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Fluorescent probes as markers of oxidative stress in keratinocyte cell lines following UVB exposure

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Dedicated to Professor Pietro Pratesi

Abstract

In this work we describe the development of specific markers for determination of both the membrane and intracellular damage induced by free radicals generated by UVB radiation (5–150 mJ/cm^2) in cultured keratinocytes. This using simple, specific and sensitive fluorescent probes: *cis*-parinaric acid (PNA) to monitor membrane lipid peroxidation and 2′,7′-dichloro-dihydrofluorescein diacetate (DCFH-DA) to evaluate the intracellular redox status, in parallel to the fluorimetric determination of the main intracellular antioxidant glutathione. To validate the methodologies, the changes in the intracellular oxidative status following exposure to low doses UVB were measured in both control and *N*-acetylcysteine-protected cells, in parallel with morphological analyses. UVB induces an early reduction of GSH inside the cell correlated with an increase in the intracellular peroxide content. The effects were time- and dose-dependent. In addition, using a sensitive fluorescent method, we quantitated the release of proteases, a family of proteolytic enzymes greatly involved in the onset/perpetuation of the free radical-induced skin damage from keratinocytes exposed to suberythemal UVB doses (5–15 mJ/cm^2). The use of these fluorescent probes provides a reliable tool to detect the early signs of damage in keratinocyte cultures (when the apoptotic phenomenon has not yet been triggered) useful for future screening of protective molecules.

Keywords: Keratinocytes; UVB radiation; Cytotoxicity; Oxidative stress; Fluorescent probes

1. Introduction

Chronic exposure of unprotected human skin to UVB radiation is known to induce an array of adverse reactions, including premature skin ageing, erythema, inflammation and photo-carcinogenesis, and this through the formation of reactive oxygen species (ROS).

Since the cutaneous absorption of UVB in the skin occurs primarily in the keratinocyte and the ability of epidermis to protect itself from oxidative stress is dependent on the antioxidant capacity of keratinocytes, keratinocyte cell lines are well suited for mechanistic and toxicological studies.

The UVB-induced damage to keratinocytes is currently assessed by measuring the extent of formation of lipid peroxidation products, malondialdehyde (MDA) or thiobarbituric acid reactive substances (TBARS): anyway both of these parameters, as they are end-point markers of membrane derangement and aspecific and poorly sensitive, become significant only at UVB doses largely over the minimal erythemal dose in man (up to 300 mJ/cm^2) [1,2].

In this context, it has been demonstrated that low UVB doses (5–40 mJ/cm^2, well below those causing skin reddening in man), induce in cultured human keratinocytes the expression of several proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukins 1α, 1β and 6 (IL-1α, IL-1β, IL-6), which promote reactions of other effector cells in the skin, e.g. leukocytes and endothelial cells [3]. Anyway, none of these markers of early UVB-induced cell damage can be used as routine tool for the evaluation of the toxic effects of UVB (and conversely the protective

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effects of photoprotective agents), because of the high cost of the analytical determinations.

With this in mind, the aim of this work was to develop new markers for the determination of both the intramembrane and intracellular damage induced by low UVB doses in cultured human keratinocytes, using simple, sensitive and specific fluorescent probes: *cis*-parinaric acid (PNA) to monitor membrane lipid peroxidation and 2′,7′-dichloro-dihydrofluorescein diacetate (DCFH-DA) to monitor the overall intracellular antioxidant status. This in parallel to the fluorimetric determination of glutathione, the main intracellular antioxidant.

In addition, since it has been demonstrated that ultraviolet exposure leads to sustained elevation of matrix metalloproteinases [4], a family of proteolytic enzymes which contribute to skin photoaging, and in skin carcinoma to the spreading of metastatic cells by specifically degrading skin collagen and elastin, in the last part of this work we have investigated whether protease release (fluorescence-based assay) could be used as bioanalytical marker of early keratinocytes damage by low UVB doses.

2. Experimental

2.1. Chemicals and apparatus

The organic solvents used were of analytical grade (E. Merck, Bracco, Milan, Italy). DMEM (Dulbecco's modified Eagle's Medium), phenol red-free DMEM, L-glutamine, sodium pyruvate, penicillin-streptomycin solution, DCFH-DA, *o*-phthalaldehyde (OPA), *N*-acetyl-L-cysteine (NAC), ethylenediamine-tetraacetic acid (EDTA), metaphosphoric acid and 2-phenyl-1,2-benzisoselenazol-3[2*H*]-one (ebselen) were purchased from Sigma (Milan, Italy); 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), PNA (9,11,13,15 *cis*, *trans*, *trans*, *cis*-octadecatetraenoic acid) and the EnzChek™ protease assay kit (E-6638) were from Molecular Probes (Società Italiani Chimici, Rome, Italy); fetal calf serum, trypsine-EDTA solution and phosphate buffer solutions (PBS) from Hyclone (Celbio, Milan, Italy); porcine pancreatic elastase (130 U/mg protein), NADH and the in situ cell death detection kit, POD from Boehringer Mannheim Italia (Roche, Milan, Italy).

Spectrophotometric and fluorimetric determinations were performed with a computer-aided Perkin–Elmer Lamdba 16 spectrophotometer and a computer-aided Perkin–Elmer LS50B luminescence spectrometer (Perkin–Elmer, Monza, Italy). Light and fluorescence microscopy studies were done with a Zeiss Axioplan Fluorescence Microscope with fluorescein isothiocyanate (FITC) filter.

2.2. Cell irradiation

The NCTC 2544 human keratinocyte cell line [5] (Flow Laboratories, Irvine, UK) was cultured at 37°C in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine and antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin). Cultures were maintained at 37°C in a humidified 5% CO_2 atmosphere. For UV experiments, cells were seeded at a density of 5×10^4/cm^2 and cultured in 9.4 cm^2 cell plastic culture dishes until 80–90% confluence. UVB radiation was carried out with a parallel bank of two Philips TL20W/12 fluorescence tubes (Sara s.r.l., Castellanza (Va), Italy) emitting a continuous spectrum between 280 and 320 nm with a peak emission at 312 nm. Fluence rate at the site of cell irradiation (25 cm from UVB source) was of 0.85 mW/cm^2 and the UVB doses employed ranged from 5 to 150 mJ/cm^2 (delivered within 12–180 s), as measured with a Vilber Lourmat VLX-3W radiometer (UVB probe, 312 nm).

Before irradiations, cell monolayers were washed three times with prewarmed PBS and exposed to UVB in the presence of PBS. After irradiation, PBS was replaced by serum-phenol red free DMEM (K-DMEM) and incubated for different time periods (4, 8 and 24 h). Sham-control cells were subjected to the same procedure without UV exposure.

Cell viability was determined by the trypan blue exclusion assay [6] and by measuring the LDH leakage [7], expressed as ratio of LDH values in supernantant/(supernatant + cell suspension). Protein determinations were performed by a modified Lowry method [8], using bovine serum albumin as a standard.

2.3. Intracellular oxidation: DCF and GSH assays

Stock solutions of DCFH-DA (3.34 mM) were prepared in absolute ethanol purged with nitrogen and stored at −20°C in 500 μl aliquots. Immediately after irradiation, the cell cultures were incubated with 10 μM DCFH-DA in PBS for 30 min at 37°C. Thereafter the medium was discarded and the cell monolayer washed three times with prewarmed PBS to remove not-incorporated DCFH-DA and then incubated with K-DMEM. At 4, 8 and 24 h post-irradiation, the media were discarded, the cells washed twice with prewarmed PBS and then gently removed by scraping into 3 ml of PBS. The cellular suspension was then transferred into 1 ml thermostated fluorescence cuvettes (25°C) equipped with a magnetic stirrer and the fluorescence intensity of the probe (λ_{exc} 502 nm; λ_{em} 520 nm; band widths 5 nm) recorded. The fluorescence intensity values at all the observation times were correlated to protein content and expressed as % increase of fluorescence in respect to the controls. To confirm intracellular localization of the fluorescent probe, UVB-irradiated

and sham-control cells were also examined by fluorescence microscopy (original magnification 1:250).

When NAC was used as supplement according to Steenvoorden and Beijersbergen van Henegouwen [9], keratinocytes were preincubated for 30 min before irradiation with a 20 mM NAC solution in PBS. The medium was then removed, the monolayer washed and treated as described above.

The intracellular GSH content was determined by the method of Hissin and Hilf [10] suitably modified. Briefly, cells were washed twice with PBS and scraped into 2.5 ml of 0.1 M phosphate buffer (pH 8) containing 5 mM EDTA. Aliquots (1.5 ml) of the cell suspension were collected, deproteinized with 350 μl of a 25% solution of metaphosphoric acid, homogenized by gentle sonication at 0°C and then centrifuged for 10 min at 18 000 rpm. Aliquots (0.7 ml) of the supernatant were added to 3.3 ml of phosphate-EDTA buffer, vortexed and 500 μl of this solution transferred to the reaction mixture constituted by 4.4 ml of phosphate-EDTA buffer and 100 μl of OPA (1 mg/ml in methanol). Fluorescence was measured after incubation at 25°C for 30 min, at 350 nm (λ_{exc}, band width 5 nm) and 420 nm (λ_{em}, band width 5 nm). The GSH content was calculated as nmol/mg protein and expressed as % consumption versus sham-control cells.

2.4. Membrane peroxidation: PNA assay

Stock solutions of PNA (3.34 mM) were prepared in absolute ethanol purged with nitrogen and stored in 1 ml aliquots in screw-top vials at −20°C. Immediately after irradiation, the cell cultures were incubated with 10 μM PNA (dispersed in K-DMEM) for 30 min at 37°C. Cell cultures were then treated as described for DCFH-DA experiments and the fluorescence intensity of the probe (λ_{exc} 312 nm; λ_{em} 455 nm; band widths 5 nm) recorded at 25°C as previously described [11].

2.5. Protease release

Protease activity was determined in culture media from UVB-exposed and sham-control cells by the EnzCheck™ protease assay kit. Immediately after irradiation with 5–50 mJ/cm^2, the cell cultures were incubated with prewarmed phenol red free DMEM containing 0.05 mg/ml substrate (BODIPY FL casein). After 24 h, the supernatant was transferred into 1 ml cuvette and the fluorescence intensity (λ_{exc} 485 nm, band width 5 nm; λ_{em} 530 nm, band width 10 nm) recorded. Spontaneous BODIPY FL casein degradation was checked by incubating the substrate for 24 h without the cells. Protease release in the supernatant was determined as elastase activity (mU/mg protein) on a standard curve obtained by incubating for 24 h the substrate with different amounts of elastase (0.001–1 mU/ml). The data were expressed as % release in respect to sham-control cells.

2.6. Morphological analysis

Cell morphology was evaluated by staining with May-Grünwald and apoptotic cells were identified by light microscopy according to Schwarz et al. [12]. Internucleosomal DNA fragmentation was confirmed by the TUNEL (TdT-mediated dUTP nick end labelling) assay using an in situ cell death detection kit. Stained cells were analyzed under light microscopy. The amount of cells staining positive for TUNEL was calculated as percentage of the total cells: at least 100–150 cells were counted for each experimental group.

2.7. Statistical analysis

The data were expressed as mean ± SD of six independent experiments. Statistical analysis was done using one-way analysis of variance (ANOVA) combined with the Tukey's test or by the Student's *t*-test. The 5% level of statistical significance was used in all experiments.

3. Results and discussion

3.1. Cell viability

The viability of cultured keratinocytes, determined by Trypan blue exclusion and LDH leakage 4 and 8 h after UVB irradiation from 10 to 150 mJ/cm^2, was always over 90% and not significantly different in respect to the sham-control cells. At the last observation time (24 h) the viability was reduced to 76.3 ± 4.6 and 65.7 ± 3.2% only at the highest doses (100 and 150 mJ/cm^2). The fluorescent probes DCFH-DA and PNA and the substrate for protease assay (BODIPY FL casein) by themselves did not affect the cell viability.

3.2. GSH content

One of the most important endogenous defense mechanism against UV-induced ROS is the tripeptide glutathione (GSH). It directly scavenges radicals by hydrogen transferring, acts as cofactor for the enzyme GSH–peroxidase, which scavenges peroxides, and finally regenerates vitamins E and C. On the basis of this evidence and taking into account its cytosolic location, we monitored the GSH levels as a first index of intracellular oxidative stress.

As shown in Fig. 1, UVB irradiation induced at all the observation times a dose-dependent GSH depletion (immediately after UVB irradiation GSH content was always in the range of the controls for all the doses). At the first observation time (4 h) GSH content was only

slightly affected, since maximal reduction, to 75.45 ± 1.8% was observed at 150 mJ/cm^2 UVB exposure (taking as 100% the GSH levels in sham-control cells, 76.9 ± 4.3 nmol/mg protein). The decrease was more pronounced after 8 h: GSH was in fact significantly reduced to 84.00 ± 3.25% at 10 mJ/cm^2, to 76.13 ± 1.39% at 25 mJ/cm^2, to 58.43 ± 2.30%, 49.05 ± 2.80% and 46.24 ± 2.30% at 50, 100 and 150 mJ/cm^2, indicating a time-dependent damage of the intracellular antioxidant pool. Maximal decreases were observed at 24 h: GSH levels fell to 64.1 ± 0.88% (10 mJ/cm^2), to 51.8 ± 1.20% (25 mJ/cm^2) and to 26.28 ± 0.20% (50 mJ/cm^2). No determinations were made at higher UVB doses, because of the lower viability of the cells. The depletion of GSH in keratinocytes exposed to 50 mJ/cm^2 (24 h) was significantly restrained by pretreatment of the cells with 20 mM NAC (Fig. 2): GSH levels were more than doubled in respect to irradiated cells and close to those of untreated sham-control cells. This indicates that the cytoplasmic machinery involved in the enzymatic resynthesis of GSH, whose levels in supplemented control cells are increased by 50%, is not impaired by this UVB dose.

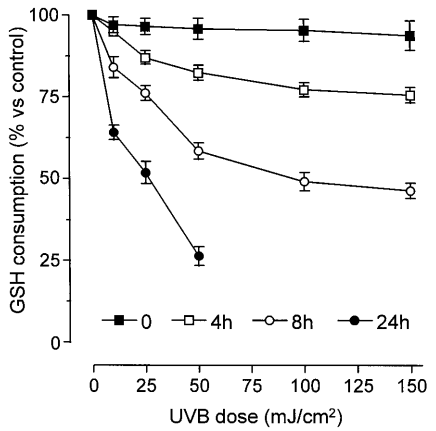

Fig. 1. Time-dependent depletion of GSH in UVB-irradiated keratinocytes. All the post-irradiation values, except 10 mJ/cm^2 (4 h), are statistically different from the controls ($P < 0.001$). GSH content in sham-control cells (100%) = 76.9 ± 4.3 nmol/mg protein.

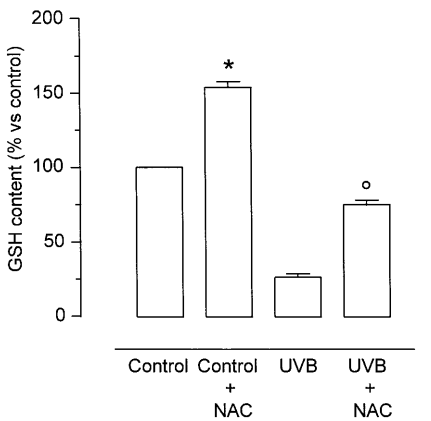

Fig. 2. Effect of *N*-acetylcysteine (NAC) supplementation on GSH levels in control and UVB-irradiated (50 mJ/cm^2) keratinocytes. Keratinocytes were preincubated for 30 min before irradiation with 20 mM NAC in PBS, and GSH determinations were made 24 h post-irradiation. Values are expressed as % vs. sham-control cells (*$P < 0.001$ vs. sham-control cells; °$P < 0.001$ vs. irradiated cells).

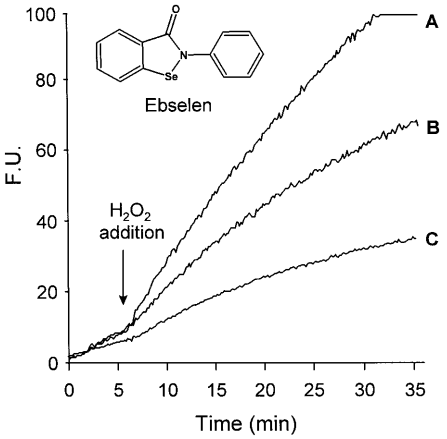

Fig. 3. Dose-dependent effect of ebselen on H_2O_2-driven oxidation of DCFH in control keratinocytes. Sham-control cells were exposed to 1 mM H_2O_2 in the absence (A) or in the presence of 50 μM (B) or 100 μM (C) ebselen. Ebselen was preincubated for 15 min before H_2O_2 addition.

3.3. Intracellular oxidation: DCF assay

Since GSH depletion can induce ROS accumulation, we checked in parallel the intracellular oxidative status by a fluorimetric assay, previously applied for quantitation of picomolar concentrations of hydrogen peroxide [13] and peroxinitrite [14] in endothelial and glial cell cultures. The assay is based on the ability of the non-polar, non-fluorescent DCFH-DA to diffuse through the cell membrane and to be deacetylated by cytosolic esterases to form the polar, non-fluorescent dichlorodihydrofluorescein (DCFH). This last is trapped inside the cell where, by reacting with ROS, it gives rise to the formation of the fluorescent derivative dichlorofluorescein (DCF).

The method was validated first by exposing intact keratinocytes to 1 mM hydrogen peroxide (Fig. 3) and monitoring the increase of fluorescence due to oxidation of DCFH and by the use of the prototype antioxidant ebselen (50, 100 μM), a compound with glutathione peroxidase-like activity, which dose-dependently inhibits the hydrogen peroxide-induced fluorescence formation.

UVB caused a dose-dependent accumulation of DCF at all the observation times post-irradiation (Fig. 4). Four hours post-irradiation the DCF fluorescence was detectable (10.22 ± 1.08% increase versus controls) only at 25 mJ/cm^2 and significantly increased with the UVB dose: 28.2 ± 0.3% at 50, 35.4 ± 4.3% at 100 and 47.3 ±

5.3% at 150 mJ/cm^2. Pretreatment of the cells with 20 mM NAC counteracts the oxidative process, since an almost complete protection was observed at 50 and 100 mJ/cm^2, with a reduction of DCF formation to 12.2 ± 3.4% at 150 mJ/cm^2.

The oxidative process was more pronounced at 8 h post-irradiation and clearly dose-dependent: the DCF fluorescence increased by 33.8 ± 1.7% at 25 mJ/cm^2, by 73.3 ± 3.2% at 50 mJ/cm^2 (23.5 ± 3.2% in the presence of NAC) and by 133.0 ± 12.2% and 164.7 ± 11.4% at 100 and 150 mJ/cm^2. Also at the highest UVB doses, NAC supplementation significantly restrained the oxidative burst (85.4 ± 5.8 and 101.2 ± 13.7%).

As shown in Fig. 2, the more striking increase in DCF fluorescence in cells exposed to low UVB doses was observed at 24 h: 7.7 ± 1.6% at 10 mJ/cm^2, 57.7 ± 4.5% at 25 mJ/cm^2 and 112.7 ± 11.1 at 50 mJ/cm^2. The

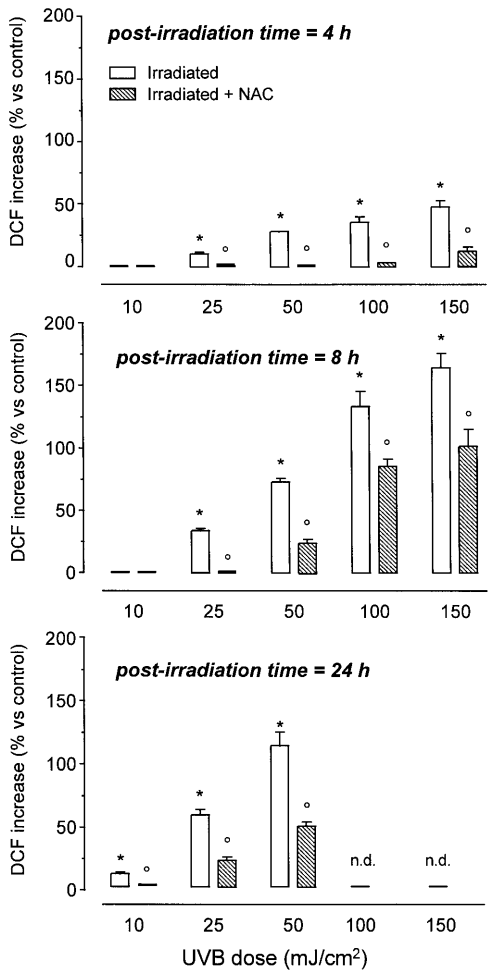

Fig. 4. Dose-dependent increase of DCF fluorescence intensity in UVB-irradiated keratinocytes and protective effect by NAC at the different post-irradiation times. Keratinocytes were preincubated for 30 min before irradiation with 20 mM NAC in PBS. Values are expressed as % increase vs. sham-control cells (*$P < 0.001$ vs. sham-control cells; °$P < 0.001$ vs. irradiated cells).

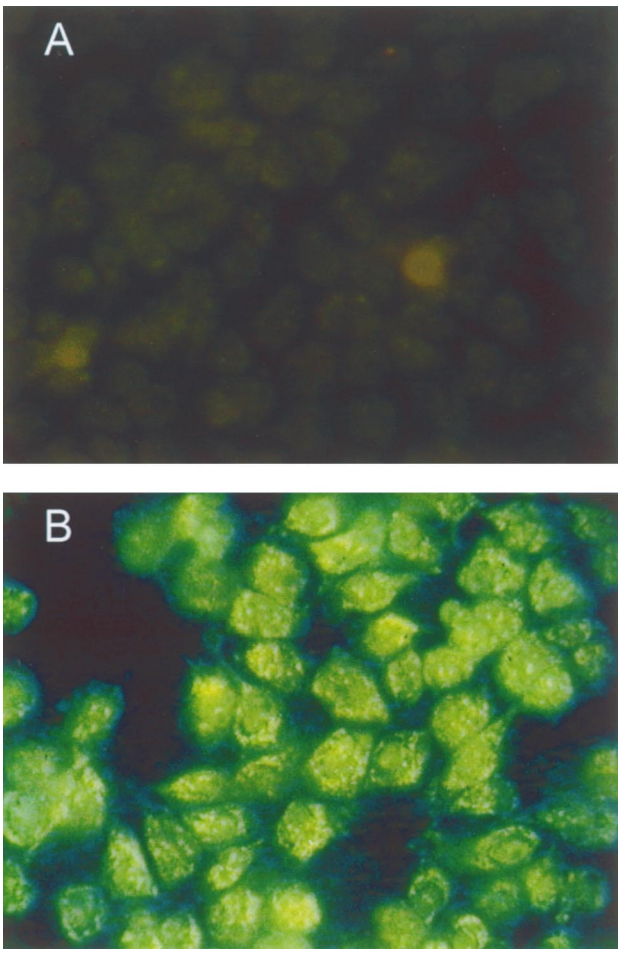

Fig. 5. Fluorescence microscopy analysis of intracellular DCF formation in unirradiated (A) and UVB-irradiated keratinocytes (50 mJ/cm^2, B). Cells were analysed 24 h post-irradiation (original magnification 1:250).

samples protected with NAC showed a trend to reduction in DCF accumulation similar to that evidenced at the first observation times. This protective effect is due to an enhanced intracellular availability of NAC, which can act as a direct radical scavenging agent and/or as a precursor for the ex-novo synthesis of GSH.

Fluorescence microscopy examination of the UVB-irradiated cells (50 mJ/cm^2; 24 h) (Fig. 5(B)) shows that all cells were strongly fluorescent, with a fairly uniform distribution of the dye, to confirm the spreading of the oxidative burst within the cell.

As shown in Fig. 6, there was a strict inverse correlation ($r^2 = 0.9548$; $P < 0.0001$) between intracellular GSH levels and DCF fluorescence formation, to indicate that ROS accumulation within the keratinocyte is strictly dependent on the content of the main endogenous antioxidant. This makes the DCF assay a reliable and specific marker of early intracellular oxidative damage.

3.4. Membrane lipid peroxidation: PNA assay

PNA, a naturally occurring fatty acid which specifically incorporates into the phospholipid bilayer, was used as an index of membrane lipid peroxidation. The intact conjugated tetraene structure of PNA makes it fluorescent only when incorporated within a lipid environment. Oxidative disruption of the double bonds is directly translated into an irreversible loss of fluorescence, and this is the basis for the use of PNA as a sensitive and specific indicator of cumulative oxidation of conjugated double bonds of membrane lipids. Also in this case, the assay was validated in intact, unirradiated keratinocytes using as radical inducer a water-soluble (extracellular) azo-initiator (AAPH), which generates peroxyl radicals in aqueous phase at a constant rate. In the presence of different concentrations of the radical generator, the probe dose-dependently loses its initial fluorescence (Fig. 7), reflecting the extent of oxidation in the membranes. At all the UVB doses we never observed PNA oxidation at 4 and 8 h post-irradiation; at the maximal dose of 150 mJ/cm^2, a slight but significant PNA oxidation (15.4 $\pm$ 3.2%) occurred only 24 h post-irradiation (data not shown). These results indicate that in keratinocytes membrane peroxidation is an event which starts when the cell has already lost the intracellular redox control and for this reason it must be considered in this model as a late marker of toxicity. This in agreement with the results of other authors [1,2], which demonstrated that the lipid peroxidation process by UVB is significant only at high doses (i.e. 10 times greater than those used in the present study), when the oxidant species generated overwhelm the intracellular defense systems.

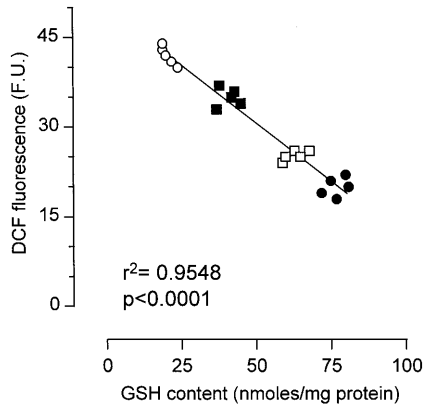

Fig. 6. Scatter plot of GSH levels and DCF formation in controls and UVB-irradiated keratinocytes. Controls (●); 10 mJ/cm^2(□), 25 mJ/cm^2 (■), 50 mJ/cm^2 (○).

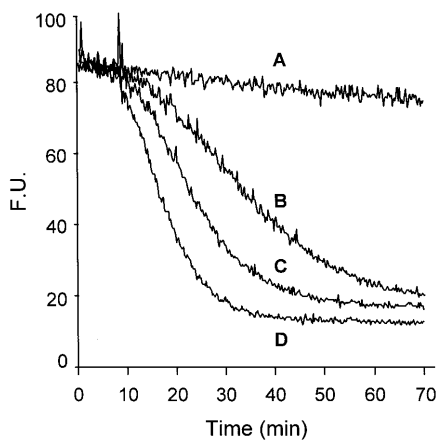

Fig. 7. Time-course of Parinaric acid (PNA) fluorescence decay induced by the radical initiator AAPH in unirradiated keratinocytes. A = Control; B = 1 mM AAPH; C = 2 mM AAPH; D = 4 mM AAPH.

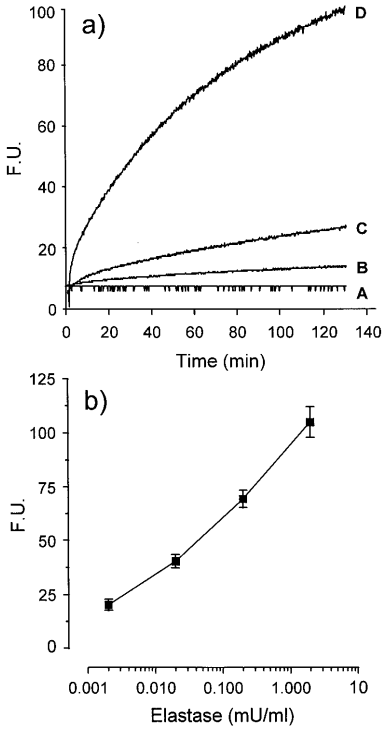

Fig. 8. Fluorimetric determination of elastase activity. (a) Kinetics of substrate hydrolysis (A = control; B = 2 mU/ml elastase; C = 20 mU/ml; D = 200 mU/ml); (b) calibration curve: fluorimetric measurements determined after 24 h incubation at 37°C, pH 7.4.

3.5. Protease release

The release of proteases in cell supernatants was also determined using a fluorescence-based assay which measures the activity of a wide variety of metallo- and serine-proteases. The assay relies on the use of an aspecific substrate (casein) that is labeled with a green fluorescent dye deeply embedded in the protein, making the fluorescence dye totally quenched. Protease-catalyzed hydrolysis of the substrate relieves the quenching, yielding a marked increase in fluorescence that is proportional to protease activity. The method was first validated in cell free systems using porcine pancreatic

elastase as proteolytic enzyme: as shown in Fig. 8(a), which reports the kinetics of substrate hydrolysis, the increase in the fluorescence units is strictly dependent on elastase activity; the protease activity calibration curve for studies in keratinocytes (Fig. 8(b)) was calculated by fluorescence measurements at a fixed time (24 h). Fig. 9 shows the effects of low UVB doses on extracellular protease activity, expressed as % versus sham-control cells: protease activity in cell supernatants was significantly increased, by $23.2 \pm 5.5\%$ already at 5 mJ/cm^2, by $33.2 \pm 4.1\%$ at 10 mJ/cm^2 and by $44.2 \pm 7.3\%$ at 15 mJ/cm^2. At higher doses (25–50 mJ/cm^2) the release of proteases was significantly restrained, with a trend towards the controls. This behavior, currently under investigation by Western blot analysis, might be due to inactivation of the enzyme proteins by the oxidative burst or to an overproduction and secretion of the endogenous inhibitors of metalloproteases (TIMP). Whatever might be the explanation, these results confirm the assumption that protease release is a reliable marker of early damage induced by UVB radiation, sensitive already at 5 mJ/cm^2, an UVB dose (0.1 MED) that is equivalent to a 2–3 min solar irradiation on a summer day which causes no perceptible skin reddening.

3.6. Cell morphology and TUNEL-positive cell counting

When keratinocytes were exposed up to 25 mJ/cm^2, no significant morphological alterations were observed in respect to sham-control cells (Fig. 10(A)). By contrast, in 50 mJ/cm^2-exposed cells (incubated for 24 h) cell morphology was markedly modified (Fig. 10(B)), since marginal condensation of the chromatin and nuclear fragmentation, typical signs of the apoptotic process, were observed in $20 \pm 1.5\%$ of the irradiated cells.

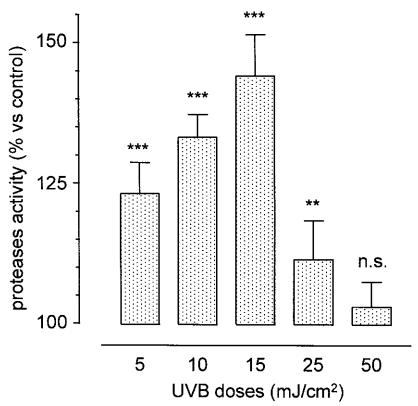

Fig. 9. Extracellular proteases activity in UVB-irradiated keratinocytes. Proteases activity, expressed as % vs. sham-control cells, was determined 24 h post-irradiation. ***$P < 0.001$; **$P < 0.01$; n.s. not significant vs. controls.

This value was confirmed by TUNEL assay ($22 \pm 1.8\%$), which preferentially labels DNA strand breaks generated during apoptosis [15]. Less than 2% of unirradiated control cells were TUNEL-positive. NAC pretreatment not only preserved normal cell morphology (Fig. 10(C)), but also reduced the percentage of TUNEL-positive cells to levels even below those of unirradiated cells, to indicate that NAC behaves as an effective inhibitor of the indicators of apoptosis in irradiated keratinocytes.

4. Conclusions

The results of this study point to the use of some easy to handle and low cost fluorescent probes with specific cellular compartmentalization which in human keratinocyte cell lines allow the detection of the oxidative stress at the early stage of damage, i.e. that produced by suberythemal UVB doses (0.1 MED–1 MED), when the apoptotic phenomenon is absent or only at the beginning. It is well known that peroxide generation precedes both DNA fragmentation and morphological changes typical of apoptosis, and that pharmacological agents that block the production of free radicals such as NAC, trolox, or ebselen inhibit radiation-induced apoptosis [16].

In particular the DCF probe, here applied for the first time to study the intracellular oxidative status of keratinocytes after UVB irradiation, properly coupled with the GSH profile, furnishes a clear picture of the time-course of the decay (and of the recovery) of the cytosol antioxidant pool. The fact that GSH levels were almost completely recovered in NAC-pretreated keratinocytes exposed to UVB, confirm from one side the validity of the analytical approach here proposed, as evidenced also by morphological analysis, and from the other the role of cysteine derivatives as UV radiation protectors.

The release of proteases in keratinocytes, although still under investigation, deserves credit as early marker of UVB-induced damage: suberythemal UVB doses are now considered the *primum movens* of premature skin aging, as they increase the levels of matrix metalloproteinases, the primary mediators of connective tissue damage, in the absence of any skin reddening [4].

In addition, the use of these fluorescent probes seems to be highly suited for the screening of new antioxidants/photoprotective agents of natural and synthetic source, since it allows to establish their specific cellular localization and their efficacy to counteract the oxidative process generated by UV radiation. Finally, it can be used as a tool to test the phototoxic potential of pharmaceutical/cosmetic substances acting specifically on the skin.

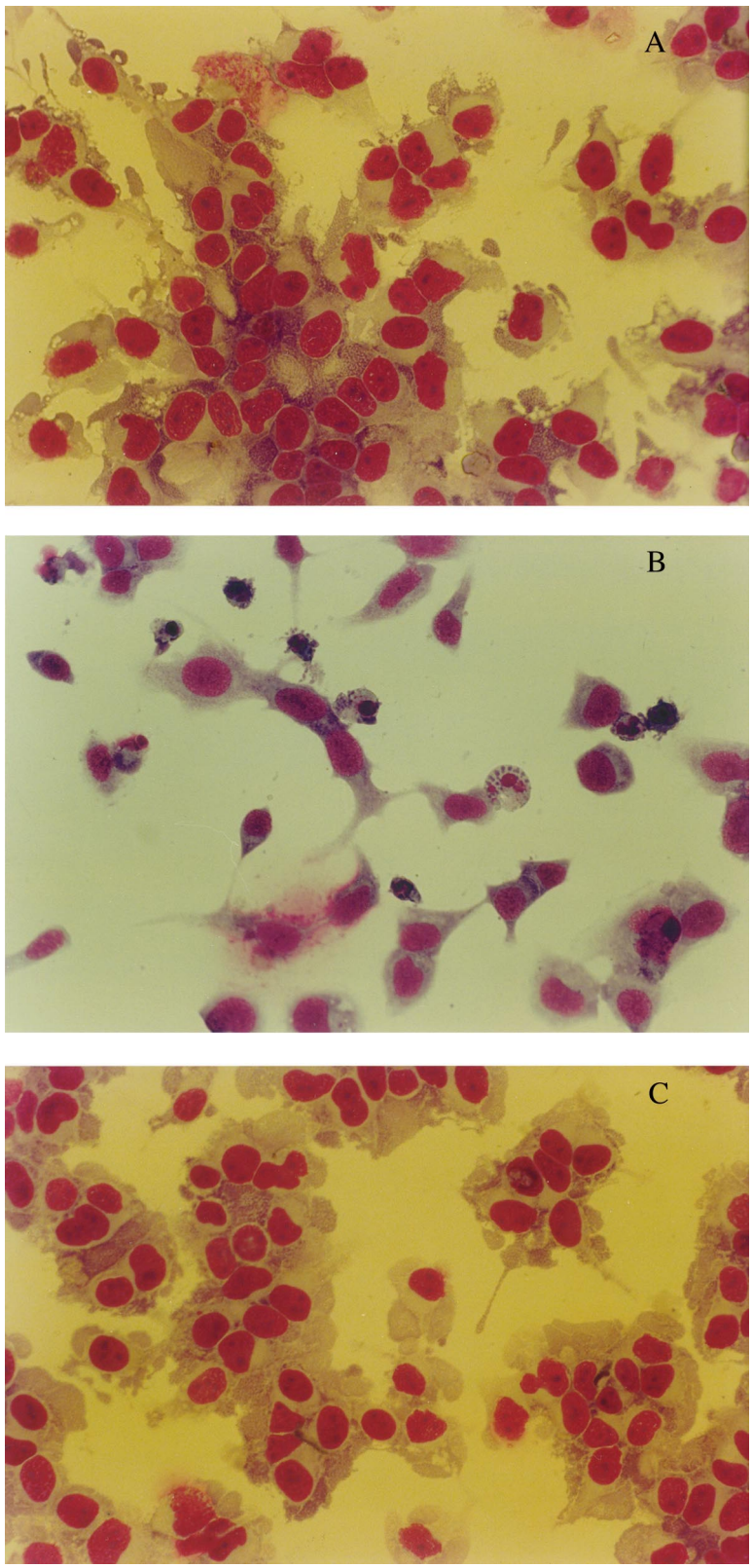

Fig. 10. Morphological analysis by light microscopy of controls (A), irradiated cells (50 mJ/cm^2, B) and NAC-irradiated keratinocytes (C). Original magnification 1:300.

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References

[1] K. Punnonen, A. Puntala, C.T. Jansen, M. Ahotupa, UVB irradiation induced lipid peroxidation and decreases antioxidant enzyme activities in human keratinocytes in vitro, Acta Derm. Venereol. 71 (1991) 239–273.
[2] M.S. Stewart, G.S. Cameron, B.C. Pence, Antioxidant nutrients protect against UVB-induced oxidative damage to DNA of mouse keratinocytes in culture, J. Invest. Dermatol. 106 (1996) 1086–1089.
[3] M. Brauchle, J.O. Funk, S. Werner, Ultraviolet B and H_2O_2 are potent inducers of vascular endothelial growth factor expression in cultured keratinocytes, J. Biol. Chem. 271 (1996) 21793–21797.
[4] 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, N. Engl. J. Med. 337 (1997) 1419–1428.
[5] V.P. Perry, K.K. Sanford, V.J. Evans, G.W. Hyatt, W.R. Earle, Establishment of epithelial cells from human skin, J. Natl. Cancer Inst. 18 (1957) 707–717.
[6] K. Punnonen, T. Puustinen, C.T. Jansén, Ultraviolet B irradiation induced changes in the distribution and release of arachidonic acid, dihomo-γ-linolenic acid and eicosapentaenoic acid in human keratinocytes in culture, J. Invest. Dermatol. 87 (1987) 611–614.
[7] B. Tebbe, S. Wu, C.C. Geilen, J. Eberle, V. Kodeljia, C.E. Orfanos, L-Ascorbic acid inhibits UVA-induced lipid peroxidation and secretion of IL-1α and IL-6 in cultured human keratinocytes in vitro, J. Invest Dermatol. 108 (1997) 302–306.
[8] M.A. Markwell, S.M. Haas, N.E. Tolbert, L.L. Bieber, Protein determination in membrane and lipoprotein samples: manual and automated procedures, Methods Enzymol. 72 (1981) 296–303.
[9] D.P.T. Steenvoorden, G.M.J. Beijersbergen van Henegouwen, Cystein derivatives protect against UV-induced reactive intermediates in human keratinocytes: the role of glutathione synthesis, Photochem. Photobiol. 66 (1997) 665–671.
[10] P.J. Hissin, R. Hilf, A fluorimetric method for determination of oxidized and reduced glutathione in tissues, Anal. Biochem. 74 (1976) 214–226.
[11] R. Maffei Facino, M. Carini, G. Aldini, M.T. Calloni, E. Bombardelli, P. Morazzoni, Sparing effect of procyanidins from Vitis vinifera on vitamin E: in vitro studies, Planta Med. 64 (1998) 343–347.
[12] A. Schwarz, R. Bhardwaj, Y. Aragane, K. Mahnke, H. Riemann, D. Metze, T.A. Luger, T. Schwarz, Ultraviolet-B-induced apoptosis of keratinocytes: evidence for partial involvement of Tumor Necrosis Factor-α in the formation of sunburn cells, J. Invest. Dermatol. 104 (1995) 922–927.
[13] J. Royall, H. Ischiropoulos, Evaluation of 2′,7′-dichlorofluorescin and dihydro-rhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells, Arch. Biochem. Biophys. 302 (1993) 348–355.
[14] H. Possel, H. Noack, W. Augustin, G. Keilhoff, G. Wolf, 2,7-Dihydrochlorofluorescein diacetate as a fluorescent marker for peroxinitrite formation, FEBS Lett. 416 (1997) 175–178.
[15] Y. Gavrieli, Y. Sherman, S.A. Ben-Sasson, Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation, J. Cell. Biol. 119 (1992) 493–501.
[16] N. Ramakrishnan, J.F. Kalinich, D.E. McClain, Ebselen inhibition of apoptosis by reduction of peroxides, Biochem. Pharmacol. 51 (1996) 1443–1451.