Interleukin-12 suppresses ultraviolet radiation-induced apoptosis by inducing DNA repair

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Induction of apoptosis of keratinocytes by ultraviolet (UV) radiation is a protective phenomenon relevant in limiting the survival of cells with irreparable DNA damage. Changes in UV-induced apoptosis may therefore have significant impact on photocarcinogenesis. We have found that the immunomodulatory cytokine IL-12 suppresses UV-mediated apoptosis of keratinocytes both *in vitro* and *in vivo*. IL-12 caused a remarkable reduction in UV-specific DNA lesions which was due to induction of DNA repair. In accordance with this, IL-12 induced the expression of particular components of the nucleotide-excision repair complex. Our results show that cytokines can protect cells from apoptosis induced by DNA-damaging UV radiation by inducing DNA repair, and that nucleotide-excision repair can be manipulated by cytokines.

One of the hallmark events of exposure to ultraviolet B radiation (UVB, 290–320 nm) is the induction of apoptotic cell death of keratinocytes, the results of which are evident within the epidermis as sunburn cells (SCs)[1]. The discovery that the tumour suppressor gene *p53* is intimately linked with UVB-induced apoptosis gave rise to the idea that SC formation is a protective mechanism for limiting survival of cells with irreparable DNA damage[2]. The crucial importance of UVB-mediated DNA damage in UVB-induced apoptosis has been substantiated by several studies[3,4], although other pathways, for example activation of death receptors[5–7] and autocrine or paracrine release of death ligands[8–10], may also be involved. Because of its protective function, alterations in UVB-induced apoptosis may have a profound impact in the induction of skin cancer, the most common malignancy in people of western European origin, both in the United States and in Europe[11].

The proinflammatory mediator interleukin-1 (IL-1) was recently found to protect keratinocytes from apoptosis induced by the death ligands TRAIL (tumour necrosis factor-related apoptosis-inducing ligand) or Fas/CD95 ligand by activating the transcription factor NF-κB[12,13]. In contrast, UV-induced apoptosis was not prevented by IL-1 but was even enhanced[13]. Although the mechanisms responsible for these diverse actions remain to be determined, this indicated that UV-induced apoptosis can be influenced by cytokines. As several of these mediators are released during UV exposure[14], we studied the effects of various cytokines on UV-induced apoptosis.

Here we report that the immunomodulatory mediator IL-12 inhibits UVB-induced apoptosis of keratinocytes. Surprisingly, UVB-induced DNA damage was significantly reduced on application of IL-12, and this seems to be due to induction of DNA repair. As far as we are aware, this is the first demonstration of induction of DNA repair by a cytokine protecting cells from apoptosis induced by DNA-damaging UVB radiation. It also shows that nucleotide-excision repair can be influenced by cytokines.

Results

IL-12 reduces UVB-induced apoptosis *in vitro*. Exposure of the epithelial cell line KB to UVB resulted in apoptosis, which was significantly reduced when cells were preincubated with IL-12 2 h before irradiation (Fig. 1a). Similar results were obtained with normal human keratinocytes, the spontaneously transformed keratinocyte cell line HaCaT and the haematopoietic cell lines Jurkat and THP-1 (data not shown). However, IL-12 did not protect from apoptosis induced by γ-irradiation (Fig. 1b).

To exclude the possibility that IL-12 only delays apoptosis but does not rescue cells, the colony-forming efficiency of KB cells and normal human keratinocytes after UVB irradiation was determined. Preconfluent dishes of cells were either left unirradiated or exposed to UVB in the absence or presence of IL-12. After exposure to UVB, IL-12 partially rescued more colonies compared to the UVB-only group (Fig. 1c). However, the survival rates induced by IL-12 were not as pronounced as in the DNA fragmentation ELISA (enzyme-linked immunoassay), which might be due to the fact that the ELISA is evaluated rather soon after UVB exposure. Nevertheless, the colony forming assay indicates that IL-12 enables long-term survival of UVB-exposed keratinocytes and KB cells.

IL-12 reduces UVB-induced DNA damage *in vitro*. As the severity of DNA damage is one of the major determinants for a cell to undergo apoptosis upon UVB exposure[2,3], the impact of IL-12 on the amount of UVB-specific DNA lesions was determined. UVB induces two types of DNA lesions, (6–4)-photoproducts (the numbers refer to the sites on the molecules at which the products occur) and cyclobutane pyrimidine dimers (CPDs), the latter being predominant[15]. Southwestern dot-blot analysis using an antibody directed against CPDs revealed that in DNA samples extracted 3.5 h after UVB exposure, UVB-induced DNA damage was significantly reduced upon IL-12 treatment (Fig. 2). In samples extracted 10 min after UVB, equal amounts of CPDs were observed, irrespective of whether the cells were pretreated with IL-12 or not. This

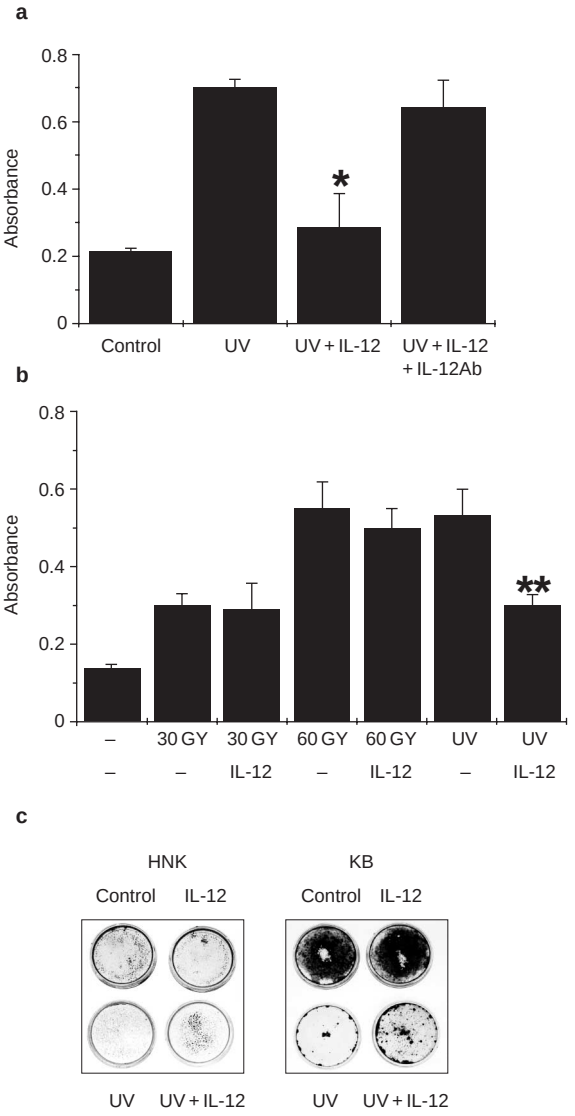

Figure 1 **IL-12 inhibits UVB-induced apoptosis. a,** KB cells (2×10^5 ml^{-1}) were exposed to UVB radiation (250 J m^{-2}). Two hours before UVB exposure, recombinant human IL-12 (50 ng ml^{-1}) was added (UV + IL-12). IL-12 activity was blocked by simultaneous addition of a neutralizing anti-IL-12 antibody (UV + IL-12 + IL-12Ab). Control cells were left untreated. Sixteen hours after irradiation, apoptosis was examined by determining nucleosomal DNA fragmentation using an apoptosis-determination kit. The rate of apoptosis is reflected by increase of absorbance, as shown on the *y*-axis. *$P < 0.05$ (UV + IL-12 versus UV). **b,** KB cells (2×10^5 ml^{-1}) were exposed to γ-irradiation (30 or 60 Gy) or to UVB (250 J m^{-2}). Two hours before irradiation, recombinant human IL-12 (50 ng ml^{-1}) was added. Control cells were left untreated. Sixteen hours after irradiation apoptosis was determined. **$P < 0.05$ (UV + IL-12 versus UV). **c,** Normal human keratinocytes (HNK) and KB cells were supplemented with either 50 ng ml^{-1} IL-12 or an equal amount of diluent (Control) for 2 h before UVB irradiation (250 J m^{-2}). Cells were replated, and supplemented with complete medium containing IL-12 or diluent. Colonies were stained after 21 (HNK) and 18 days (KB), respectively. Data show one of three independent experiments.

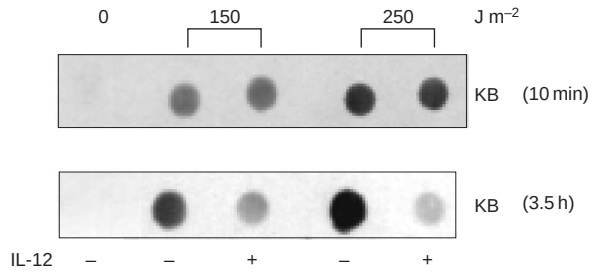

Figure 2 **IL-12 reduces UVB-induced DNA damage.** KB cells were exposed to 150 or 250 J m^{-2} UVB in the absence of presence of IL-12 (50 ng ml^{-1}). Control cells were left untreated. Ten minutes or 3.5 h after UVB exposure, genomic DNA was extracted and analysed by southwestern dot-blot using an antibody against CPD. Data shown are from one of three independent experiments.

observation excludes the possibility that the reduction of DNA damage by IL-12 is due to a filtering effect.

IL-12 reduces UVB-induced DNA damage and sunburn cell formation *in vivo*. To elucidate the *in vivo* relevance of these findings, the backs of C3H/HeN mice were exposed to UVB. One group received IL-12 intracutaneously (i.c.) 3 h before UVB. Sixteen hours later, biopsies were taken and the presence of CPDs was assessed by immunohistochemistry using the antibody directed against CPDs. In UVB-exposed samples, CPDs were detected in almost any keratinocyte (Fig. 3c), but the amount of DNA lesions was remarkably reduced in animals that had received IL-12 before UVB (Fig. 3d). In contrast, in biopsies obtained immediately after UVB exposure, no differences in the staining pattern were observed between UVB-irradiated mice that were injected with IL-12 (Fig. 3b) or with saline (Fig. 3a).

As the formation of SCs is primarily a consequence of DNA damage, the effect of IL-12 on UVB-induced apoptosis *in vivo* was determined. IL-12 or an equal volume of saline was injected i.c. into the backs of C3H/HeN mice. After 3 h the backs were UVB irradiated, and punch biopsies taken 16 h later were analysed for the number of SCs. Mice given i.c. injection of IL-12 had significantly reduced numbers of SCs compared with mice exposed to UVB but not treated with IL-12 (Fig. 4). As a variety of cells in the skin can function as a source of IL-12[16,17], we tested whether endogenously released IL-12 confers protection in IL-12 knockout mice, in which the *p40* gene (encoding the IL-12 p40 subunit) is knocked out[18]. The number of apoptotic keratinocytes was significantly higher in the knockout mice than in wild-type mice (Fig. 5).

IL-12 affects nucleotide-excision repair. The fact that the amounts of UVB-induced DNA damage were the same in the IL-12-treated and untreated samples immediately after UVB exposure, but were reduced by IL-12 at later time points both *in vitro* and *in vivo*, implies that IL-12 might remove UVB-induced DNA lesions by inducing DNA repair. UVB-mediated DNA damage is repaired by nucleotide-excision repair (NER), in which an incision into the damaged site is made; the damage is then excised and a single-strand break is created[19]. Repair DNA synthesis and ligation then complete the repair process. Single-strand breaks can be visualized on an individual cell basis by the 'comet' assay, as relaxed and broken DNA fragments migrate faster in an electric field, forming comet-like bands on the gel[20]. Comet formation has been observed after exposure to ionizing radiation, various genotoxic agents and UVB[21–23]. Comets induced by ionizing radiation and chemotherapeutic agents such as bleomycin are regarded as a direct consequence of the DNA-breaking properties of these agents. As no single-strand breaks can be detected at the UVB doses used in this study, UVB-induced comets result from repair incisions at DNA photoproducts[20]. Therefore, the length of UVB-induced comets correlates both with the severity of DNA damage and with the capacity for DNA repair. Thus, when UVB is the DNA-damaging agent, the comet assay can be used as a proxy for the efficacy of NER. Comets were detected in UV-irradiated KB cells (Fig. 6). Comet formation was enhanced in cells which were exposed to IL-12 before UV irradiation. The finding

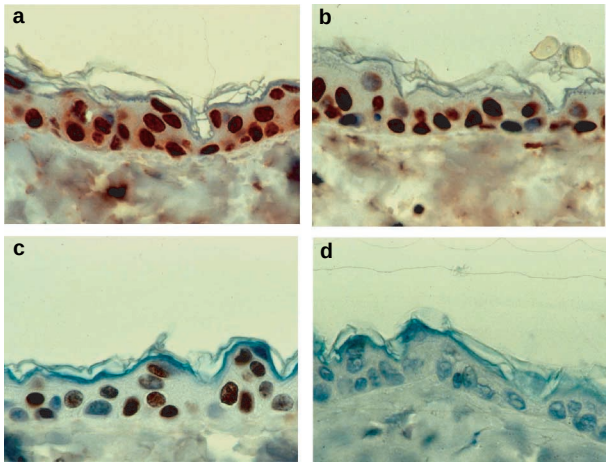

Figure 3 **IL-12 reduces UVB-induced DNA damage *in vivo*.** C3H/HeN mice were irradiated with UVB (500 J m^{-2}) on the shaved back. **a–d**, Three hours before UVB exposure, 100 ng IL-12 (**b**, **d**) or equal volumes (100 µl) of saline (**a**, **c**) were injected i.c. After 15 min (**a**, **b**) and 16 h (**c, d**), respectively, biopsies were taken and stained with an antibody against CPD. Data shown are from one of two independent experiments.

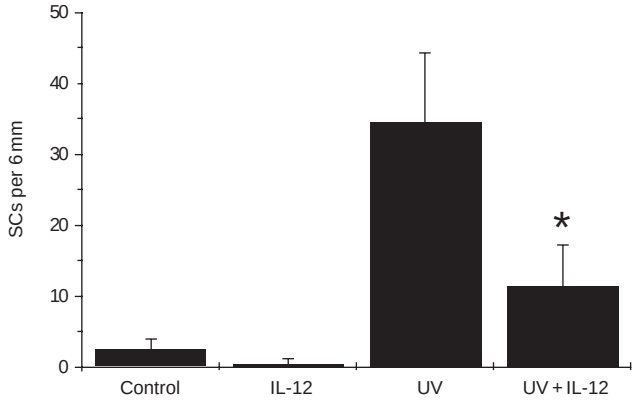

Figure 4 ***In vivo* reduction of SCs by IL-12.** C3H/HeN mice were irradiated with UVB (3,000 J m^{-2}) on the shaved back. Three hours before UVB exposure, IL-12 (100 ng) was injected i.c. Biopsies were taken 16 h later and SCs counted per 6-mm length of epidermis. The mean number (± s.d.) of SCs from four independent experiments is shown. *$P <$ 0.001 vs UV.

Table 1 IL-12 affects the expression of NER genes

Genes analysed†	Relative expression‡ (%)		
Treatment*	IL-12	UVB	UV+IL-12
XPC	126 ± 90	68 ± 40	179 ± 35
XPF	95 ± 34	31 ± 10	56 ± 35
XPG	80 ± 33	66 ± 18	157 ± 50
RPA(p14)	137 ± 75	118 ± 34	178 ± 99
RPA(p32)	104 ± 32	83 ± 25	133 ± 16
RPA(p70)	106 ± 27	95 ± 37	117 ± 32
DDB1	87 ± 33	70 ± 20	102 ± 33
DDB2	110 ± 24	95 ± 20	149 ± 27
hHR23B	91 ± 24	75 ± 20	108 ± 26

The data is from four independent experiments (mean ± s.d.).
* The expression of NER genes was studied in cells which were left untreated, exposed to UVB (250 J m^{-2}) in the absence of presence of IL-12 (50 ng ml^{-1}), or treated with IL-12 alone. After 4 h, RNA was extracted and RNase protection assay carried out.
† RNA (10 µg) was hybridized with the radiolabelled antisense RNA probe set hNER-1.
‡ The expression level of each gene of interest was quantified using phosphorimager analysis and normalized with the expression of GAPDH or L32. The level of mRNA of unirradiated control cells was assumed to represent basal expression and was arbitrarily set at 100% relative expression.

that IL-12 reduces the amounts of CPD and at the same time enhances comet length suggests that IL-12 might induce NER.

NER is a highly complex process involving multiple factors[19]. We assessed whether IL-12 affects the expression of NER genes by using the RNase protection assay. The set of genes analysed included the NER genes *XPG, DDB1, XPC, XPF, RPA*(*p70*), *DDB2, hHR23B, XPA, RPA*(*p32*) and *RPA*(*p14*), with the ribosomal protein *L32* and *GAPDH* (glutaraldehyde phosphate dehydrogenase) as standards for normalization. The expression level of each gene was quantified by phosphorimager analysis and normalized with the expression of *GAPDH* or *L32*. Although the genes differed in their expression rates in response to UVB and/or IL-12, most were downregulated by UVB. Treatment of cells with IL-12 before UVB exposure not only prevented UVB-mediated downregulation, but even enhanced gene expression in comparison to untreated cells (Table 1), suggesting that IL-12 might induce NER through induction of NER gene expression. In contrast, IL-12 did not affect the expression of components of the base-excision repair complex (data not shown).

Xpa knockout mice were used to confirm that IL-12 reduces UVB-induced DNA damage by inducing NER. These mice are severely deficient in NER as the result of the disruption of *Xpa*[24], and after UVB exposure we found a higher number of SCs than in wild-type mice exposed to the same UVB dose, confirming previous findings[25]. Although IL-12 significantly reduced SC numbers in UVB-irradiated wild-type mice, it had no significant effect on SCs in the *Xpa* knockouts (Fig. 7). To consolidate these findings, peripheral blood mononuclear cells were obtained from a patient suffering from xeroderma pigmentosum (XP) and from a healthy control and were exposed to UVB in the absence or presence of IL-12. At 3.5 h after irradiation, DNA was extracted and the amounts of CPD determined. UVB-induced DNA damage was reduced after IL-12 treatment in the healthy cells, whereas IL-12 did not reduce the amounts of CPD in cells from the XP patient (Fig. 8). Taken together, these results confirm that IL-12 reduces UVB-mediated DNA damage through induction of NER.

Discussion

Non-melanoma skin cancer is the most common and most rapidly increasing type of cancer in people of western European extraction in the US and Europe[11]. UVB radiation is the principal causative agent because of its ability to induce DNA lesions which, if not immediately removed, can give rise to mutations and skin cancer[2]. UVB-induced DNA lesions are removed by NER. The importance and efficacy of NER is shown by the drastically enhanced rate of skin cancer in patients suffering from XP, which is caused by defects in various components of the NER system[26].

Induction of apoptosis is an additional protective mechanism as it eliminates cells that were unable to repair the DNA damage completely[1]. Therefore, dysregulation of UV-induced apoptosis will enhance the risk of photocarcinogenesis. There are several ways of inhibiting UV-induced apoptosis — by overexpressing heat-shock proteins[27] or anti-apoptotic proteins[28,29], by disrupting p53 function[2] or by blocking death-receptor activation[5,8–10], for example. As these allow the survival of cells carrying DNA damage, they may give rise to mutations and skin cancer.

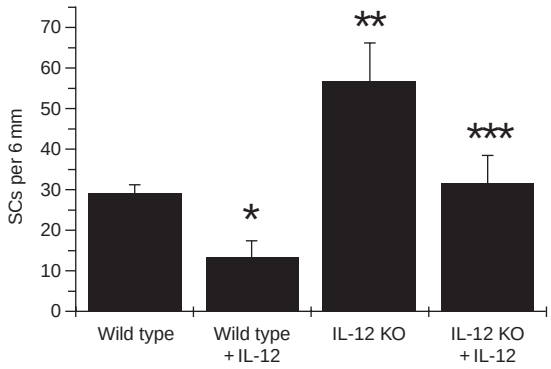

Figure 5. Increased number of SCs in IL-12 knockout mice. IL-12/p40 knockout mice (KO) and wild-type control mice (C57BL/6) were irradiated with UVB (750 J m^{-2}) on the shaved back. Biopsies were taken 16 h after UV exposure and SCs counted per 6-mm length of epidermis (mean ± s.d.). Data shown are from one of two independent experiments. *P < 0.001; **P < 0.001 vs wild type; ***P < 0.001 vs IL-12 knockout.

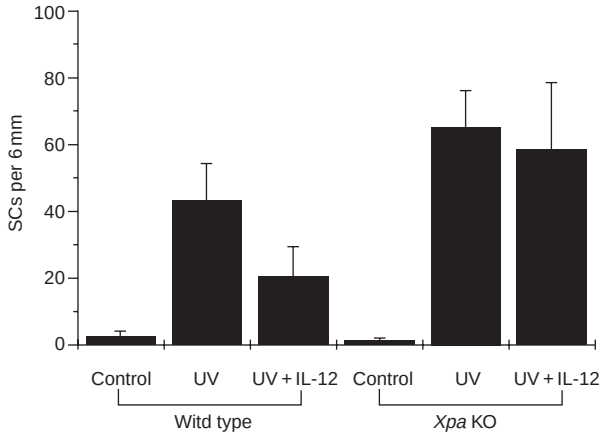

Figure 7 **IL-12 does not reduce the number of SCs in *Xpa* knockout mice.** *Xpa* knockout mice (KO) and wild type control mice (C57BL/6) were irradiated with UVB (3,000 J m^{-2}) on the shaved back. Biopsies were taken 16 h after UV exposure and SCs counted per 6-mm length of epidermis (mean ± s.d.). One representative of three independent experiments is shown. *P < 0.001 vs wild-type UV.

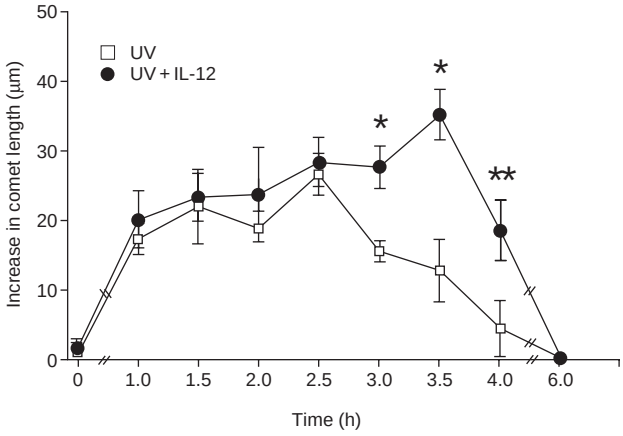

Figure 6 **IL-12 enhances comet length in UV-exposed cells.** KB cells were exposed to 250 J m^{-2} in the absence or presence of IL-12 (50 ng ml^{-1}). Immediately after irradiation (0 h) and at various time points, cells were collected and the comet assay carried out. The *y*-axis shows increase in comet length (in μm), which represents the differences between comet lengths in the treated and untreated samples. Data show the mean ± s.d. of three independent experiments. *P < 0.01; **< 0.05 vs UV.

Here we provide the first evidence that IL-12 inhibits SC formation. However, in contrast to the approaches mentioned above, inhibition of UVB-mediated apoptosis by IL-12 seems to be due to a remarkable reduction in UVB-induced DNA damage. The fact that there was no difference in the amount of DNA lesions immediately after UVB irradiation but a difference at later times can only be explained by enhanced removal of photoproducts. The involvement of NER was confirmed by the comet assay and by the RNase protection assay. There are two main findings from the RNase protection assay: first, many of the NER genes are downregulated by UVB; second, this downregulation is compensated for by IL-12. In addition, several NER components are induced above their baseline levels, an interesting observation as NER is thought to be poorly regulated. The inducing effect of IL-12 was most pronounced for the NER genes *DDB2*, *RPA(p32)*, *RPA(p14)*, *XPC* and *XPG*. The *XPG* gene encodes an endonuclease which is involved in incision[30]. The XPG-mediated 3′ incision precedes the 5′ incision caused by XPF[31]. In addition, XPG appears to have a structural function in the assembly of the NER DNA–protein complex, in which interactions with the transcription factor TFIIH and RPA may be important[32]. RPA is a multifunctional single-stranded (ss)-DNA-binding protein complex composed of subunits of relative molecular mass (M_r) 70,000 (70K), 34K and 14K (ref. 33). RPA binds to DNA through the 70K subunit, whereas the 34K and 14K subunits are primarily responsible for protein–protein interactions[34]. DDB2 (p48) is a 48K subunit of the DDB (damaged DNA-binding) factor, which is involved in damage recognition during NER[35]. DDB2 seems to be under the control of *p53* and may facilitate the recognition of lesions that are poorly recognized by the XPC–hHR23B complex[36].

The involvement of NER is also supported by the observation that IL-12 did not protect cells from apoptosis caused by γ-irradiation, as double-strand breaks induced by γ-irradiation are not repaired by NER[37]. Furthermore, it seems unlikely that IL-12 indirectly affects the status of NER, for example by altering the cell cycle, as IL-12 did not cause a change in the cell cycle (data not shown). Although the detailed mechanism by which IL-12 induces NER gene expression remains to be determined, induction of transcription seems to be essential, as the effect of IL-12 in reducing DNA damage is lost in the presence of the transcription inhibitor actinomycin D (data not shown). The causal relationship between the protective effect of IL-12 and NER was finally shown by the fact that IL-12 was not protective in *Xpa* knockout mice nor in cells from a patient with XP.

An alternative way of reducing UV-mediated DNA damage is the application of exogenous DNA-repair enzymes. The bacterial DNA-repair enzyme T4 endonuclease V (T4N5) can be delivered into cells by liposomes[38]. Topically applied T4N5 liposomes penetrate the skin, increase the removal of CPDs and reduce the incidence of skin cancer[39]. In accordance with the accelerated removal of DNA lesions by T4N5, SC formation was also found to be reduced[40]. In contrast to the external application of DNA-repair enzymes, IL-12 affects the cell's own NER system. A similar phenomenon was recently described using thymidine dinucleotides (pTpT), which enhance the repair of UV-induced DNA damage in a *p53*-dependent manner[41]. It seems unlikely that the effect of IL-12 described here is mediated by *p53*, as the effect was also observed in HaCaT cells, which carry two *p53* mutations[42].

IL-12 is a classical immunomodulatory cytokine and among its many biological activities the most important are its capacity to

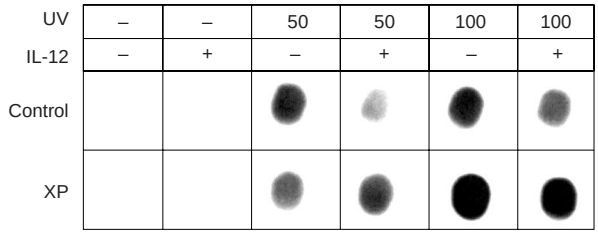

Figure 8 **IL-12 does not reduce UVB-induced DNA damage in XP cells.** Peripheral blood mononuclear cells were obtained from a patient suffering from XP and from a healthy volunteer (Co). Cells were exposed to UVB radiation (50, 100 J m^{-2}) in the absence of presence of IL-12 (50 ng ml^{-1}). Three and a half hours after UVB exposure, genomic DNA was extracted and subjected to southwestern dot-blot analysis using an antibody against CPD.

activate natural killer cells and its crucial role in the generation of an immune response of the T helper 1 type[43]. It is highly unlikely that the reduction in SCs is due to activation of phagocytic cells by IL-12, as it is characteristic of SCs that they are never associated with macrophages within the epidermis[1]. Despite this fact, we cannot absolutely exclude the possibility that, besides the induction of NER, IL-12 may also induce other effects *in vivo* which may support keratinocyte survival.

On the basis of its activities known so far, there was no reason to suspect that IL-12 would affect NER. IL-12 is known to prevent UVB-mediated immunosuppression and to restore immune competence after UVB exposure[44]. Impairment of the immune system by UVB is mediated in large part by the immunosuppressive cytokine IL-10, the release of which is induced by UVB[45]. There is compelling evidence that DNA damage is the primary trigger for IL-10 release upon UVB exposure[46]. Schmitt *et al.*[47] recently reported that IL-12 inhibits UVB-induced IL-10 release[47], which is in good agreement with our findings. Therefore, we can speculate that IL-12, through its capacity to remove DNA damage by NER, might counteract UVB-induced immunosuppression.

NER is the main mechanism of repair of UVB-induced DNA damage in mammalian cells. Thus, it is of primary importance to identify agents that can promote NER activity. We have identified IL-12 as one such agent, showing that cytokines can protect cells from apoptosis induced by DNA-damaging UVB through the induction of DNA repair. As far as we are aware, this is the first demonstration of induction of DNA repair by a cytokine protecting cells from apoptosis induced by DNA-damaging UVB radiation. It also shows that nucleotide-excision repair can be influenced by cytokines. It remains to be determined whether constitutive overexpression of IL-12 in the epidermis *in vivo* will not only be associated with a reduction in the number of SCs but, even more important, also with a reduced risk of developing skin cancer after chronic UVB exposure. Taking into account the possibility that not all cells with damaged DNA may be eliminated by apoptosis, reducing UVB-mediated DNA damage should be beneficial. Studies using transgenic mice overexpressing IL-12 in the basal epidermis may give the answer. Moreover, topically applied IL-12 might have potential as a therapeutic agent for preventing UV-induced skin cancer. □

Methods

Cells

The epidermoid carcinoma cell line KB (American Type Culture Collection) was cultured in RPMI culture medium containing 10% FCS and 1% glutamine. Irradiation with UV light was carried out as described previously[3] using a bank of six fluorescent bulbs (TL12, Philips) which emit most of their energy within the UVB range (290–320 nm) with an emission peak at 313 nm. Subconfluent cells were exposed through PBS. Cells were γ-irradiated by exposure to a cobalt-60 source.

Detection of cell death

A cell-death-detection ELISA (Boehringer Mannheim) was used to detect DNA fragmentation; mono- and oligonucleosomes released into the cell cytoplasm are detected by biotinylated anti-histone- and peroxidase-coupled anti-DNA antibodies. Optical density is shown on the *y*-axis as mean ± s.d. of triplicates.

To determine the colony-forming efficiency, normal human keratinocytes and KB cells were supplemented with either 50 ng ml^{-1} IL-12 or an equal amount of diluent for 2 h before UV irradiation. After irradiation (250 J m^{-2}), cells were trypsinized, replated at different densities and supplemented with complete medium containing IL-12 or diluent. The cultures were monitored daily, and when colonies were easily visible, cells were stained with crystal violet.

Southwestern dot-blot analysis

Genomic DNA was isolated from 10^6 cells according to the DNA extraction protocol from Biozym Diagnostik. Genomic DNA (2 µg) was transferred to a positively charged nylon membrane by vacuum dot-blotting and fixed by baking the membrane for 15 min at 80 °C. A monoclonal antibody directed against thymine dimers (Kamiya Biomedical Company) was used for southwestern analysis. Detection was carried out with a horseradish peroxidase-conjugated anti-mouse antibody.

Comet assay

A commercial reagent kit for single cell gel electrophoresis assay (Travigen) was used. Briefly, after irradiation and incubation, nonadherent cells were centrifuged and the pellet resuspended in 50 µl medium. Adherent cells were detached using trypsin–EDTA. Cells were washed in cold PBS and resuspended at 10^5 per ml in cold Ca^{2+}- and Mg^{2+}-free PBS. Cells were mixed with melted LMA agarose at a ratio of 1/10 (v/v) and 75 µl was pipetted onto Comet slides. The slides were placed on a flat surface at 4 °C in the dark for 10 min and then immersed in pre-chilled lysis solution for 30 min. The taped slides were then immersed in alkali solution for 60 min at room temperature in the dark. Slides were washed twice with 1 × TBE buffer and placed into a horizontal electrophoresis apparatus and electrophoresis carried out at 1 V cm^{-1} for 10 min. Diluted SYBR Green (50 µl), a fluorescent stain, was placed onto each circle of dried agarose. DNA comets were visualized with a fluorescence microscope. Duplicate experiments were carried out with each sample. Comet lengths were measured manually using an ocular scale. Seventy-five randomly selected cells were scored for every sample.

RNase protection assay

Total RNA was extracted using the Total RNA Isolation Kit (Pharmingen) and assayed for specific mRNA content using the Riboquant MultiProbe RNase Protection Assay System (Pharmingen). Briefly, 10 µg of RNA from each sample was hybridized with the radiolabelled antisense RNA probe set hNER-1. After hybridization, free probe and remaining ssRNA were digested with RNase. The protected probes were purified, resolved on 5% denaturing polyacrylamide gels, and quantified by autoradiography using a Fujix BAS1000 phosphoimager and MacBAS software version 1.01 (Fuji). The intensity for each gene of interest was measured, and values were normalized to values for GAPDH and L32. Normalized counts for each sample were expressed as the percentage of the respective normalized counts of the untreated control, which were arbitrarily set as 100%.

In vivo treatment of mice

C3H/HeN and C57BL/6 mice were purchased from M and B A/S (Denmark). IL-12(*p40*) knockout mice were kindly provided by I. Förster, Munich. *Xpa* knockout mice were generated at the RIVM, Bilthoven, The Netherlands[27]. Mice were exposed on their shaved back to UVB using TL12 lamps. Punch biopsies were taken from the irradiated and control areas at different time points after UVB exposure. Recombinant murine IL-12 (100 ng) or an equal volume (100 µl) of physiological saline solution were injected i.c. 3 h before irradiation.

Immunohistochemical staining for dimers

Biopsies were fixed in 7% buffered paraformaldehyde, dehydrated and embedded in paraffin. After deparaffinization with xylol and ethanol at decreasing concentrations, sections were immersed in an aqueous solution containing 0.1 M citric acid–0.1 M sodium citrate and subsequently microwave-treated for 15 min to unmask antigenic epitopes. Endogenous peroxidase activity was blocked by incubation with 0.1% H_2O_2 and 0.6% sodium acid in PBS for 20 min. After rinsing with PBS, nonspecific binding sites were blocked with 2% BSA for 30 min at room temperature followed by incubation with the primary antibody diluted in 1% BSA overnight. The anti-thymidine-dimer antibody was used at a dilution of 1:2000. Staining was by an indirect immunoperoxidase technique using the following reagents: PBS, peroxidase-conjugated anti-mouse antibody (undiluted; Dako, Hamburg, Germany), 0.01% H_2O_2, 3-amino-9-ethylcarbazole (Sigma). Negative controls consisted of the omission of the first antibody.

Evaluation of SC formation

Biopsies were taken from the areas of the skin exposed to UV, fixed in formaldehyde and embedded in paraffin. Tissue sections (5 µm) were stained with haematoxylin and eosin, and the number of SCs (defined as apoptotic cells within the epidermis having a shrunken eosinophilic cytoplasm and a condensed nucleus) was counted throughout the epidermis of sections per sample using a 1 cm × 1 cm grid inserted in a conventional microscope. SCs per 6-mm length of epidermis were counted. At least five fields of each sample were evaluated and the number of SCs (mean ± s.d.) calculated. Student's *t*-test was used to test the significances of the differences.

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