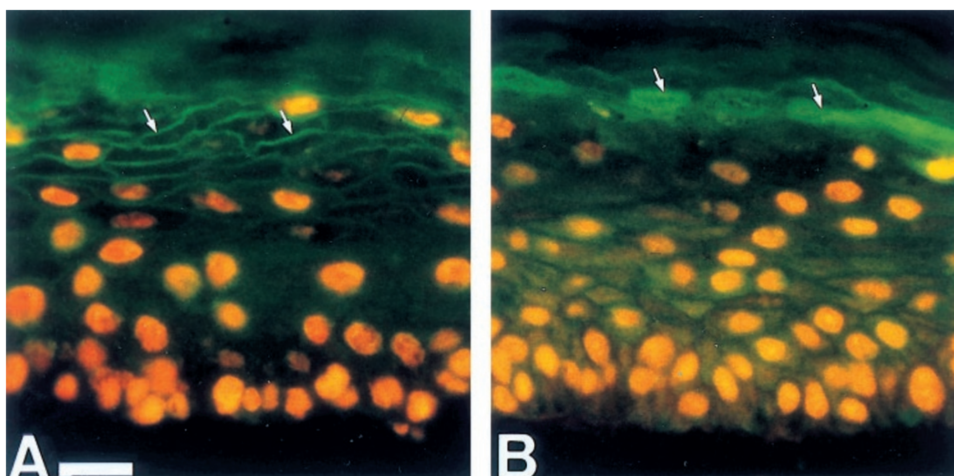

Figure 4 UVA irradiation induced subcellular redistribution of loricrin. Immunolabelling using anti loricrin antiserum. (**A**) Sham irradiated sample. (**B**) Sample irradiated with 30 J/cm^2 UVA (320–400 nm). Note that the classical loricrin peripheral staining of granular cells is cytoplasmic in the UVA exposed skin (arrows). Scale is 50 μm

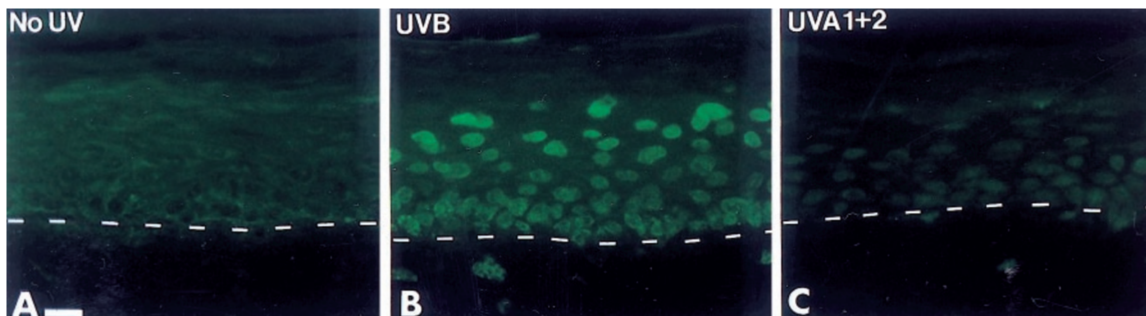

Figure 5 Absence of significant CPD induction after UVA irradiation. Immunodetection of pyrimidine dimers using H3 monoclonal antibody. (**A**) Sham-irradiated sample. (**B**) 50 mJ/cm^2 UVB-irradiated positive control. (**C**) 30 J/cm^2 UVA (320–400 nm) irradiated sample. The UVB positive control shows fluorescent nuclei. UVA exposed skin shows a very slight staining. Scale is 30 μm

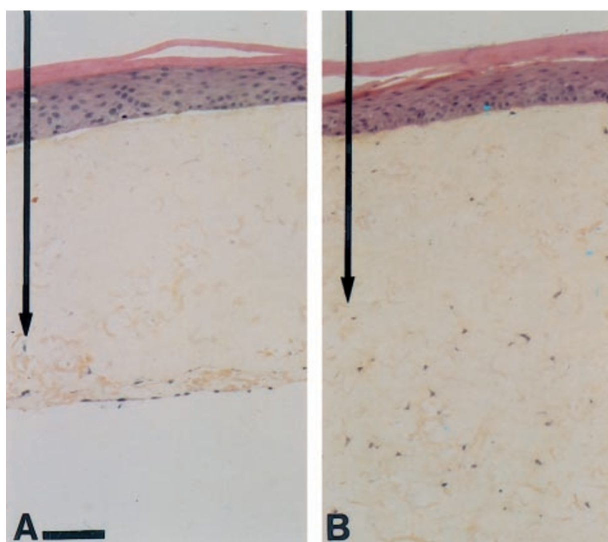

Figure 6 Biological assay of UVA effects in the dermal compartment. (**A**) Classical reconstructed skin composed of a 0.25 mm thick dermal equivalent; (**B**) Reconstructed skin using a 0.80 mm thick dermal equivalent, 48 h after 25 J/cm^2 UVA (320–400 nm). Note that the effects into the dermal compartment corresponds to approximately the same depth in all conditions, allowing the survival of the deepest fibroblasts. Scales are 100 μm

Interstitial collagenase is a UVA dermal maker

The secretion of MMP1 in culture medium after UVA irradiation was measured using ELISA assay. Figure 8A shows that UVA exposure induced an increase in the level of MMP1. The assay was also performed using samples from which the epidermis was removed immediately after UVA exposure and revealed a similar increase (Figure 8B), which suggests that the major part of MMP1 released in culture medium was produced by dermal fibroblasts.

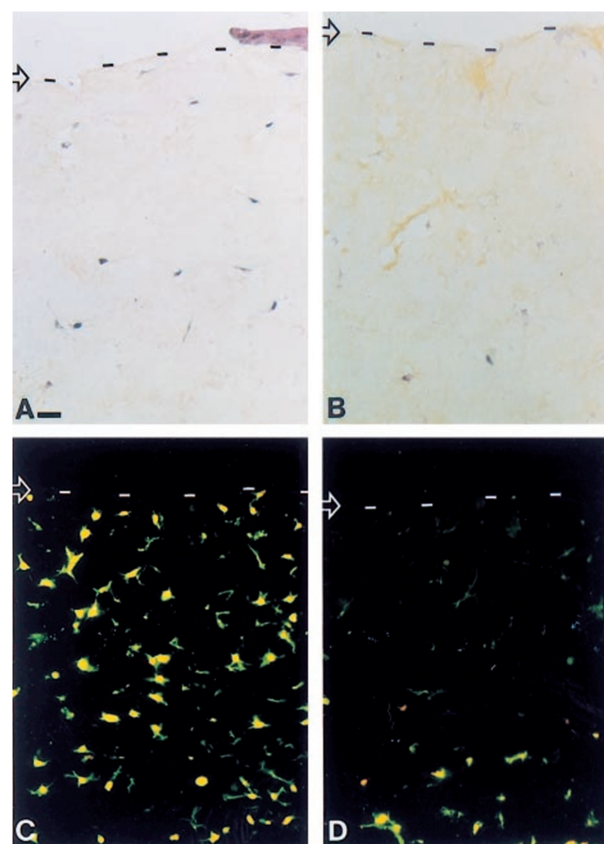

Figure 7 UVA dermal effects can be obtained independently of the epidermal response. The epidermis of the reconstructed skin has been removed immediately after UVA exposure. Histology (**A,B**) or immunolabelling using anti-vimentin antibody (**C,D**) have been performed 48 h after UVA irradiation (320–400 nm). (**A,C**) Sham irradiated sample. (**B,D**) 48 h after 25 J/cm^2 UVA. The disappearance of superficial fibroblasts is observed even in the absence of epidermis during the 48 h following UVA exposure. Scale is 30 μm

Dermal 'regeneration' occurred within 2 weeks after UVA exposure

After UVA-BED exposure, a step by step dermal recovery takes place. The epidermis that was not significantly altered during the first 48 h did not show obvious further modifications. Interestingly, histological observations showed a progressive recolonization of superficial dermis by deeply located surviving cells. This process began with a progressive proliferation of deep fibroblasts and migration through the

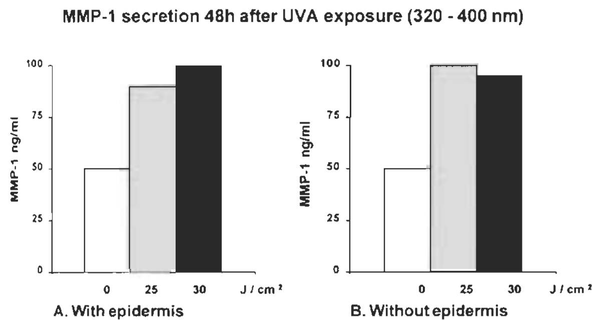

Figure 8 Interstitial collagenase ELISA assay. Culture media were collected 48 h after UVA irradiation (320–400 nm). The period post irradiation has been performed with epidermis (**A**) or without epidermis (**B**). The values represent the mean of three samples. The S.E.M. was less than 10% for all data

dermis from the deepest part to the top. After 10–14 days the dermal equivalent showed a similar cell density when compared to the sham-irradiated sample. Anti-vimentin immunostaining supported these histological observations (Figure 9). Forty-eight hours after UVA irradiation, the upper part of the dermis was devoid of fibroblasts and appeared as a negative zone just underneath the epidermis (Figure 9A and B). On days 3, 4 and 7 this zone decreased in size (in depth), the deeper part of the dermis was recolonized before the uppermost part (Figure 9C–E). By day 10 (Figure 9F) and 14 (not shown) the whole dermis contained fibroblasts, suggesting that recolonization was achieved.

Proliferation of dermal fibroblasts was followed by BrdU incorporation. Positive dermal fibroblasts were not found in sham-irradiated samples (data not shown). In contrast, positive cells were identified 7 days after UVA exposure (Figure 10). Characterization of these recolonizing fibroblasts was assessed by immunostaining using antibodies directed against antigens of the extracellular matrix. An increase in the deposition of fibronectin and procollagen I was clearly detected in the dermal equivalent during the recolonization period (Figure 11).

Discussion

In a previous work, we demonstrated that skin reconstructed *in vitro* was suitable to study early damages due to a single UVB exposure (DNA lesions, keratinocyte apoptosis) and also subsequent alteration and recovery of the tissue (Bernerd and Asselineau, 1997). The present work focused on the response of reconstructed skin after either 25 J/cm^2 UVA or 30 J/cm^2 UVA1 irradiation, which is a dose close to the human UVA Minimal Erythemal Dose (MED) (Lavker *et al*, 1995a,b; Lowe *et al*, 1995). The first observation was that UVA failed to significantly induce the classical UVB damages. Sunburn cells were not found, and immunodetection of pyrimidine dimers (CPD) revealed only a weak signal after exposure to a full UVA spectrum (320–400 nm) and none after only UVA1. This is in agreement with the UV action spectrum of CPD induction, which showed a decreased efficiency with increasing wavelengths (>300 nm) (Freeman *et al*, 1989). Moreover, UVA is more efficient in inducing other DNA lesions deriving from oxidative stress, such as single or double strand breaks (Peak *et al*, 1987; Peak and Peak, 1991; Tyrell and Pidoux, 1989). The major tissue target of UVB was the epidermis resulting in significant alterations in the keratinocyte differentiation process. On the contrary, UVA exposure did not lead to drastic structural alterations of the epidermis, but only slight morphological changes within the upper layers. UVA induced a redistribution of subcellular localization of loricrin, from the cell periphery, where it is incorporated in the forming cornified envelope (Magnaldo *et al*, 1992; Steinert and Marekov, 1995), to the whole cytoplasm. It is tempting to relate this effect to UVA induction of cell membrane damages (Gaboriou *et al*, 1993). In addition, the results obtained during post irradiation time after the epidermis was removed suggests that keratinocytes are not required for the UVA dermal response.

The major result in the present study is that UVA effects are targeted to the dermis. Regarding the induction of the apoptotic process, keratinocytes are not affected by UVA, while superficial dermal fibroblasts are drastically altered, suggesting a differential cell type sensitivity to UVA radiation. Previous studies regarding the UVA-induced oxidative stress response indicated that fibroblasts offered less resistance than keratinocytes (Applegate *et al*, 1995; Morlière *et al*, 1991; Moysan *et al*, 1995; Niggli and Applegate, 1997). Additionally, keratinocytes express constitutively high levels of heme oxygenase-2 and ferritin (Applegate *et al*, 1995; Applegate and Frenk, 1995), which are involved in the defense response to oxidative stress (Vile and Tyrrell, 1993) and may increase the resistance of keratinocytes to UVA radiation. Considering the fact that keratinocytes also represent the most superficial cell type of our body, which is exposed to multiple external aggressions, it is tempting to hypothesize that they are genetically programmed for stress resistance. In that sense, the presence of high amounts of keratin polypeptides in the keratinocytes (Fuchs, 1996), as well as a normal keratinocyte differentiation program leading to cornified envelope formation (Reichert *et al*, 1993; Steinert and Marekov, 1995), may provide these cells with the appropriate equipment to build an efficient physical barrier.

The UVA delayed response corresponds to a dynamic process of recolonization of the dermis by spared fibroblasts from the deeper dermis. Skin reconstructed *in vitro* can thus be considered as a suitable model to study dermal fibroblasts responses. Several features of fibroplasia known to occur during *in vivo* wound repair are indeed found, such as fibroblast proliferation, migration, and new extracellular matrix deposition, like fibronectin and procollagen I (Clark, 1991). Interestingly, migrating fibroblasts exhibited an increased level of Keratinocyte Growth Factor (KGF) mRNA (data not shown), thus completing the phenotype of wound healing fibroblast (Werner *et al*, 1992). In addition, MMP 1 production after UVA exposure may facilitate the migration of fibroblasts inside the collagen matrix during the recolonization phase. Other studies showed secretion of matrix degrading enzymes after UVA irradiation (Petersen *et al*, 1995; Sharffetter *et al*, 1991). All together these results show that this 'simplified' dermis is able to 'regenerate', thus providing an attractive model for connective tissue repair *in vitro*.

How can the UVA-induced alterations described in this study could be related to the photoaging process? Histological examination of photoaged skin reveals two distinct dermal zones. First, a subpapillary area called 'grenz zone' in human photoaged skin (Lavker, 1995) or 'repair zone' in animal models of photoaging (Kligman *et al*,

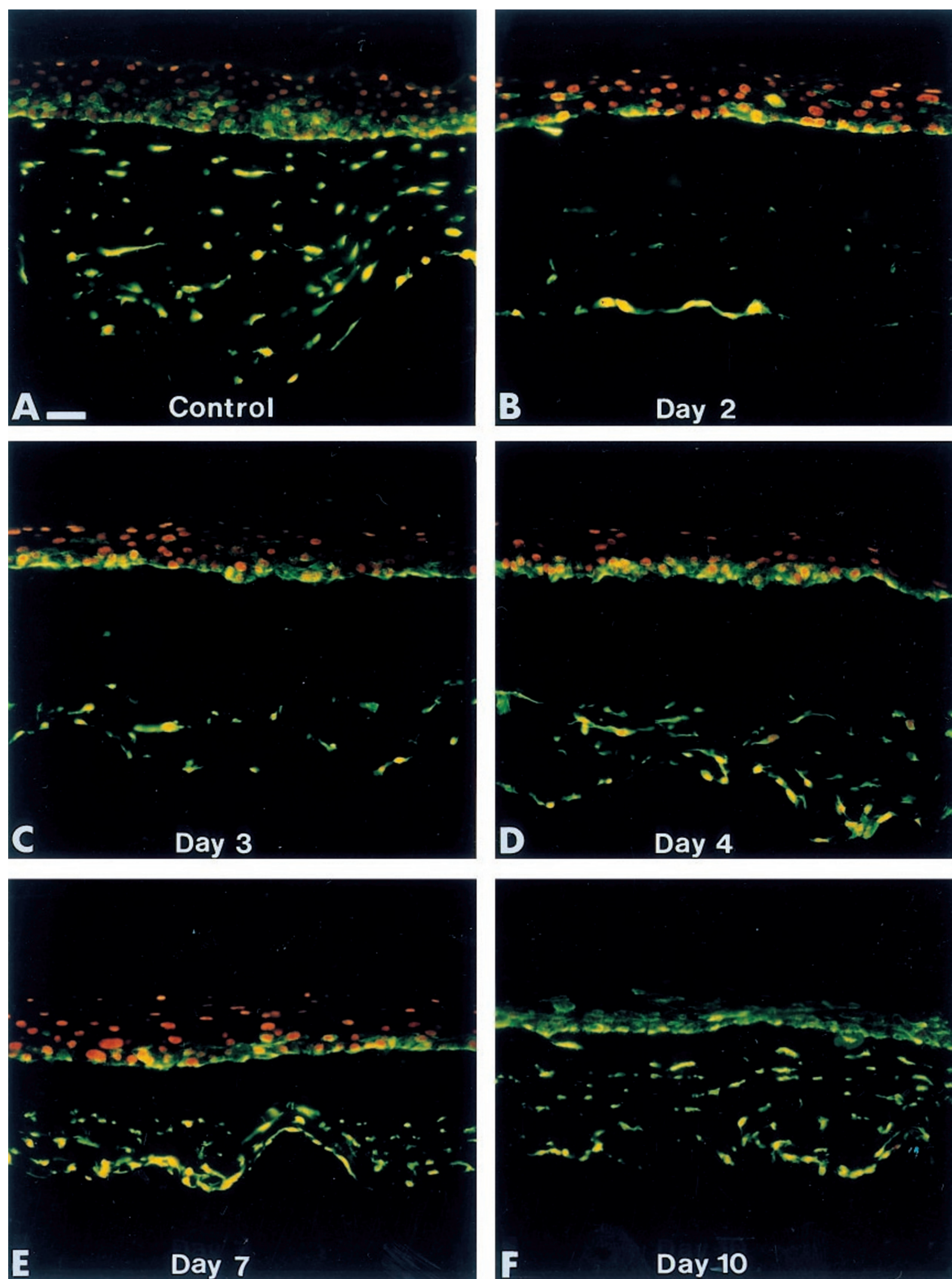

Figure 9 Recolonization of the superficial dermis by deep fibroblasts. Anti-vimentin immunostaining. (**A**) Sham irradiated sample. (**B – F**) Samples irradiated with 25 J/cm^2 UVA (320 – 400 nm) and observed up to 10 days after. The time (days) after the UVA exposure is indicated at the bottom. Note that the presence of vimentin positive fibroblasts in the dermal equivalent progressively increases from the bottom to the top. After 10 days the distribution of fibroblasts closely resembles that of the control. Scale is 60 μm

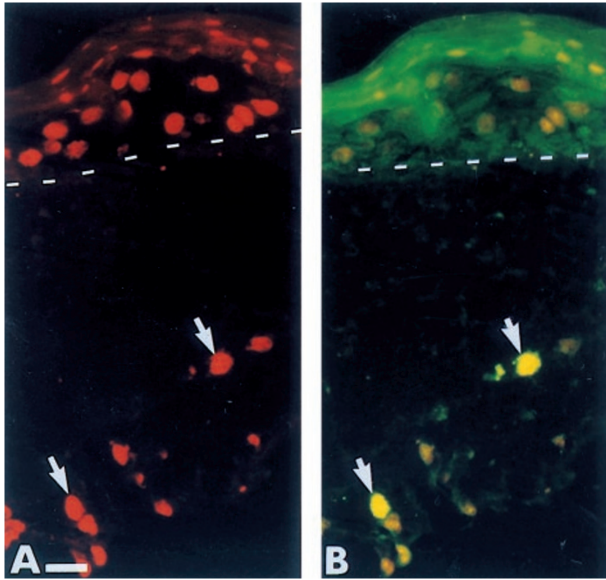

Figure 10 BrdU immunostaining 7 days after UVA exposure (320–400 nm, 25 J/cm^2). (**A**) Propidium iodide. (**B**) Anti-BrdU immunostaining. Note that positive nuclei are observed in deep fibroblasts recolonizing the dermal equivalent. Scale is 30 μm

1982); second, underneath this superficial area, a zone where elastotic material is present (Kligman, 1995; Lavker, 1995; Yaar and Gilchrest, 1995). In the *in vitro* reconstructed skin model, the disappearance of fibroblasts in the superficial dermis after UVA exposure could be correlated with the first zone that is progressively recolonized. To support this idea, in the *in vivo* subepidermal repair zone, a new connective tissue is formed when UV exposure stops, corresponding to *de novo* synthesis of collagen, elastin and fibronectin (Kligman *et al*, 1982, 1984; Schwartz and Kligman, 1995). This phenomenon has been compared to a wound healing process. The deeper part of dermal equivalent, which is characterized by the beginning of the recolonization process and a high deposition of fibronectin, may correspond to the location where elastotic material is produced *in vivo*. High levels of fibronectin have been detected in elastotic material in human photoaged skin (Chen *et al*, 1986), as well as after UVA irradiations in the mouse model (Boyer *et al*, 1992). The collagenase production could also contribute to the decrease in total collagen content that takes place during photoaging (Warren *et al*, 1991; Fisher *et al*, 1996).

Successive UVA-induced events described above (alterations followed by repair processes), may contribute to the histological feature of photoaged skin, especially with regard to the formation of 'grenz zone' and possibly the localization of elastotic material.

Materials and Methods

Tissue culture

Keratinocyte and fibroblast cultures Human epidermal keratinocytes were isolated from skin obtained after mammary reduction and cultured as described by Rheinwald and Green (1975) on a feeder layer of Swiss 3T3 fibroblasts. Human adult dermal fibroblasts were isolated after spreading from mammary skin explants prepared for isolation of epidermal keratinocytes. The 'FMD' strain of fibroblasts corresponds to the skin from a 16-year old person.

Reconstructed skin in vitro (Asselineau *et al*, 1985). Dermal equivalents (lattices) were prepared as described previously, except that we used 'FMD' cells (Bernerd and Asselineau, 1997). Normal sized lattices were prepared using 7 ml of the collagen-fibroblast mixture (containing 10^6 cells) in a 60 mm petri dish. Thick lattices were respectively prepared using 7 ml of the mixture and 10^6 cells, 10 ml and 1.4×10^6 cells, and 14 ml and 2×10^6 cells in 35 mm petri dishes. The lattices were allowed to contract for 3 days. Adult human keratinocytes were seeded on this support and kept submerged for 7 days allowing the cells to form a monolayer. The cultures were then raised at the air-liquid interface on grids and kept 1 week to allow keratinocytes to stratify and differentiate completely.

Irradiation sources

UVA irradiations were performed using two different sources. A UVASUN 3000 (Mutzhas, Germany) was used to produce UVA1 (340–400 nm). A Solar simulator (Arcane, France) fitted with a 1000 Watts Xenon lamp filtered by a UG11 (1 mm) and a WG335 (3 mm) Schott filters delivered total UVA. UV spectra were carefully checked with an ORIEL Intaspec IV spectroradiometer.

Reconstructed skins on grids were irradiated without medium. Fresh medium was added after irradiation. Reconstructed skins were routinely incubated 1 h at 37°C in a medium containing 0.1 mg/ml bromodeoxyuridine (BrdU) (Boehringer Mannheim, Germany) before being fixed for histology or frozen in liquid nitrogen. The conditioned medium obtained 24 or 48 h after the irradiation was carefully collected for collagenase I assay.

Histology

Samples were fixed in 10% neutral formalin and treated for histology. Paraffin sections were stained with hematoxylin, eosin, saffron.

Immunostaining

Antibodies Mouse monoclonal antibodies (Mab) were against human keratin 10 (RKSE 60, Sanbio Laboratories), filaggrin (Biomedical Technologies Inc, USA), keratinocyte transglutaminase (Biomedical Technologies Inc, USA), vimentin (Monosan, The Netherlands) and BrdU (Boehringer Mannheim, Germany) and thymine dimers (H3) (Dr. Roza, Netherlands) (Roza *et al*, 1988). A monoclonal rat anti human procollagen type I was purchased from Chemicon (USA). Rabbit polyclonal antibodies were against human involucrin (Biomedical Technologies Inc, USA), loricrin (Dr. Magnaldo, France), (Magnaldo *et al*, 1992), fibronectin (Pasteur Institute, France). FITC-conjugate rabbit anti-mouse immunoglobulins or FITC-conjugate swine anti-rabbit immunoglobulins (Dako, Denmark) were used as second antibodies.

Procedure Samples were embedded in Tissue Tek (Miles, USA), frozen in liquid nitrogen and 5 μm vertical cryosections were prepared. Immunohistochemistry was performed as previously described (Bernerd and Asselineau, 1997). Detection of thymine dimers was carried out according to Roza *et al* (1991). Nuclear counterstaining using propidium iodide was carried out routinely.

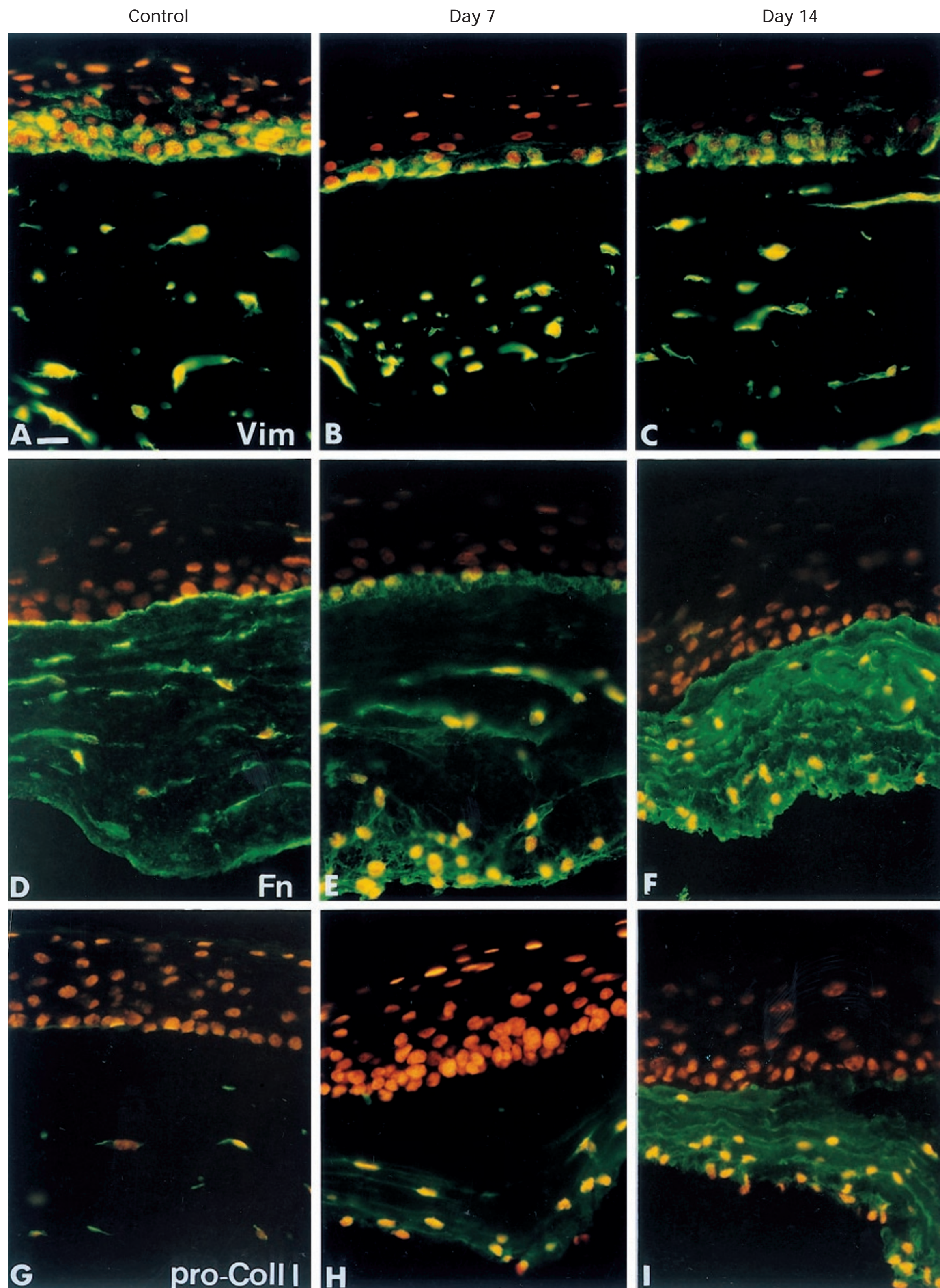

Figure 11 Characterization of recolonizing fibroblasts. (**A–C**) Anti-vimentin immunostaining (Vim); (**D–F**) Anti-fibronectin immunostaining (Fn). (**G–I**) Anti-procollagen I immunostaining (pro-Coll I). (**A,D,G**) Sham-irradiated samples observed at day 10. (**B,E,H**) 25 J/cm^2 UVA-irradiated (320–400 nm) samples observed at day 7. (**C,F,I**) 25 J/cm^2 UVA irradiated samples observed at day 14. Note that fibronectin and procollagen I deposits are clearly more important in the dermal equivalents of irradiated samples, compared to sham-irradiated samples. Scale is 30 μm

TUNEL method

TUNEL reaction was performed as described (Bernerd and Asselineau, 1997) using the In Situ Cell Death Detection Kit (Boehringer Mannheim, Germany) on 4% formaldehyde fixed frozen sections.

MMP 1 assay

The amount of secreted interstitital collagenase (MMP1) was assessed using the Biotrack MMP-1 human ELISA system (Amersham, England). Conditioned medium from sham-irradiated or UVA-irradiated reconstructed skin was collected either 24 or 48 h after the irradiation.

Proliferation estimation

Positive cells labelled with anti-BrdU was analyzed using a fluorescent microscope.

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