Molecular Responses to Photogenotoxic Stress Induced by the Antibiotic Lomefloxacin in Human Skin Cells: From DNA Damage to Apoptosis

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Photo-unstable chemicals sometimes behave as phototoxins in skin, inducing untoward clinical side-effects when exposed to sunlight. Some drugs, such as psoralens or fluoroquinolones, can damage genomic DNA, thus increasing the risk of photocarcinogenesis. Here, lomefloxacin, an antibiotic from the fluoroquinolone family known to be involved in skin tumor development in photoexposed mice, was studied using normal human skin cells in culture: fibroblasts, keratinocytes, and Caucasian melanocytes. When treated cells were exposed to simulated solar ultraviolet A (320–400 nm), lomefloxacin induced damage such as strand breaks and pyrimidine dimers in genomic DNA. Lomefloxacin also triggered various stress responses: heme-oxygenase-1 expression in fibroblasts, changes in p53 status as shown by the accumulation of p53 and p21 proteins or the induction of *MDM2* and *GADD45* genes, and stimulation of melanogenesis by increasing the tyrosinase activity in melanocytes. Lomefloxacin could also lead to apoptosis in keratinocytes exposed to ultraviolet A: caspase-3 was activated and *FAS-L* gene was induced. Moreover, keratinocytes were shown to be the most sensitive cell type to lomefloxacin phototoxic effects, in spite of the well-established effectiveness of their antioxidant equipment. These data show that the phototoxicity of a given drug can be driven by different mechanisms and that its biologic impact varies according to cell type. ***Key words: DNA damage/fluoroquinolone/melanogenesis/p53/phototoxicity/skin cells. J Invest Dermatol 121:596–606, 2003***

Cutaneous photosensitivity reactions are of increasing concern in dermatology. Such problems are more and more frequent as today's lifestyle is often associated with exposure to sunlight. Photoreactive chemicals or drugs sometimes trigger skin reactions under exposure to normally harmless doses of sunlight. For example, antibiotics, psoralens, nonsteroidal anti-inflammatory drugs, and tranquilizers have been described as inducing such unwanted side-effects (Allen, 1993; Elmets, 1993). The activating wavelengths are most often in the ultraviolet A (UVA) range (320–400 nm). Chemical mechanisms of photosensitizing effects have been well described (see Kochevar, 1995, for a review). Although clinical reactions associated with phototoxicity are relatively well characterized (exaggerated sunburn, presence of vesicles, hyperpigmentation, eczema), the corresponding mechanisms need to be further investigated at cellular and molecular levels.

According to the literature, a great variety of lesions can give rise to photogenotoxicity. First, adducts to DNA can be formed by a direct interaction between the photosensitizer and DNA. The best reported examples are furocoumarins (see Averbeck, 1994, for a review). When the energy of its excited state is close to that of thymine, the photosensitizer can transfer the absorbed photonic energy to DNA. If the thymine reaches its triplet excited state, it can form a covalent link with an adjacent pyrimidine: a pyrimidine dimer is then produced, even at wavelengths where this damage is less likely to occur. Such a process was reported for pyrido-psoralen in the range of solar wavelengths (Andreu Guillo *et al*, 1996). But the most frequent process, or at least the most commonly reported, is the photosensitization via oxidative reactions. In this case, the sensitizer, once in its excited state, reacts with oxygen and generates reactive oxygen species (ROS) such as singlet oxygen $^1O_2$, superoxide anion ($O_2^{\cdot-}$), and hydroxy radical ($OH^{\cdot}$). As a consequence, a photosensitizer can generate a relatively intense local oxidative stress producing a large variety of DNA damage. Many phototoxic drugs have been shown to induce single strand breaks in DNA; some psoralen structures (Sage *et al*, 1989; Oroskar *et al*, 1993), antibiotics (Martinez and Chignell, 1998), anti-inflammatory drugs (Artuso *et al*, 1991; Agapakis-Causse *et al*, 2000), and porphyrins (Kochevar and Dunn, 1990) for instance were reported to nick DNA under light exposure. Various types of oxidative damage to purines and pyrimidines are also commonly induced upon photooxidative stress, depending on the nature of the ROS involved (Cadet *et al*, 1997, for a review).

In this work, we have studied the effects of the phototoxic antibiotic fluoroquinolone lomefloxacin on cultured human skin cells upon UVA exposure. Photochemical studies have shown that, when exposed to UVA, lomefloxacin could generate ROS (Martinez *et al*, 1998) as well as fluoride and reactive carbene after photolysis (Martinez *et al*, 1997). Lomefloxacin was previously reported to induce breaks and oxidative damage to DNA (Chetelat *et al*, 1996; Reavy *et al*, 1997; Marrot *et al*, 2001; Sauvaigo *et al*, 2001) and, more recently, to photosensitize pyrimidine dimers (Traynor

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Abbreviation: CPD, cyclobutane pyrimidine dimer.

and Gibbs, 1999; Sauvaigo *et al*, 2001). Its ability to damage lipids was also described (Condorelli *et al*, 1996) and, finally, its role as a photocarcinogenic compound was questioned (Klecak *et al*, 1997). Here, we have characterized some of the biologic effects resulting from lomefloxacin-induced photogenotoxic stress. Using cultured human fibroblasts, keratinocytes, and melanocytes, we first confirmed the ability of lomefloxacin to induce DNA damage such as strand breaks and pyrimidine dimers. Furthermore, we have shown that the stress gene heme-oxygenase-1 (*HO-1*) was induced. The activation of the p53 pathway was also demonstrated either by the accumulation of the protein p53 or by its ability to *trans*-activate some of its target genes such as *P21*, *GADD45*, and *MDM2*. Lomefloxacin could also stimulate melanogenesis under UVA exposure as shown by the increase of tyrosinase activity in normal human melanocytes. Finally, in human keratinocytes, lomefloxacin could trigger apoptosis. Interestingly, clear differences in responses were observed according to cell type. Keratinocytes were the most sensitive whereas melanocytes seemed to be naturally protected by their endogenous melanin.

MATERIALS AND METHODS

Chemicals Culture media for human cells were from Clonetics (Walkersville, MD). Phosphate-buffered saline (PBS) was from Gibco-BRL (New York, NY). The fluoroquinolone lomefloxacin was from Sigma (St Louis, MO).

Monoclonal antibodies directed against cyclobutane thymine dimers were obtained from TNO (Leiden, The Netherlands). Agarose for the comet assay was the low melting Incert Agarose from FMC (Rockland, MA). Excell gels sodium dodecyl sulfate (SDS) from Amersham (Amersham, Buckinghamshire, UK) were used for SDS polyacrylamide gel electrophoresis (PAGE). Nitrocellulose membranes and polyvinylidene fluoride membranes (Hybond, Amersham) were used for protein or DNA transfer, respectively.

Biologic systems Normal human fibroblasts, keratinocytes, and Caucasian melanocytes were neonatal cells from Clonetics or Cascade (Portland, OR). Fibroblasts and keratinocytes were cultured as generally reported (Boyce and Ham, 1983). Melanocytes were cultured in conditions where they can respond to α-melanocyte-stimulating hormone and UV irradiation as described by Im *et al* (1998). When necessary, the number of viable cells was assessed by the 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT) assay as described by the supplier (Roche, Mannheim, Germany) or by cell counting (for tyrosinase activity analysis for instance) using a cell counter (Beckman Coulter, Fullerton, CA).

Light sources and spectral measurement The light source was a solar UV simulator from Oriel (Stratford, CT) equipped with a 1000 W xenon short arc lamp, a dichroic mirror, and an appropriate cut-off filter for UVA (WG335/3 mm cut-off filter). The beam size was 152×152 mm. The incident UV spectrum was analyzed with a spectroradiometer (Instaspec IV, Oriel). Such a spectral power distribution was relatively close to that of UVA in sunlight. Our experiments were thus performed in realistic conditions of irradiation. The average irradiance was measured by integrating the area under the spectrum. Spectroradiometry gave the following fluences: 9 W per m^2 for UVA2 (320–340 nm) and 68.5 W per m^2 for UVA1 (340–400 nm). Thus exposures of 15 min or 30 min correspond to doses of 70 kJ per m^2 or 140 kJ per m^2, respectively.

Irradiation procedure Cells were incubated for 30 min with lomefloxacin in the dark and then exposed to UVA in the presence of lomefloxacin in PBS. When exposure was followed by post treatment incubation, cells were rinsed and covered again with the initial growth medium without lomefloxacin. When the comet assay was performed immediately after exposure, cells were first embedded in an agarose–PBS microgel and irradiated in cold PBS (4°C) in the presence of lomefloxacin.

Experiments were performed at least twice for each cell type.

Comet assay The comet assay was performed as described by Alapetite *et al* (1996). Immediately after irradiation, the 0.5% agarose slide with embedded cells was placed into the lysis buffer (NaCI 2.5 M; ethylenediamine tetraacetic acid (EDTA) 100 mM; Triton X-100 1%; Tris 10 mM pH 10) for 1 h 30 min at 4°C and then washed and equilibrated in alkaline buffer (0.3 M NaOH, 1 mM EDTA). The electrophoresis was performed for 20 min at 25 V and 300 mA in the same buffer in the presence of 1% dimethyl sulfoxide. After neutralization in Tris buffer (pH 7.5), DNA was stained with ethidium bromide (2 μg per ml) and the comets were examined and photographed with a fluorescence microscope. For the quantification, a mean tail moment for 50 analyzed nuclei was calculated using software image analysis (Comet 4.0, Kinetic Imaging, Silver Spring, CO).

Semiquantitative RT-PCR The RNeasy mini kit (Qiagen, Hilden, Germany) was used to produce preparations of total RNA from skin cells as described by the supplier.

First strand cDNA synthesis and PCR were performed using a RoboCycler Gradient 96 Temperature Cycler with Hot Top Assembly (Stratagene, La Jolla, CA). First strand cDNA was synthesized using 5 μg of total RNA as template by extension of 300 ng oligo-dT and the final concentrations of reagents in a 50 μL reaction volume as described by the supplier (ProSTAR First Strand RT-PCR kit, Stratagene). Specific primers to amplify the *GAPDH*, *MDM2*, *GADD45*, and *HO-1* genes were from Genset (Evry, France) and *FAS-L* gene from R&D Systems (Minneapolis, MN). PCR thermocycling conditions for gene expression were set up as follows: 1 cycle of activation at 95°C for 15 min; followed by 25–35 cycles at 94°C for 30 s, 25–35 cycles at 55–62°C for 30 s, and 25–35 cycles at 72°C for 1 min; followed by a final primer sequence extension at 72°C for 10 min.

The sense and antisense primer sequences used were the following (annealing temperature in parentheses): *GAPDH*, 5′-CCACCCATGGCAAATTCCATGGCA-3′ and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (60°C); *MDM2*, 5′-GGCAGGGGAGAGTGATACAG-3′ and 5′-GTCTACATACTGGGCAGGGC-3′ (58°C); *GADD45*, 5′-GGAAGTGTCCAGCAAAGCCC-3′ and 5′-GTCATCTATCTCCGGGCCCC-3′ (58°C); *HO-1*, 5′-CCGCAACCCGACAGCATGCC-3′ and 5′-GCCGCTTCACATAGCGCTGC-3′ (62°C). *FAS-L* (human FasL Primer Pair, from R&D Systems) was amplified using 2 units of Faststart Taq DNA polymerase with an activation at 95°C for 4 min. PCR samples (10 μL) were analyzed on an ethidium-bromide-stained 1% agarose gel. The relative amounts of RT-PCR products were quantified using a bioimaging system Chemigenius (Syngene, Cambridge, UK). In order to rigorously evaluate the modulation of gene expression, we controlled that *GAPDH* expression remained unchanged in fibroblasts and in keratinocytes exposed to UVA in the presence of lomefloxacin (data not shown). As a practical consequence, in all experiments dealing with gene induction presented below, quantification of RT-PCR products was systematically standardized using *GAPDH* expression level measured simultaneously.

Quantitative assessment of thymine dimers Immediately after exposure to UVA and lomefloxacin, DNA was prepared as previously described (Eveno *et al*, 1995). Purified DNA was then loaded on a polyvinylidene fluoride membrane (Hybond-P, Amersham) and subjected to immuno-detection of thymine dimers using monoclonal antibodies as reported elsewhere (Otto *et al*, 1999). Quantification was obtained by scanning the blots on a Fluor-Imager (Molecular Dynamics, Sunnyvale, CA).

Preparation of nuclear extracts and western blot analysis of p53 and p21 proteins At 24 h post exposure, cells were harvested and proteins of the nucleus were extracted according to the method of Lin and Benchimol (1995). Equal amounts of proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Western blot analysis was carried out according to Super-Signal Westpico western blot system instructions (Pierce, Rockford, IL) using the anti-p53 antibody clone OP43 or anti-wafl clone OP64 (Oncogene, Boston, MA).

Assessment of caspase-3 activation Caspase-3 activation was assessed using the CaspACE colorimetric assay (Promega, Madison, WI) as described by the supplier. This test is based on the ability of activated caspase-3 to cleave the peptide Asp-Glu-Val-Asp labeled with p-nitroaniline and to release this chromophore.

Tyrosinase activity Tyrosinase activity was assayed spectrophotometrically by following the oxidation of L-dihydroxyphenylalanine (L-DOPA) to dopachrome at 475 nm (Takahashi and Parsons, 1992). Cells were washed with PBS and lyzed with 200 μL of 1% Triton-X100/PBS. After sonication and vibration, 150 μL of 5 μM L-DOPA was added to 50 μL lysis buffer (or boiled cell lysate). The absorbance values were compared with a standard curve obtained with mushroom tyrosinase (Sigma). The standard curve was linear within the range of experimental values, and there was no increase in absorbency in the control wells.

RESULTS

Photocytotoxicity and photogenotoxicity of lomefloxacin under UVA exposure Cells (fibroblasts, keratinocytes, or melanocytes) were exposed to UVA for 30 min in the presence of increasing lomefloxacin concentrations. Photocytotoxicity was measured 24 h post treatment using the MTT assay. **Figure 1** shows that lomefloxacin plus UVA treatment produced a dose-dependent phototoxic effect in keratinocytes, whereas fibroblasts and melanocytes were either slightly affected or not affected at all. At low concentrations (1 μM or 5 μM) keratinocytes did not grow, whereas at 10 μM they were killed and some cells started to detach 24 h post treatment. Photocytotoxicity could also be observed with fibroblasts and melanocytes but at higher concentrations (over 25 μM), melanocytes being the most resistant cell type (data not shown).

Photoinduction of DNA damage by lomefloxacin was first studied using the comet assay, a simple and visual as well as quantitative technique for measuring DNA breakage in the nuclei of individual cells (Tice *et al*, 2000). Necrosis and apoptosis can induce nonspecific DNA breakage, however, and must be avoided when the comet assay is carried out. Moreover, UVA radiation was also reported to induce breaks in genomic DNA (Alapetite *et al*, 1996; Marrot *et al*, 1999). Thus, in order to lower UVA-induced DNA breakage and to prevent cell death, exposure time was limited to 15 min in comet assays performed here. In such conditions, we controlled that cell integrity was not affected when the comet assay was carried out (immediately after UVA exposure) using the two dyes carboxy-fluorescein diacetate and ethidium bromide as previously described (Strauss, 1991) (data not shown).

Figure 2(*a*) shows the typical aspects of comets for each cell type (fibroblasts, keratinocytes, and melanocytes) after treatment with 20 μM lomefloxacin and a 15 min UVA exposure. In **Fig 2(*b*)**, mean tail moments (based on the comet tail length and its fluorescence intensity) were calculated for increasing lomefloxacin concentrations. A dose-dependent induction of DNA breakage was observed, and here again keratinocytes were the most sensitive cell type. In contrast, melanocytes were less affected under these experimental conditions.

Photoproduction of cyclobutane pyrimidine dimers (CPD) by lomefloxacin exposed to UVA was previously reported in cultured cells using the comet assay after T4 endonuclease V digestion (Traynor and Gibbs, 1999). T4 endonuclease V can also cleave DNA at abasic sites, however, and we preferred to use here a more specific assay based on immunochemical detection of CPD. As our goal was to check whether lomefloxacin could actually produce CPD within genomic DNA of cells, we treated fibroblasts and keratinocytes with relatively high lomefloxacin concentrations (50 μM and 100 μM) without considering photocytotoxicity. DNA was extracted immediately after UVA exposure and CPD were assessed using dot-blot analysis. **Figure 3** shows that CPD were formed after treatment by lomefloxacin plus UVA exposure in both keratinocytes and fibroblasts, keratinocytes being clearly more susceptible. Indeed, 3- to 4-fold more CPD were induced in keratinocytes than in fibroblasts. At present, there is no obvious explanation for such a difference between fibroblasts and keratinocytes. It is worth noting, however, that keratinocytes were also more sensitive than fibroblasts to CPD induction by UVA alone in our experimental conditions.

The next series of experiments focused on specific cell responses. Here our goal was now to mimic realistic phototoxicologic conditions likely to be encountered in human skin and to study the effects of subphototoxic exposures, checking for a possible threshold level above which a specific stress response could be induced.

This is why lower amounts of lomefloxacin were used (up to a maximum of 10 μM) but with longer UVA exposure (30 min) knowing that the UVA fluence from our solar simulator was close to that of sunlight.

Lomefloxacin plus UVA treatment induced the stress gene *HO-1* Lomefloxacin was reported to induce oxidative damage probably due to ROS production during UVA exposure. It was thus expected that it might trigger responses commonly related to oxidative stress. This was demonstrated here by showing the induction of *HO-1* gene up to a maximum of 5- fold *versus* unirradiated background (**Fig 4**). *HO-1* was previously reported to be overexpressed when fibroblasts were exposed to UVA, and *HO-1* induction is considered as part of an adaptive response to oxidative stress (Vile *et al*, 1994). In keratinocytes, however, *HO-1* was shown to be barely expressed (Applegate *et al*, 1995). Similar behavior was observed in our experiments. In fibroblasts *HO-1* induction reached a peak 4 h post treatment, and decreased 24 h after. As expected, no significant *HO-1* expression could be detected in keratinocytes (not shown).

Stimulation of p53 pathway by lomefloxacin plus UVA exposure When DNA is damaged, specific signaling pathways can be stimulated in response to genotoxic stress in order to maintain homeostasis. Among them, the tumor suppressor gene

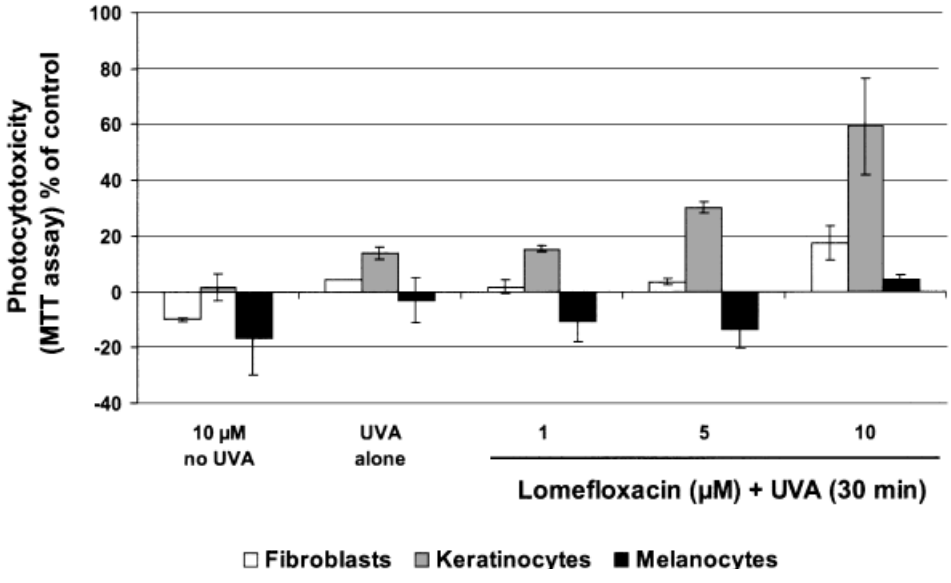

Figure 1. Photocytotoxic impact of lomefloxacin on fibroblasts, keratinocytes, and melanocytes assessed by the decrease of cell viability 24 h post treatment (MTT colorimetric assay).

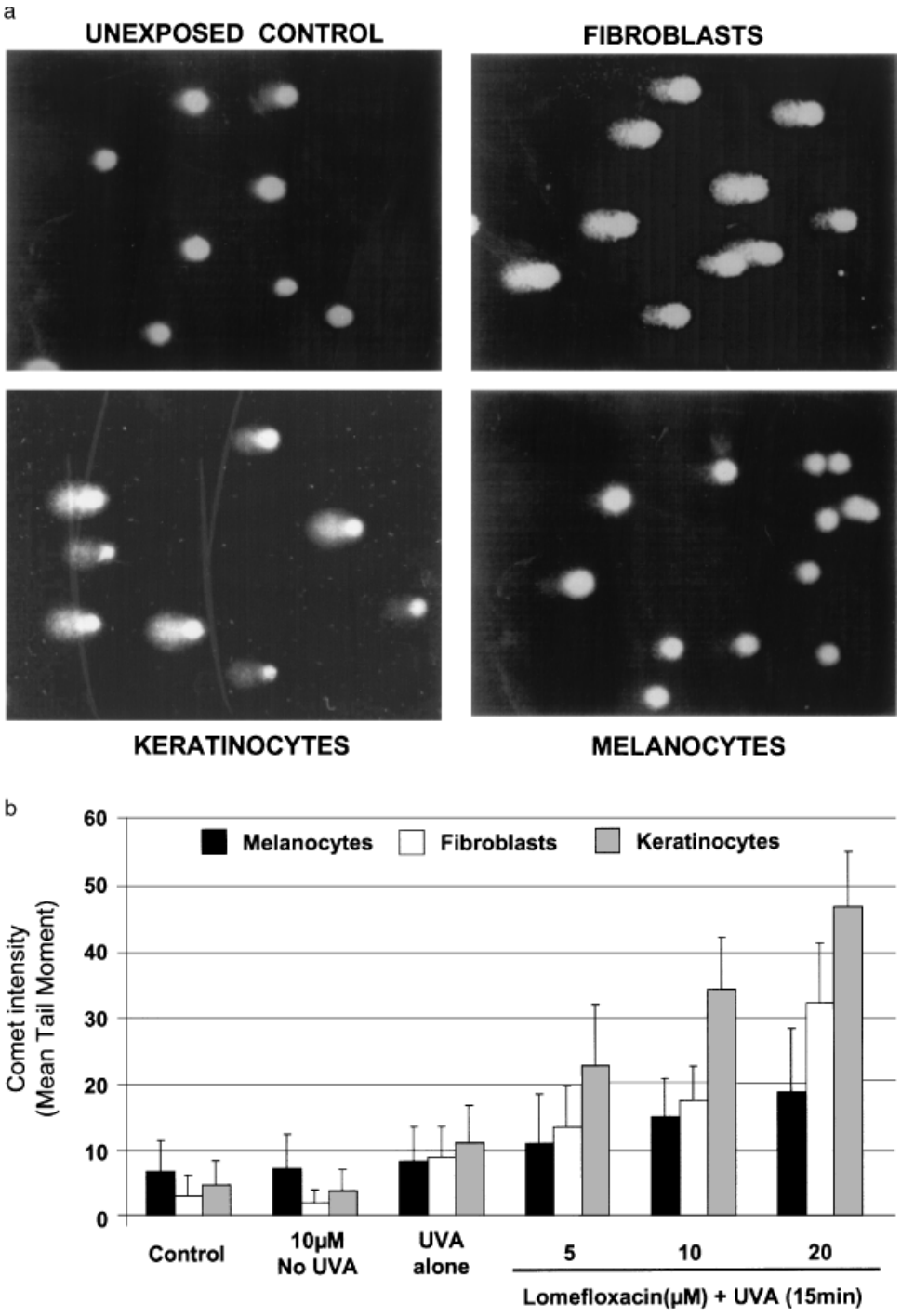

Figure 2. DNA breakage induced by lomefloxacin in nuclei of cells exposed to UVA. (*a*) Typical comets observed immediately after exposure to UVA (15 min) of cells treated by lomefloxacin (20 μM). The figure shows aspects of nuclei in unexposed control (identical for all cell types and for UVA alone), fibroblasts, keratinocytes, and melanocytes. (*b*) Quantification by image analysis (mean tail moments of 50 nuclei, bars represent standard deviation) of comets observed after exposure to UVA (15 min) of skin cells treated with increasing lomefloxacin concentrations.

p53 plays a major role in protecting genome integrity (Hall and Lane, 1997; Levine, 1997).

Figure 5 confirms that in cells treated with lomefloxacin and exposed to UVA, p53 was stabilized as detected by western blot analysis and densitometry of the autoradiography. p53 accumulated differently in amount and kinetics according to cell type. Interestingly, in keratinocytes, p53 stabilization was the highest 24 h post exposure for the three concentrations studied, whereas in fibroblasts it was relatively similar for 4–24 h post treatment for 10 μM but it did not occur for 1 μM. This difference in kinetics and intensity could reflect the higher level of CPD in genomic DNA of keratinocytes as it is well established that the delay for CPD repair can exceed 24 h in skin (Young *et al*, 1996). Moreover, in melanocytes, p53 never accumulated at a significant rate, even at 10 μM lomefloxacin. This result is consistent with the low genotoxic effect in pigment cells observed above using the comet assay.

Figure 5 shows that the p21 protein accumulated as well 8 h and 24 h post exposure in keratinocytes and fibroblasts, suggesting that p53 was controlling the cell cycle in response to lomefloxacin-induced DNA damage. In keratinocytes, however, a decrease in p21 accumulation was observed 24 h after exposure to 5 μM and more clearly to 10 μM lomefloxacin, suggesting that induction of the apoptotic pathway replaced cell cycle arrest in such stress conditions.

MDM2 gene contains a p53 binding site and its expression increases in the presence of wild-type p53 (see Lane and Hall, 1997, for a review). It is thus an interesting marker to

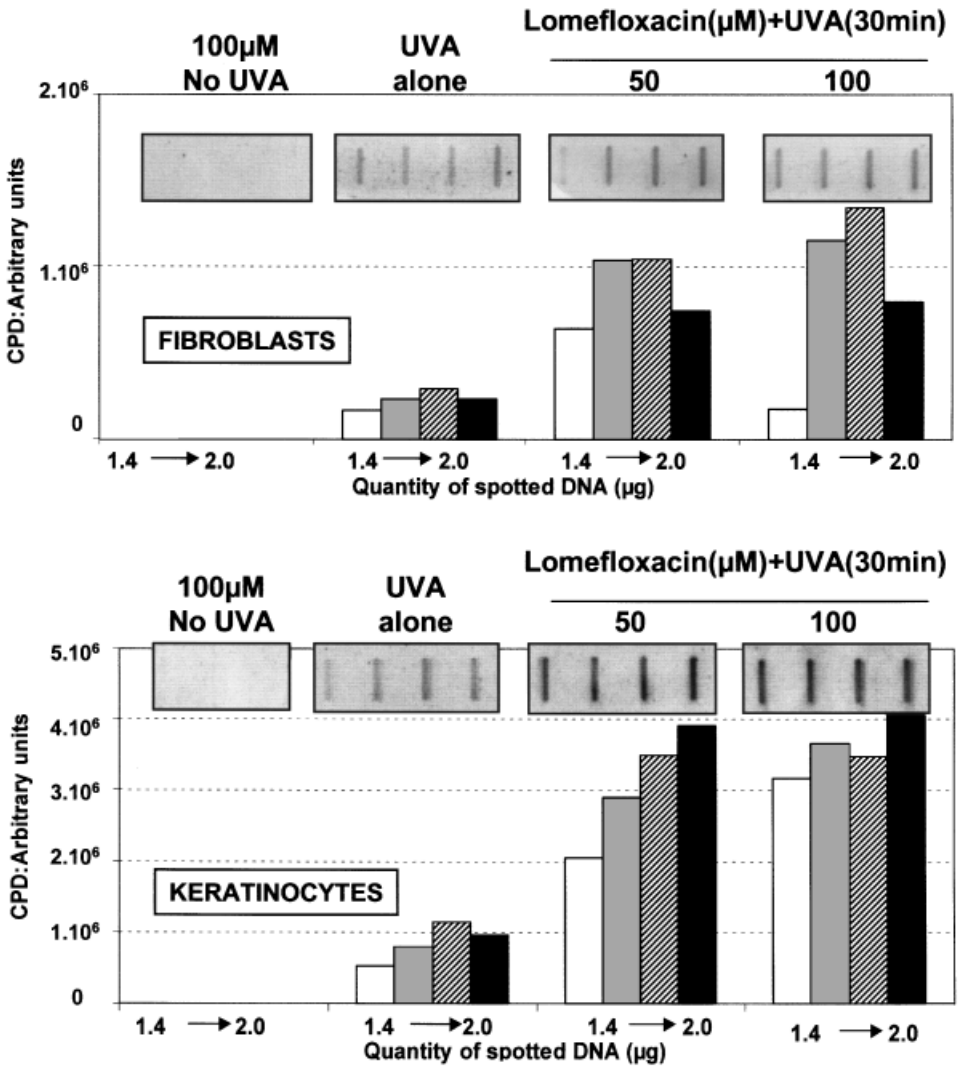

Figure 3. Detection of thymine dimers by dot-blot analysis in genomic DNA isolated from fibroblasts (*upper part*) or from keratinocytes (*lower part*) immediately after treatment by lomefloxacin (50 and 100 μM) and exposure to UVA (30 min). The amounts of DNA spotted on the membrane were 1.4, 1.6, 1.8, and 2 μg.

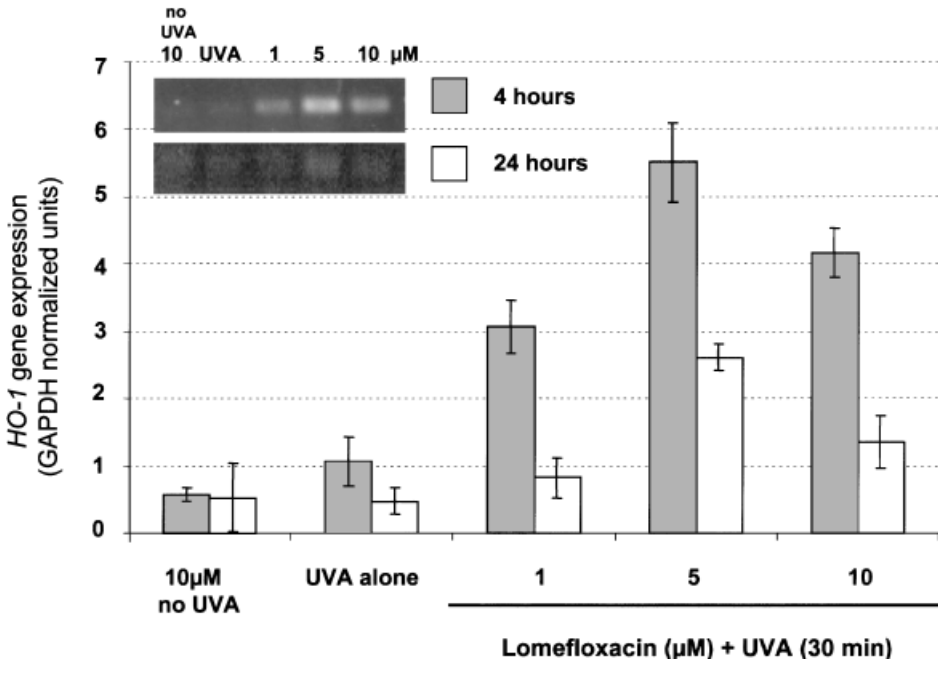

Figure 4. Expression of *HO-1* gene in fibroblasts treated by lomefloxacin and exposed to UVA (30 min) assessed at 4 h and 24 h post treatment. Quantification (mean value from two independent experiments, bars represent standard deviation) was standardized with *GAPDH* expression measured simultaneously.

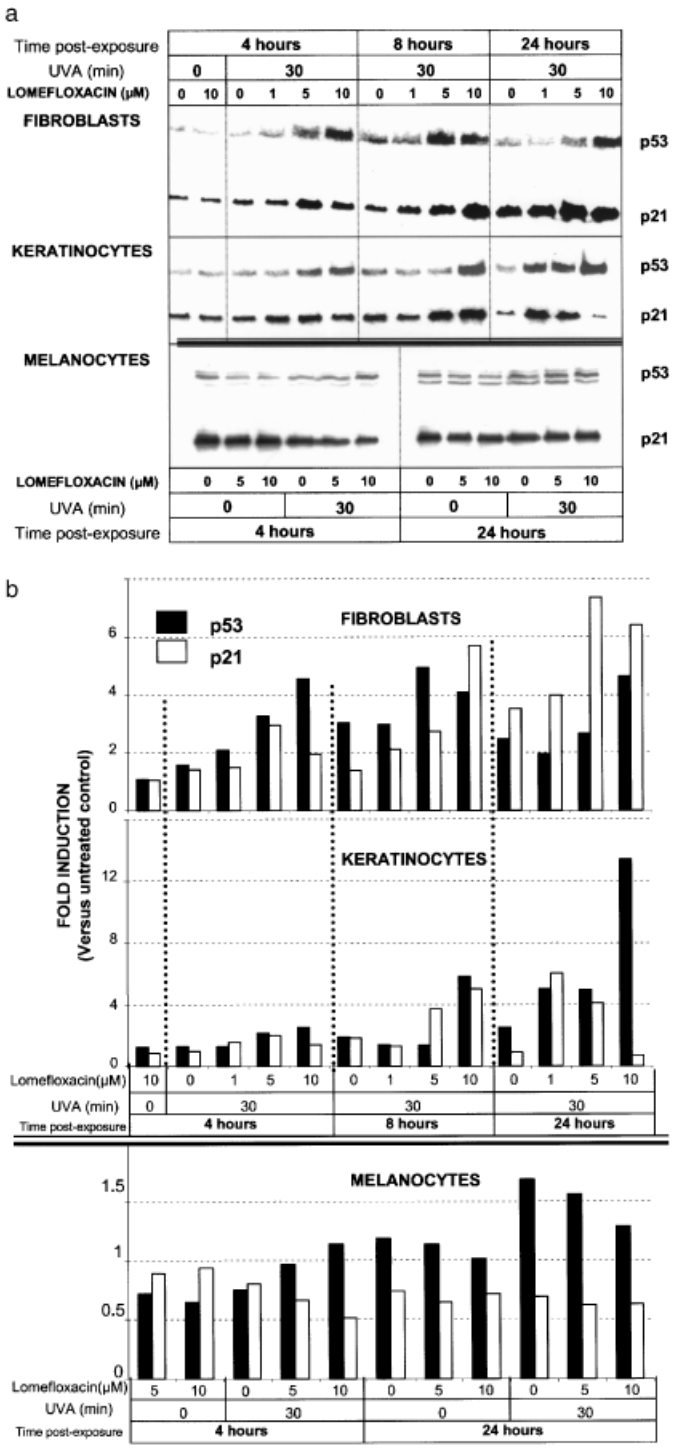

Figure 5. P53 status in cells treated by lomeflaxacin and exposed to UVA. (*a*) Western blot detection of p53 and p21 proteins in fibroblasts, keratinocytes, and melanocytes treated with lomefloxacin and exposed to UVA (30 min). Proteins were extracted from nuclei 4 h, 8 h (except for melanocytes), and 24 h post exposure. (*b*) Densitometric analysis of p53 and p21 bands scanned from the western blot shown in (*a*). Values represent the ratio between assays and untreated control for each cell type.

demonstrate the activation of the p53 pathway. **Figure 6** shows that in fibroblasts and in keratinocytes *MDM2* was induced 24 h post treatment whereas no significant expression was observed after 4 h. This result strongly suggests that lomefloxacin exposed to UVA not only stabilized p53 protein but also led to activation of the p53 pathway. **Figure 7** confirms this hypothesis, taking *GADD45* expression as another endpoint to assess p53 activation. *GADD45* is generally reported to be *trans*-activated by p53 in response to DNA damage induction. In this study, the induction was stronger in keratinocytes than in fibroblasts and, in both cases, was detectable from 4 h to 24 h post exposure although less intensely in fibroblasts after 24 h.

Exposure to lomefloxacin plus UVA can trigger apoptosis in human keratinocytes Apoptosis can be an ultimate response to various stresses. It involves a large number of biochemical processes such as caspase activation or Fas/Fas-L interaction.

Caspases are proteins that play an essential role during apoptotic cell death: once activated, they cleave various essential cellular components. Caspase-3 has a central role in this process and is considered as the main activator of apoptotic DNA fragmentation. Its involvement in UV-induced apoptosis has been previously described (see Cohen, 1997, for a review).

FAS-L expression was previously reported to participate in DNA-damage-induced apoptosis and is induced in response to different kinds of stress (Leverkus *et al*, 1997; Pinkoski and Green, 1999). Moreover, it was recently published that, in skin, sunburn cell formation required Fas and Fas-L interaction (Hill *et al*, 1999). Using both biologic markers, we observed that keratinocytes (but not fibroblasts) underwent apoptosis after UVA exposure in the presence of lomefloxacin. **Figure 8** shows that caspase-3 was activated from 8 h post exposure (peak of activation) up to 24 h using 10 μM lomefloxacin plus UVA. It can be noticed that a slight activation could even be detected with 5 μM lomefloxacin. In **Fig 9**, *FAS-L* gene induction confirmed the stimulation of apoptosis in keratinocytes and this induction occurred as early as 8 h post treatment as observed with caspase-3. Here again, no significant changes could be observed in fibroblasts even with increasing lomefloxacin concentration up to 20 μM (not shown).

Exposure to lomefloxacin plus UVA can stimulate pigmentation in human melanocytes Melanogenesis is considered as a defense mechanism against various stresses and it was proposed that DNA damage can trigger pigmentation (Eller *et al*, 1996). **Figure 10** shows that exposure to lomefloxacin plus UVA clearly changed tyrosinase status within melanocytes from two Caucasian donors having different basal tyrosinase activity. Five days after treatment with lomefloxacin (up to 10 μM), stimulation of dopa-oxidase activity of tyrosinase was observed. In parallel, a decrease in cell proliferation assessed by cell counting was evidenced, probably as a consequence of growth arrest as no cell death was observed (see **Fig 1**). This finding is consistent with the assumption that pigmentation can be a peculiar defense mechanism of melanocytes against phototoxic processes.

DISCUSSION

This study shows that lomefloxacin could induce DNA breaks, produce pyrimidine dimers via photosensitization, and lead to cell growth arrest or cell death. In addition, striking differences were observed depending on cell type for each endpoint recorded. Keratinocytes were the most sensitive cells, melanocytes the most resistant ones, and fibroblasts showed intermediate sensitivity (see **Table I**).

As DNA damage was assessed immediately after treatment, these results cannot be explained by differences in DNA repair kinetics, at least if one considers the nucleotide excision repair pathway. Repair of pyrimidine dimers would take more than 24 h to be completed (Young *et al*, 1996); it is thus unlikely that significant differences would occur during the 30 min UVA exposure. Moreover, neonatal cells were used here, which excluded a potential relationship with repair efficacy as a function of age of the donor (Goukassian *et al*, 2000), even if some individual

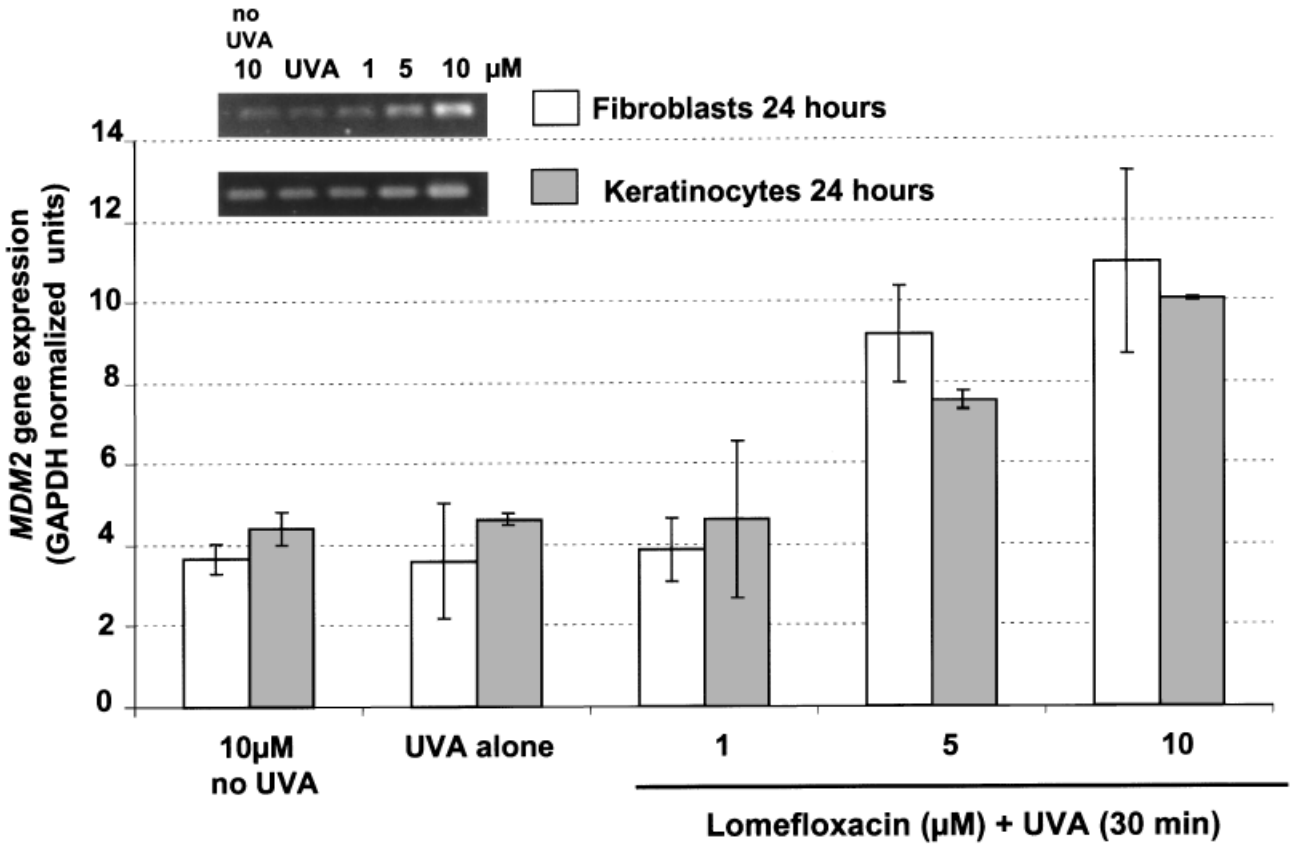

Figure 6. Expression of the *MDM2* gene in fibroblasts and keratinocytes 24 h after treatment with lomefloxacin and UVA (30 min). Quantification (mean value from two independent experiments, bars represent standard deviation) was standardized with *GAPDH* expression measured simultaneously.

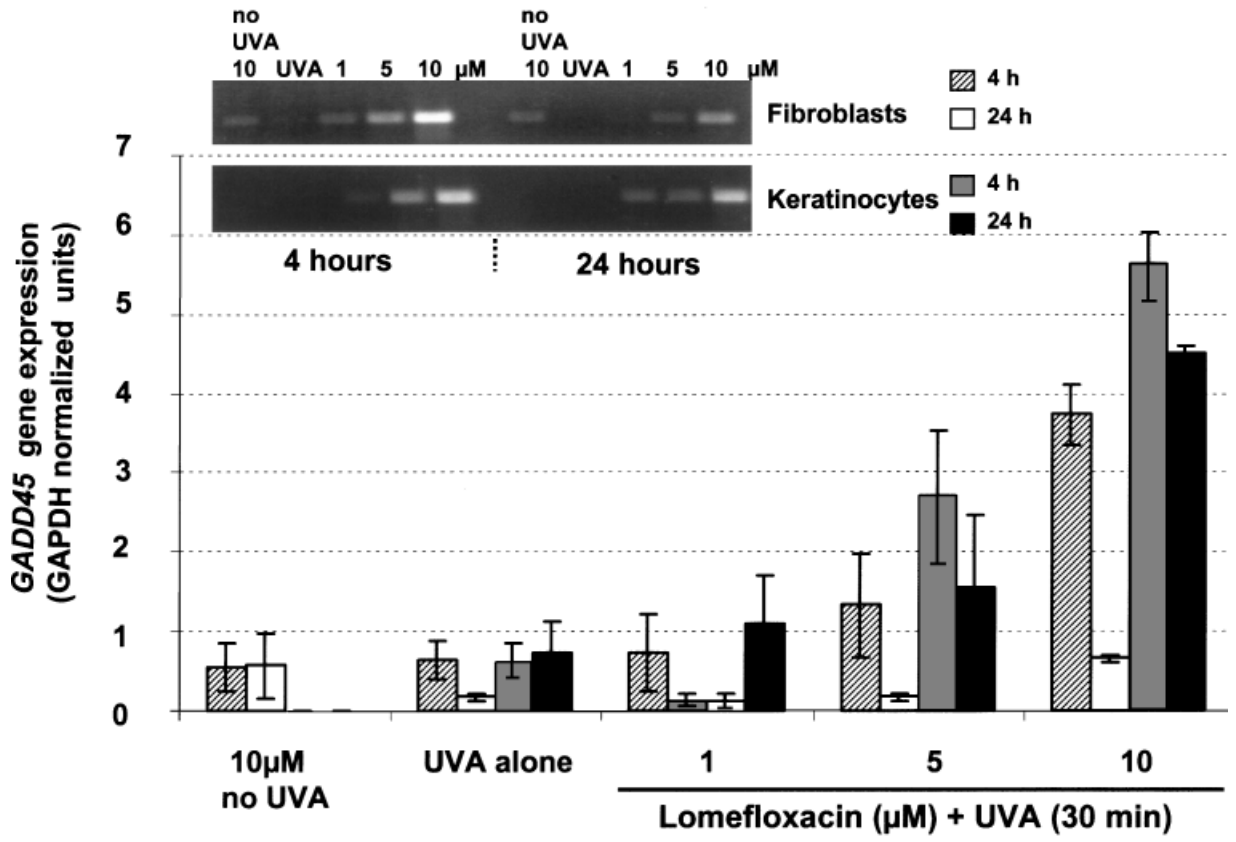

Figure 7. Expression of *GADD45* gene in fibroblasts and keratinocytes 4 h and 24 h after treatment with lomefloxacin and UVA (30 min). Quantification (mean value from two independent experiments, bars represent standard deviation) was standardized with *GAPDH* expression measured simultaneously.

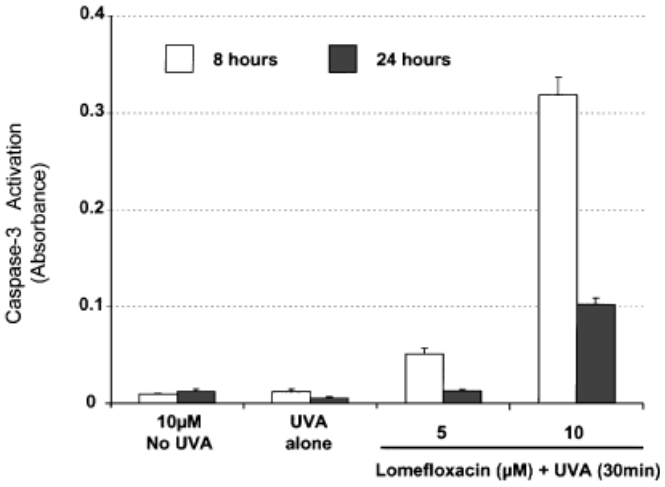

Figure 8. Activation of caspase-3 in keratinocytes treated with lomefloxacin and exposed to UVA (30 min). Proteins were extracted from cells 8 h and 24 h post exposure and assessed for caspase-3 activity using a colorimetric assay as described in *Materials and Methods.*

differences may exist (Bykov *et al*, 1999). It appears more likely that lomefloxacin uptake, bioavailability within cells (in particular in the nucleus), or potential metabolic activation (or inactivation) may play a role. In fact, it was recently published that microsomal metabolism of ciprofloxacin (a fluoroquinolone comparable in structure to lomefloxacin) generated free radicals (Gürbay *et al*, 2001). Lomefloxacin mainly accumulates in lysosomes (Ouedraogo *et al*, 1999), but the fraction that escapes these organelles and that is capable of inducing damage elsewhere is still unknown. It would be interesting to study "dark" effects of lomefloxacin during longer preincubation times or the effects of exogenous metabolic activation.

This study also showed that endogenous cellular defense against photosensitized lomefloxacin-induced oxidative stress varied from one cell type to another. The most obvious difference was associated with the presence of melanin. Melanin is considered as a double-edged sword because, on one hand, it can be activated by light and produce reactive species but, on the other hand, it behaves as a scavenger of free radicals (Hill *et al*, 1997). Moreover melanin can bind various organic molecules (Mars and Larsson, 1999) including fluoroquinolones (Fukuda *et al*, 2000). The fact that melanocytes appeared more resistant in our experiments is therefore not really surprising. Once bound to melanin, lomefloxacin is no longer available (or less available) for interacting with DNA in the nucleus, but one cannot exclude that this storage could finally be deleterious, if the drug is released from the pigment as previously reported for the fluoroquinolone sparfloxacin (Hamanaka *et al*, 1999). Melanocytes were sensitive to the stress produced by lomefloxacin exposed to UVA, however, as melanogenesis was stimulated. Tyrosinase activity (evaluated in our experiments by dopa-oxidase activity), which controls melanogenesis, can be modulated by post-translational modifications of the enzyme in response to exogenous genotoxic stimuli. It was recently suggested that activation of melanogenesis could be controlled by p53 (Nylander *et al*, 2000; Khlgatian *et al*, 2002). But tyrosinase is also influenced by the redox status within cells and O_2 was shown to be a better substrate than O_2 (Wood and Schallreuter, 1991; Schallreuter *et al*, 1994). Production of ROS by photosensitized lomefloxacin could thus have a direct impact on melanogenesis.

The striking difference observed between keratinocytes and fibroblasts is a bit more unexpected. First, it was reported elsewhere that the same UVB dose induced less CPD in keratinocytes than in fibroblasts (Otto *et al*, 1999). Opposite results are observed here, but mechanisms of direct CPD induction by UV and CPD photosensitization by lomefloxacin might be different. A tight interaction between the drug (or its photoproducts) and DNA is necessary in order to ensure the transfer of the photonic energy. Why such an interaction could be facilitated in keratinocytes remains to be elucidated.

DNA strand breakage detected by the comet assay immediately after exposure was higher in keratinocytes than in fibroblasts. Assuming that most of this breakage was due to the cleavage of the DNA phosphodiester backbone by ROS, one might question the antioxidant status in each cell type. Published data show that the epidermis (or keratinocytes) has better antioxidant defenses than dermis (or fibroblasts). In fact, glutathione, antioxidant enzymes such as superoxide dismutase, or constitutive heme-oxygenase-2 contents are higher in epidermal cells (Shindo *et al*, 1994; Applegate *et al*, 1995; Niggli and Applegate, 1997; Leccia *et al*, 1998) although glutathione peroxidase is sometimes present at higher concentrations in fibroblasts from different human donors (Moysan *et al*, 1995). Thus, the endogenous level of antioxidants cannot explain our results.

One could also think that repair of DNA oxidative lesions occurred faster in keratinocytes resulting in a large induction of incisions in DNA (or of alkaline-sensitive sites such as abasic sites) in a short time. It is well established that most oxidative damage (such as 8-oxo-guanine) is processed by the base excision repair

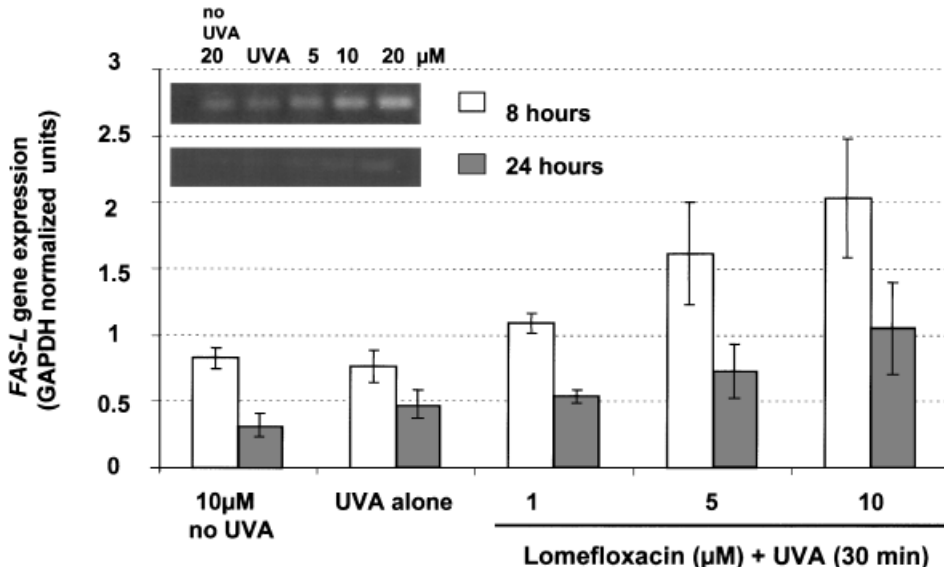

Figure 9. Expression of the *FAS-L* gene in keratinocytes treated with lomefloxacin and exposed to UVA (30 min) assessed 8 h and 24 h post exposure. Quantification (mean value from two independent experiments, bars represent standard deviation) was standardized with *GAPDH* expression measured simultaneously.

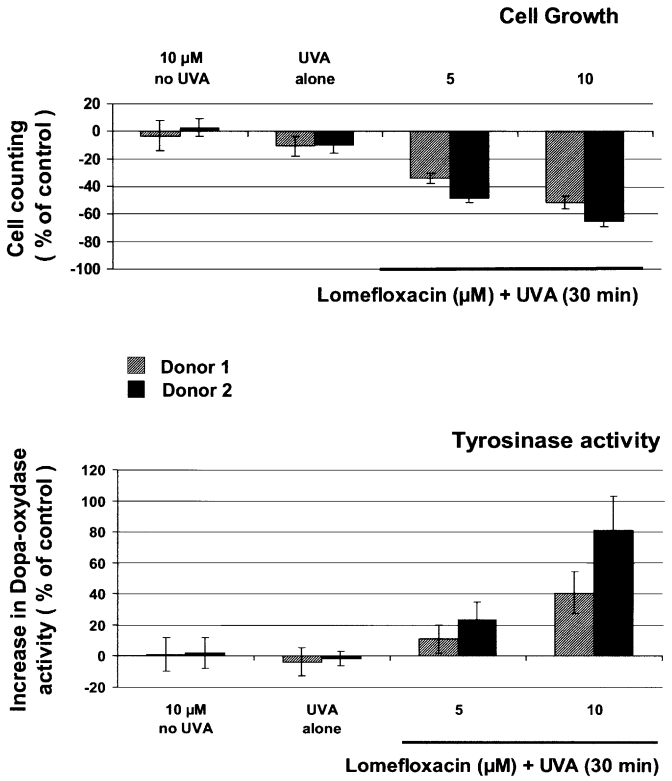

Figure 10. Impact of lomefloxacin and UVA (30 min) on cell growth and dopa-oxidase activity of endogenous tyrosinase in melanocytes from two Caucasian donors 5 d post exposure.

Table I. Summary of responses observed in the three human cell types photosensitized by lomefloxacin and UVA

	Keratinocytes	Fibroblasts	Melanocytes
MTT assay	High photocytotoxicity	Low photocytotoxicity	No photocytotoxicity up to 25 µM
Comet assay	+ + +	+ +	+
CPD formation	+ +	+	ND
p53 accumulation	+ + (particularly at 24 h)	+	0
p21 accumulation	+ (0 at high concentrations at 24 h)	+	0
MDM2 induction	+ +	+ +	ND
GADD45 induction	+ + (4 h and 24 h)	+ (4 h)	ND
HO-1 induction	0	+ (4 h)	ND
FAS-L induction	+	0	ND
Caspase-3 activation	+ (8 h)	0	ND
Tyrosinase activation	ND	ND	+

ND, not done.
+ to + + + corresponds to increasing responses.

pathway, which is completed faster than nucleotide excision repair. It is thus likely that DNA incisions start to be induced by repair enzymes during UVA exposure. In line with this, comets observed immediately after treatment were more intense in keratinocytes. But we also noticed that p53 accumulation and *GADD45* expression lasted much longer in keratinocytes than in fibroblasts. Oxidative DNA photolesions induced by UVA-exposed lomefloxacin were probably shortly repaired whereas bulky lesions, such as CPD, were repaired much more slowly. It is thus possible that a higher induction of both types of DNA lesion combined with differences in repair kinetics could influence the behavior of keratinocytes in our experiments.

Finally, the marked photocytotoxicity of lomefloxacin in keratinocytes could be explained by the ability of these cells to undergo apoptosis as shown by caspase-3 activation and *FAS-L* induction. In fact, p53 was reported to trigger apoptosis in highly damaged cells, depending, however, on a variety of factors such as biochemical environmental conditions and cell type (Polyak *et al*, 1997). As it was observed in this study that the p53 pathway was triggered, p53-dependent stimulation of apoptosis might be stimulated by UVA-photosensitized lomefloxacin. The induction of CPD could also greatly contribute to apoptosis, as it does in epidermis when sunburn cells are formed after UVB exposure. In fact, the formation of sunburn cells (apoptotic keratinocytes) requires Fas and Fas-L interaction: Fas and Fas-L may serve as external pro-apoptotic sensors of DNA-damage-mediated cellular proof-reading (Hill *et al*, 1999) and DNA damaging agents have been shown to induce expression of *FAS-L* via the activation of AP1, a transcription factor for various stress genes. Moreover, it was reported that photosensitized lomefloxacin could also induce a marked delayed destabilizing effect on mitochondrial membrane (Ouedraogo *et al*, 2000) and it is established that the alteration of mitochondrial membrane is an important step in apoptosis development (see Kroemer and Reed, 2000, for a review). The activation of caspase-3 followed by keratinocyte cell death could result from this process. Interestingly, lomefloxacin was reported to trigger apoptosis in retinal cells of albino BALB/c mice but not in pigmented DBA/2 mice under UVA exposure (Shimoda and Kato, 1999). In addition to the possible protective role of melanin previously discussed, these published data also underline that apoptosis could occur in response to lomefloxacin phototoxicity.

In addition to this information about molecular processes, our results also raise the question of the biologic endpoints used for phototoxicity assessment. Reliable evaluation of photocytotoxicity is needed for early detection of the photodamaging potential of photo-unstable compounds. Neutral Red uptake using 3T3 fibroblasts was recently validated as an alternative to animal testing in this regard (Spielmann *et al*, 1998). Our data suggest that cell death cannot be the sole endpoint to assess phototoxicity, however, as significant amounts of DNA lesions (enough to activate stress genes) could be induced at subphotocytotoxic doses. These results also stress the fact that photosensitivity can vary from one cell type to another. For instance, normal human keratinocytes, which are the first and prevalent targets of xenobiotics in skin, may respond differently compared to fibroblasts (normal human cells or 3T3 cell line) in culture.

Finally, the choice of suitable references for phototoxicity assessment is an important issue. 8-Methoxy-psoralen (8-MOP) can be regarded as a very suitable molecule because therapeutic use gave rise to numerous clinical studies about its phototoxicity and its possible implication in photocarcinogenesis (Gasparro *et al*, 1998; Lindelöf *et al*, 1999). According to its chemical structure, however, 8-MOP interacts in a very peculiar way with DNA. It induces photolesions such as bulky mono-adducts and interstrand crosslinks that have a strong genotoxic impact. As a consequence, the molecular responses due to lesions such as strand breaks or oxidative damage could not be studied using 8-MOP. 8-MOP can thus hardly be the only reference when one wants to assess the phototoxicologic potential of various chemicals. In this respect, lomefloxacin appears to provide another interesting and complementary candidate: it also increases the risk of photocarcinogenesis in mice (Klecak *et al*, 1997), but it induces DNA damage of a type that is more frequently encountered in phototoxicity. Considering the increasing pattern of our understanding of the mechanisms involved in its photoreactivity/phototoxicity, lomefloxacin might be a convenient reference, together with 8-MOP, to assess the phototoxic potential of new drugs.

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