Differences in Pyrimidine Dimer Removal between Rat Skin Cells In Vitro and In Vivo

Erik Mullaart, Paul H. M. Lohman, Ph.D., and Jan Vijg, Ph.D.
The TNO Institute for Experimental Gerontology, and the TNO Medical Biological Laboratory, Rijswijk, The Netherlands

Pyrimidine dimers, the most abundant type of DNA lesions induced by ultraviolet light (UV), are rapidly repaired in human skin fibroblasts in vitro. In the same cell type from rats, however, there is hardly any removal of such dimers. To investigate whether this low capacity of rat skin cells to repair lesions in their DNA is an inherent characteristic of this species or an artifact due to cell culturing, we measured the removal of UV-induced pyrimidine dimers from rat epidermal keratinocytes both in vitro and in vivo. Epidermal keratinocytes in vitro were unable to remove any dimers over the first 3 h after UV-irradiation, while only about 20% was removed during a repair period of 24 h. In this respect, these cells were not different from cultured rat fibroblasts. In contrast to the results obtained with keratinocytes in vitro, we observed a rapid repair of pyrimidine dimers in UV-irradiated keratinocytes in vivo over the first 3 h; this rapid repair phase was followed by a much slower repair phase between 3 and 24 h. These results are discussed in terms of the possibility that mammalian cells are able to switch from one DNA repair pathway to another. J Invest Dermatol 90:346–349, 1988

The persistency of ultraviolet (UV) induced pyrimidine dimers in cultured rodent cells is well documented [1–5]. This persistency is not likely to be due to an inherent DNA repair defect of rodent cells, as can be deduced from data on the survival of such UV-irradiated cells, which is not lower than that of human cells under comparable conditions [2,4]. In addition, the amount of unscheduled DNA synthesis performed by rodent cells over a given time period is too high to match the low number of dimers removed [1,4,5]. Interestingly, it has been shown by Peleg et al [6], and more recently by La Belle and Linn [7], that early passage embryonic mouse cells remove pyrimidine dimers well. These findings suggest that in rodents the excision repair pathway(s), similar to those via which UV damage is repaired in human cells, can be activated but are not always fully utilized, possibly due to changes in the expression of specific DNA repair genes during embryonic development [6].

Recently, we showed that UV-induced pyrimidine dimers in early passage fibroblasts from rat embryos were as persistent as those in cells from adult rats [8]. In this regard it is conceivable that rat fibroblasts lose their capacity to remove dimers upon establishment into culture. In order to test this possibility, it is necessary to measure the induction and removal of pyrimidine dimers in cells in vivo, in comparison to the same cell type cultured in vitro. In principle, such a comparative study can be performed by using the UV-endonuclease enzymatic method [9]. However, a serious disadvantage of this method is its dependence on radioactively labeled DNA for the analysis of the breaks induced by the enzyme, which involves centrifugation through alkaline sucrose gradients. This limits its application to growing cells cultured in vitro, the DNA of which can be labeled during S-phase with radioactive precursors. Modifications that circumvent the need of detection by radioactivity and therefore allow application of the alkaline sucrose gradient centrifugation technique to nondividing cells or freshly isolated tissues, are based on the detection of DNA in the gradient fractions by fluorescent staining [10,11]. Unfortunately, this modification makes the method rather time-consuming, because the DNA in each gradient fraction has to be precipitated, stained, and spectrophotometrically quantified. An alternative is the detection of UV-endonuclease sensitive sites by alkaline agarose gels [12].

Recently, a rapid and sensitive method for the determination of nonradioactively labeled alkaline sucrose gradient DNA profiles was developed [13]. It is based on the fractionation of gradients in the wells of plastic microtiter plates and the subsequent covariant labeling of DNA adsorbed to the walls by reaction of the guanines with N-acetoxy-2-acetylaminofluorene (N-AcO-AAF). The treated DNA is then quantified by means of an enzyme-linked immunosorbent assay (ELISA) [14] with specific antibodies of high affinity towards dG-AAF. The method was found suitable for the accurate and sensitive detection of pyrimidine dimers induced in vitro as well as in vivo in rat skin cells by irradiation with UV. Further improvement was obtained when antibodies with a high affinity for DNA became available, which made the direct immunochromatographic detection of DNA possible without the necessity of prelabelling.

The availability of the above described method allows an unbiased comparison of the repair of pyrimidine dimers in skin cells in vitro with that in vivo. Here we show that epidermal cells in vivo repair pyrimidine dimers rapidly, whereas in the same cell type in
in vitro these UV-induced lesions are as persistent as in cultured fibroblasts.

**MATERIALS AND METHODS**

**Cell Isolation and Culture** Epidermal keratinocytes were isolated and cultured as described by Rheinwald and Green [15], with some modifications. A skin biopsy specimen was taken from the back of a narcotized rat (female WAG/Rij), and the epidermis was separated from the dermis by overnight floating (with the horny layer up) on 0.25% trypsin, 5 mM EDTA in PBS at 4°C. After this period the epidermis was removed and an epidermal cell suspension was obtained after thorough disaggregation by pipetting up and down. The cells were seeded on top of a feeder layer of lethally irradiated (20 Gy of 60Co gamma rays) rat fibroblasts. The cells were cultured in Dulbecco's modification of Eagle's medium (DEEM, Gibco) supplemented with 10% fetal calf serum, hydrocortisone (0.4 ng/ml), and cholera toxin (0.1 nM). Epidermal growth factor (10 ng/ml) was added after 2 days. The cells were used for repair experiments within 10 days after isolation.

Rat fibroblasts were obtained from the dermis of the same skin biopsy specimens as described earlier [5] and used between passage 5 and 10 as an exponentially dividing population [5].

**Determination of Pyrimidine Dimers after Irradiation In Vitro** The keratinocytes were labeled with [3H]thymidine (1 μCi/ml, 56.5 mCi/m mole, Amersham) and the fibroblasts with [3H]thymidine (1 μCi/ml, 25 Ci/m mole, Amersham). After 3 days of labeling and 1 day of incubation in nonradioactive medium, the medium was removed and the cells were washed with PBS. The cells were irradiated on ice with UV-B by using a Philips TL-20/12 fluorescent sunlamp at a fluence rate of 11 W/m², through a Schott 5-mm WG 305 filter (mimicking the horny layer). The fluence rate of the lamp was determined, before the filter, by means of a radiant flux meter (Hewlett Packard). During irradiation the cells were just covered with PBS containing 1 mg/ml glucose. After irradiation, the cells were incubated with fresh medium for 0, 3-, and 24-h periods, washed with PBS and immediately frozen on dry ice. The feeder layer was removed from the keratinocyte culture by rinsing thoroughly with PBS containing 0.02% EDTA before freezing. The cells were lysed in 0.5% (wt/v) SDS, 150 mM NaCl, 10 mM EDTA, 20 mM Tris (pH 7.6), and 0.5 mg/ml protease K for 1 h at 37°C. After isolation by phenol extraction, DNAs from identically treated keratinocyte and fibroblast samples were mixed, and the numbers of dimers were determined, after incubation of the DNA with a *Micrococcus luteus* UV-endonuclease, by alkaline sucrose gradient centrifugation as described earlier [9,16]. In this way the number of dimers could be compared on the basis of the two distinguishable sedimentation profiles in the same gradient.

**Determination of Pyrimidine Dimers after Irradiation In Vivo** For the determination of pyrimidine dimers in vivo, a narcotized rat (female WAG/Rij) was shaved and irradiated with the Philips sunlamp for various periods of time (up to 10 min). Skin biopsy specimens 10 mm in diameter were taken and the epidermal cells were isolated as described above. To measure the removal of pyrimidine dimers from rat skin, biopsies were performed at 0, 3, and 24 h after UV-irradiation. During the repair period the rats were kept in the dark to exclude the possibility of photoreactivation repair.

The epidermal cells were lysed in 0.5% (wt/v) SDS, 150 mM NaCl, 10 mM EDTA, 20 mM Tris (pH 7.6), and 0.5 mg/ml protease K for 3 h at 55°C under continuous rocking. After isolation of the nonradioactively labeled DNA by means of phenol extraction, the number of ESS was determined essentially as described earlier [13], but with some modifications. After incubation with the *M. luteus* extract, the DNA was treated with protease K (1 mg/ml) for 1 h at 37°C and subsequently extracted once with 2 volumes of phenol, saturated with 0.2 M Tris- HCl (pH 7.6), followed by overnight dialysis against UV-endo buffer [16] to remove the phenol. The number of ESS was determined by alkaline sucrose gradient centrifugation as described earlier [13].

**Gradient Fractionation and Detection of DNA** The procedures for fractionation, liquid-scintillation counting, and computer analysis of the DNA distributions obtained with radioactively labeled cells have been described [16]. Nonradioactive detection of DNA profiles was essentially as described earlier [13], but with some modifications. After centrifugation, the sucrose gradients were fractionated into 96-well polystyrene microtiter plates (Greiner) at 8 drops (about 200 μl) per well and neutralized by adding 60 μl of neutralization buffer (1.3 M K2HPO4, 1.7 M Na2HPO4; pH 6.9). Directly thereafter, 100 μl was taken out of each well and spotted in a new microtiter plate that had previously been coated with poly-L-lysine (1 μg/ml PBS, overnight at 4°C). Subsequently, the DNA was allowed to adsorb to the surface of the coated wells overnight at room temperature. The amount of DNA bound to the wells was detected by means of an enzyme-linked immunosorbent assay (ELISA) [14] involving a mouse monoclonal antibody against ssDNA.

**Figure 1.** Time course of the removal of ESS from the DNA of rat fibroblasts (triangles) and epidermal keratinocytes (squares) in vitro after irradiation with 4000 J/m² of UV-B. Under the experimental circumstances such as dose induces about 180 ESS/10⁹ dalton DNA. Each point is the mean of 3 determinations. Bars represent the standard deviations.

**Figure 2.** Dose-response curve for the induction of ESS by UV-B in epidermal keratinocytes in vivo. Points indicate the mean of determinations on three different rats. Bars represent the standard deviations.
RESULTS

When mammalian skin is irradiated with UV, the radiation will not penetrate deeply. In human skin, for example, only about 20%–30% of UV-B will reach the dermis; fibroblasts are thus not likely to be severely damaged [17,18]. However, the thin upper layer of epidermal keratinocytes is highly susceptible to the genotoxic effects of UV [18]. Thus, epidermal keratinocytes are the relevant cells in the skin for studies with respect to the induction and removal of UV-induced pyrimidine dimers in vivo.

Unfortunately, most studies on dimer removal in mammalian cells, including our own [5], have been performed with cultured fibroblasts. In cultured rodent fibroblasts, UV-induced pyrimidine dimers are rather persistent. It was unknown, however, whether this well-documented persistency would also be a characteristic of cultured keratinocytes. In view of the intended comparison between the in vivo and the in vitro situation we were obliged to first address this problem.

For an optimal comparison of DNA repair in fibroblasts with that in keratinocytes, these cells were separately isolated from a rat skin biopsy specimen and subsequently metabolically labeled with [3H]thymidine and [14C]thymidine, respectively. Figure 1 clearly shows that there is no difference in dimer removal between fibroblasts and keratinocytes after irradiation in vitro with UV-B. In neither of the two cell types was there any significant repair over the first 3 h, while after 24 h only 20% of the dimers was removed. These DNA repair time courses were completely identical to that found for rat fibroblasts after irradiation with UV-C [5]. The lack of difference in repair between keratinocytes and fibroblasts is in agreement with the results of Taichman and Setlow with cultured human cells [19].

For the determination of pyrimidine dimers in the DNA of epidermal keratinocytes irradiated in vivo with UV-B we used our recent modification of the UV-endonuclease assay [13]. We have shown that for rat fibroblasts, radioactivity gradient profiles coincide well with immunochemical ones [13]. Furthermore, dose-response and DNA repair time-course studies on UV-irradiated radioactive and nonradioactive rat fibroblasts using the original assay and our modified method, respectively, yielded identical results (unpublished data). As shown in Fig 2, in this present study a linear dose dependency was found when epidermal keratinocytes were irradiated in vivo with UV-B. The detection limit was about 500 J/m² of UV-B, which is relevant for human exposures as it is the equivalent of 1 – 2 times the minimal erythematous dose (18).

Subsequently, we determined the number of pyrimidine dimers in rat epidermal skin DNA, immediately after exposure to 4000 J/m² of UV-B and at 3 and 24 h thereafter. During the repair periods the animals were kept in the dark. After 3 h of repair, a substantial shift of the DNA distribution in the sucrose gradient towards a higher molecular weight was seen (Fig 3), which clearly demonstrated that epidermal keratinocytes in vivo are well able to remove pyrimidine dimers over this short time interval. The increase in molecular weight corresponds to 50%–60% removal.

Figure 4 shows the kinetics of pyrimidine dimer removal in epidermal cells in vivo after irradiation with UV-B. Although the removal of pyrimidine dimers in the epidermis was initially rapid, about 30% of the dimers originally induced were still present at 24 h after irradiation (Fig 4). The persistence of a substantial fraction of UV-induced pyrimidine dimers in rat skin was confirmed by a nonquantitative immunofluorescence assay using a monoclonal antibody against pyrimidine dimers (20); 48 h after irradiation, low levels of fluorescence were still observed in the nuclei of epidermal cells in cryostat sections (results not shown).

DISCUSSION

The results described above indicate that rat epidermal keratinocytes in vivo are able to remove 50%–60% of UV-induced dimers by dark repair within 3 h, whereas in the same cell type in vitro no significant removal occurs over this time period. Although we are not aware of any previous studies in which such a direct comparison has been made, this result is not completely unexpected. Peleg et al [6] and La Belle and Linn [7] showed that early embryonic mouse cells have the capacity to remove UV-induced pyrimidine dimers; this capacity was found to be lost after a few passages in vitro. In addition, data from Sutherland et al [21] and D'Ambrosio et al [22] indicate a more rapid repair of dimers in human skin cells in vivo than that found by numerous others, including ourselves [5,23], for human fibroblasts in culture.

On the basis of our present results, we may now definitely conclude that the capacity of rat skin cells to remove pyrimidine dimers is almost completely lost upon transfer of these cells into culture. It is not clear, however, whether the loss of the capacity for dimer removal has serious consequences for cell survival or preservation of genetic integrity. In spite of their much lower dimer-removing capability, rat fibroblasts in vitro have the same survival curve after UV-irradiation as cultured human fibroblasts (unpublished results). Therefore the question arises whether rodent cells in culture rely...
more heavily on other DNA repair pathways (such as postreplication repair) than human cells under the same circumstances. On the basis of these considerations we suggest that the rat skin cells used in our study switch from one DNA repair pathway to another during their transfer into culture, possibly in response to the greatly altered circumstances these cells have to deal with during active replication in vitro, as compared with the relatively “resting” state in the skin.

A major implication of our present findings is that one should be cautious with the extrapolation of data on DNA repair responses, obtained with cultured cells, to the living organism.

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REFERENCES


