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In contrast to their murine counterparts, normal human keratinocytes and human epidermoid cell lines A431 and HaCaT fail to express IL-10 mRNA and protein

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SUMMARY

In mice, keratinocyte-derived IL-10 is up-regulated by ultraviolet-B (UVB) radiation and plays a major role in UVB-induced immunosuppression. The present study was designed to examine whether a comparable phenomenon can be detected in man. Freshly isolated or cultured normal human keratinocytes (NHK) and keratinocyte cell lines A431 and HaCaT were stimulated with graded doses of UVB (up to 200 J/m^2) or with a variety of other stimuli. RNA was extracted at various time points post-stimulation and analysed by reverse transcriptase-polymerase chain reaction (RT-PCR) using four different IL-10-specific primer pairs and RNA from monocytes or T cells as positive controls. We failed to detect IL-10 mRNA in NHK from 40 different donors (breast, abdomen, leg, scalp, foreskin) and in A431 and HaCaT cells, irrespective of the stimulation used and despite successful stimulation. Supernatants of NHK, A431 and HaCaT cultures were negative for IL-10 protein, as tested by four different ELISAs and a bioassay. Murine keratinocytes, stimulated under comparable conditions and tested by the same techniques, displayed a strong expression of IL-10 mRNA and protein. Remarkably, an IL-10 mRNA signal could be detected in NHK after a second round of PCR amplification. Because NHK suspensions are contaminated with Langerhans cells, melanocytes and possibly fibroblasts, we tested pure populations of each individual cell type to determine the origin of this IL-10 mRNA. Our results clearly indicate that NHK, Langerhans cells and fibroblasts fail to express IL-10 and that melanocytes are the principal source of IL-10 mRNA in normal human epidermis.

Keywords IL-10 keratinocytes UVB radiation reverse transcriptase-polymerase chain reaction melanocytes

INTRODUCTION

Keratinocytes, which comprise the vast majority of epidermal cells, are able to produce a variety of cytokines and are considered as the principal source of epidermis-derived interleukins, interferons, colony-stimulating factors, tumour necrosis factors, growth factors, and many other factors (reviewed in [1]). The constitutive production of these factors by keratinocytes might be low, but can be remarkably augmented by exogenous stimuli, such as tumour promoters, endotoxin, or ultraviolet-B (UVB; 280–320 nm) radiation [1–4]. Enhanced secretion of keratinocyte-derived factors *in vivo* might affect not only local but also systemic immunological reactions and in this way the epidermis can play a significant regulatory role during immune responses. One obvious example in this respect is that UVB radiation stimulates keratinocytes to produce and secrete immunosuppressive peptide(s), causing inhibition of the induction of DTH and contact hypersensitivity [5–7].

An attractive candidate for such a UVB-induced inhibitory mediator would be the recently described IL-10. This cytokine has been implicated as an important suppressor of effector functions of T lymphocytes and antigen-presenting cells (APC) in both mice and man [8,9]. The inhibitory effect of IL-10 on T cell proliferation and cytokine production is mainly mediated via suppression of the costimulatory function of APC [10,11], although direct effects of IL-10 on T cells in the absence of professional APC have also been described [12]. Support for the hypothesis that IL-10 could play an important role in UVB-induced immunosuppression comes from

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several experimental *in vitro* and *in vivo* studies in mice. Murine keratinocytes and the spontaneously transformed murine keratinocyte cell line PAM 212 are able to synthesize IL-10 mRNA and secrete IL-10 protein [13,14], and this production was markedly increased after irradiation with UVB [14]. The T cell stimulatory function of Langerhans cells, the most prominent APC within the skin, is affected by IL-10 *in vitro*: IL-10-pretreated Langerhans cells remain effective APC for Th2, but they fail to induce Th1 cell proliferation and interferon-gamma (IFN-γ) production and rather render these Th1 cells tolerant/anergic [15]. This correlates nicely with the *in vivo* findings that UVB-induced suppression of contact sensitivity is associated with functional inactivation of skin-draining lymph node Th1 cells [16] and that intradermal injection of IL-10 inhibited hapten-induced ear swelling and up-regulation of IFN-γ, but induced hapten-specific tolerance in lymph node T cells [17,18]. Finally, injection of IL-10 mimics the suppressive effect of UVB radiation [19] and anti-IL-10 MoAb neutralizes the ability of supernatants from UVB-exposed keratinocytes to suppress the induction of DTH *in vivo* [14]. Injecting UVB-irradiated mice with anti-IL-10 MoAb immediately after UVB exposure partially abolished the UVB-induced inhibition of contact allergen-induced ear swelling [14] and prevented loss of APC function to stimulate Th1 cells [19].

At present it is not known whether in humans IL-10 is similarly involved in the mechanism of UVB-induced impairment of immune responses. The present study was to determine the capability of normal human keratinocytes (NHK), the spontaneously transformed human keratinocyte cell line HaCaT, and the human epidermoid carcinoma cell line A431 to produce and release IL-10. Here we provide evidence at mRNA level and protein level, that human keratinocytes, HaCaT cells, and A431 cells, unlike their murine counterparts, are not able to synthesize detectable amounts of IL-10, using various culture conditions and different stimuli. We further show that human melanocytes, usually contaminating NHK cultures, are the major source of IL-10 mRNA in normal epidermis.

MATERIALS AND METHODS

Isolation and maintenance of human cells, cell lines and clones

Thin sheets of normal human skin (foreskin or skin from abdomen, breast, leg or scalp) were incubated either overnight at 4°C in 0·5 mg/ml Thermolysin (Sigma, St Louis, MO) in PBS or 30 min at 37°C in 0·3% dispase (Sigma) in PBS enabling the separation of epidermis from dermis. To obtain single cells, the epidermis was treated with 0·025% trypsin (Sigma) for 10 min at 37°C. The epidermal cells were cultured in keratinocyte growth medium (KGM; Gibco, Paisley, UK). Alternatively, NHK were cultured under hydrocortisone-free conditions in Dulbecco's modified Eagle's medium (DMEM) plus Ham's F12 (Gibco; ratio 3:1) supplemented with 10% fetal calf serum (FCS; Gibco), 10 ng/ml epidermal growth factor (EGF) (Gibco), 250 ng/ml L-isoproterenol, 2 mM glutamine (Gibco) and penicillin/streptomycin. The medium was changed twice a week and cultures were split (0·25% trypsin; 10 min at 37°C) just before reaching confluency. NHK cultures were maximally split four times and experiments were carried out with 60–100% confluent cultures. The A431 cell line (American Type Culture Collection, Rockville, MD) and the HaCaT cell line [20] (kindly provided by Dr N. E. Fusenig, Heidelberg, Germany) were maintained in DMEM with 10% FCS. Keratinocytes were stimulated either with UVB irradiation (see below) or with 10 ng/ml phorbol myristate acetate (PMA; Sigma), 10 μg/ml lipopolysaccharide (LPS; Sigma; *Escherichia coli* 0127:B8), 1000 U/ml recombinant IFN-γ (Biogen, Geneva, Switzerland), 0·5 ng/ml recombinant tumour necrosis factor-alpha (TNF-α; Sigma), 50 U/ml IL-1α plus 50 U/ml Il-1β (Boehringer, Mannheim, Germany), 0·5 mM $NiSO_4$ or 10% supernatant (harvested at day 3) of peripheral blood leucocytes pulsed with 10 μg/ml phytohaemagglutinin (PHA; Difco, Detroit, MI) for 16 h. Cycloheximide (Sigma) was used at a concentration of 5 μg/ml.

Human melanocytes were derived from foreskin epidermis and cultured in Ham's F10 (Gibco) supplemented with 10 mg/ml PMA (Sigma), 0·1 mM isobutyl-methyl-xanthine (Sigma), 1% ultroser G (Sigma), 2 mM glutamine, penicillin/streptomycin [21]. Pure keratinocyte or melanocyte cultures were obtained via limiting dilution culturing and were identified on cytospins as cytokeratin (CK1; Dakopatts, Glostrup, Denmark)- and NKI-beteb (Sanbio, The Netherlands)-positive cells, respectively. Human fibroblasts were derived from foreskin dermis and purified by growth in selective medium: DMEM supplemented with 10% FCS, 2 mM glutamine and penicillin/streptomycin. Human Langerhans cells were enriched from epidermal breast skin suspensions by discontinuous density gradient centrifugation [22] and further purified by the so-called panning technique [23].

Monocytes were isolated from 500 ml peripheral blood obtained from healthy donors by centrifugation over Lymphoprep (1·077 g/cm^3; Nycomed, Oslo, Norway) and over Percoll (1·066 g/cm^3; Pharmacia, Uppsala, Sweden), followed by adherence to plastic. Monocytes were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco) with 10% FCS and were stimulated with 10 μg/ml LPS to induce significant levels of IL-10 production [24].

The house dust mite allergen Der p II-specific T cell clones JBC1 and PBA1, which have a Th2-like and Th0-like cytokine production profile, respectively [25,26], were kindly provided by R. J. J. Neerven (University of Amsterdam, The Netherlands). To induce significant IL-10 production [27], the Th2 clone was stimulated for 18 h with 10 ng/ml PMA plus 100 ng/ml CD3 MoAb (Central Laboratory Blood Transfusion Service, Amsterdam, The Netherlands), using IMDM with 5% pooled normal human serum, 2 mM glutamine, and 2×10^{-5} M β-mercaptoethanol (β-ME) as the culture medium. The Th0 clone was stimulated with a combination of CD3 plus CD28 MoAbs.

Murine epidermal cells

Murine keratinocytes were isolated from BALB/c, C57Bl/6 or Swiss mice obtained from Harlon (Zeist, The Netherlands) and used at 3–6 months of age. Ears of mice were cut off, rinsed in 70% ethanol and PBS and, subsequentely, ears were split by foreceps. After floating ear halves (dermal side down) on 0·5% trypsin in PBS for 30 min at 37°C, the epidermis was peeled off. The epidermis of one earhalf was subjected to RNA extraction and the remaining epidermis was incubated for 5 min in 1 ml PBS containing 0·25% trypsin. Trypsinization was stopped by addition of 1 ml FCS, whereafter epidermis was vigorously pipetted and sieved though gauze to obtain a single-cell suspension of epidermal cells ($\approx 10^6$ cells/ear). Murine epidermal cells were cultured in RPMI 1640 (Gibco) plus 10% FCS, penicillin/streptomycin and 5×10^{-5} M β-ME. The PAM212 cell line was maintained in DMEM with 10% FCS.

Ultraviolet irradiation

As described in detail before [28], cells were UVB-irradiated with

a bank of four Philips TL-12 lamps (Philips, Eindhoven, The Netherlands), having a light output of 1 J/m^2 per s at target distance, as monitored by an IL433 radiometer with a SEE 1240 UVB photodetector (International Light Inc., Newburyport, MA). As UVA source we used a bank of 10 TL-10R lamps (Philips), having a light output of 15 J/m^2 per s at target distance as monitored by an IL442 A radiometer with a SEE 115 UVA photodetector (International Light Inc.). UVA1 radiation was obtained by means of a combination of this UVA bank with a KV370 filter (Schott Glaswerke, Jena, Germany), having a light output of 6 J/m^2 per s at target distance. The adherent growing NHK, A431 or HaCaT cells were washed once with PBS just before irradiation, and were kept in 5 ml PBS during UVB exposure, whereafter cells were washed once again with PBS and replaced in culture medium.

Four healthy volunteers (male Caucasians; mean age $33 \pm 5{\cdot}6$ years; skin type II, informed consent) received a single total body exposure of 1·5 minimal erythemal dose (MED) using a UV6002 phototherapy cabinet (Waldmann, Schwenningen, Germany) equipped with TL-21 UVB lamps (Philips). The MED level of each individual was determined 1 week before the experiment using a small spot on the lower back. The test subjects had to avoid excessive sun exposure 3 weeks before and during the study. Blood was drawn from the antecubital vein into sterile, heparinized tubes at different time points before and after UVB treatment.

RNA extraction

Total RNA was isolated from (sub)confluent NHK, A431, or HaCaT cultures ($5–10 \times 10^6$ cells per Petri dish), from 20 to 40×10^6 monocytes, and from 50 to 100×10^6 Th2 cells by the guanidium isothiocyanate method. In brief, cells were washed with PBS just before addition of 4 M guanidium isothiocyanate containing 0·1 M β-ME, 24 mM sodium citrate, and 0·5% sodium lauryl sarcosinate. After phenol chloroform extraction and isopropanol/ethanol precipitation, the RNA pellet was dissolved in distilled water and the amount of total RNA was determined by a spectrophotometer at 260 nm and 280 nm. Alternatively, total RNA was isolated via Trizol (GIBCO). Isolation of mRNA from total RNA by biotinylated oligo(dT) primer and streptavidin-coupled magnetic beads (Promega, Madison, WI) was performed according to the manufacturer's protocol.

Northern analysis

For Northern analysis, 15 μg of total RNA were separated according to size on 1·4% agarose gels containing 6·6% formaldehyde (37%), and transferred to nylon Hybond-N$^+$ membranes (Amersham). Membranes were prehybridized in $5 \times$ SSPE ($1 \times$ SSPE: 180 mM NaCl, 10 mM NaH_2PO_4 pH 7·4, 1 mM EDTA), 1% SDS, $5 \times$ Denhardt's solution (0·1% Ficoll, 0·1% polyvinylpyrrolidone, 0·1% bovine serum albumin (BSA) fraction V), and 100 μg/ml ssDNA of herring sperm (Sigma) for 4 h at 42°C. Together with 10% dextran sulphate (Sigma) labelled probes were added to the prehybridization mix to incubate for 24 h at 42°C. The membranes were washed with $2 \times$ SSC, 1% SDS for 15 min at 42°C, with the same solution for 30 min at 65°C, and finally with 0·2 SSC, 0·1% SDS for 30 min at 65°C, whereafter the membranes were dried and exposed to XAR film (Kodak). Hind III-digested λ DNA was used as size marker. By means of stripping and rehybridization, specific RNA of IL-10, IL-1α, and β-actin could subsequently be determined on the same RNA blot.

Probes and primers

The following probes were used for Northern analysis of total RNA and Southern analysis of PCR products: 1757 bp Hind III fragment of pGEM-3 (IL-1α), 1407 bp BamHI/EcoRV fragment of pCD-SRa (IL-10; nt 0–1407), 265 bp SfcI/HaeII fragment of this 1407 bp IL-10 probe (IL-10; nt 405–660), 950 bp PstI fragment of pUC19 (β-actin). Probes were labelled with ^{32}P (740 kBq/100 ng DNA) to high specific activity by nick translation as random labelling procedure.

Four different pairs of IL-10 primers were used for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of human RNA:

1st pair: IL-10 sense primer 5′-CGCTGTCATC GATTTCTTCC-3′ (nt 403–422) and IL-10 antisense primer 5′-CAATAAGGTTTCTCAAGGGGC-3′ (nt 654–674);

2nd pair: IL-10 sense primer 5′-CTTCGAGATCTCCGA GATGC-3′ (nt 151–170) and IL-10 antisense primer 5′-TAT CCCAGAGCCCCAGATC-3′ (nt 626–644);

3rd pair: IL-10 sense primer 5′-ATGCCCCAAGCTGA GAACCAAGACCCA-3′(313–339) and IL-10 antisense primer 5′-TCTCAAGGGGCTGGGTCAGCTATCCCA-3′ (638–664);

4th pair: IL-10 sense primer 5′-TCTGAGAACAGCTG CACCC-3′ (nt 106–124) and IL-10 antisense primer 5′-CCC AGGGAGTTCACATGC-3′ (nt 351–368).

Other primers used in this study were:

β-actin sense primer 5′-AAATCGTGCGTGACATTA AGG-3′ (nt 661–681) and β-actin antisense primer 5′-CCG ATCCACACG-GAGTACTT-3′ (nt 1047–1066);

IL-1α sense primer 5′-CTGCATGGATCAATCTGT GTC-3′ (nt 143–163) and IL-1α antisense primer 5′-CCC ATGT-CAAATTTCACTGC-3′ (nt 492–511);

IL-10R sense primer 5′-GCTGAAGAACTTGGACC TGC-3′ (nt 987–1006) and IL-10R antisense primer 5′-CCTTCTCTTCAG-CACA TCTGG-3′ (nt 1414–1434).

Sequences of IL-10 and β-actin primers used for RT-PCR analysis of murine RNA and related internal probes were described elsewhere [13].

RT-PCR

Total RNA or mRNA (5 μg) was reverse transcribed using MMLV-RT (GIBCO) in a 20-μl reaction. Two microlitres of cDNA (equivalent of 500 ng RNA) were used per amplification reaction. The PCR mix contained 50 ng of each primer per 50 μl, 250 μM dNTP mix (Pharmacia), 50 mM KCl, 10 mM Tris–HCl pH 8·1, 1·5 mM $MgCl_2$, 0·01% gelatin, 1·25 unit Taq polymerase (GIBCO), and was covered with 20 μl mineral oil. Reactions were performed in 50 μl per well in microtest plates (Becton Dickinson, Mountain View, CA) in a PTC-100 thermal cycler (MJ Research Inc, Watertown, MA), using the following programme of 35 cycles: denaturation 94°C for 1 min, annealing 55°C for 1 min, extension 72°C for 1 min. Hot-start PCR (GIBCO) was performed according to the manufacturer's instructions. Twenty microlitres of each PCR reaction were loaded on 1·4% agarose gels in TBE buffer and PCR products were visualized by ethidium bromide staining. For Southern analysis of the PCR products, gels were denatured in 0·5 M NaOH, 1·5 NaCl, neutralized in 0·3 M Tris, 3 M NaCl pH 7·0, and transferred to nylon membranes. Membranes were prehybridized as described for Northern analysis (0·1% SDS) before addition of radio-labelled specific probes.

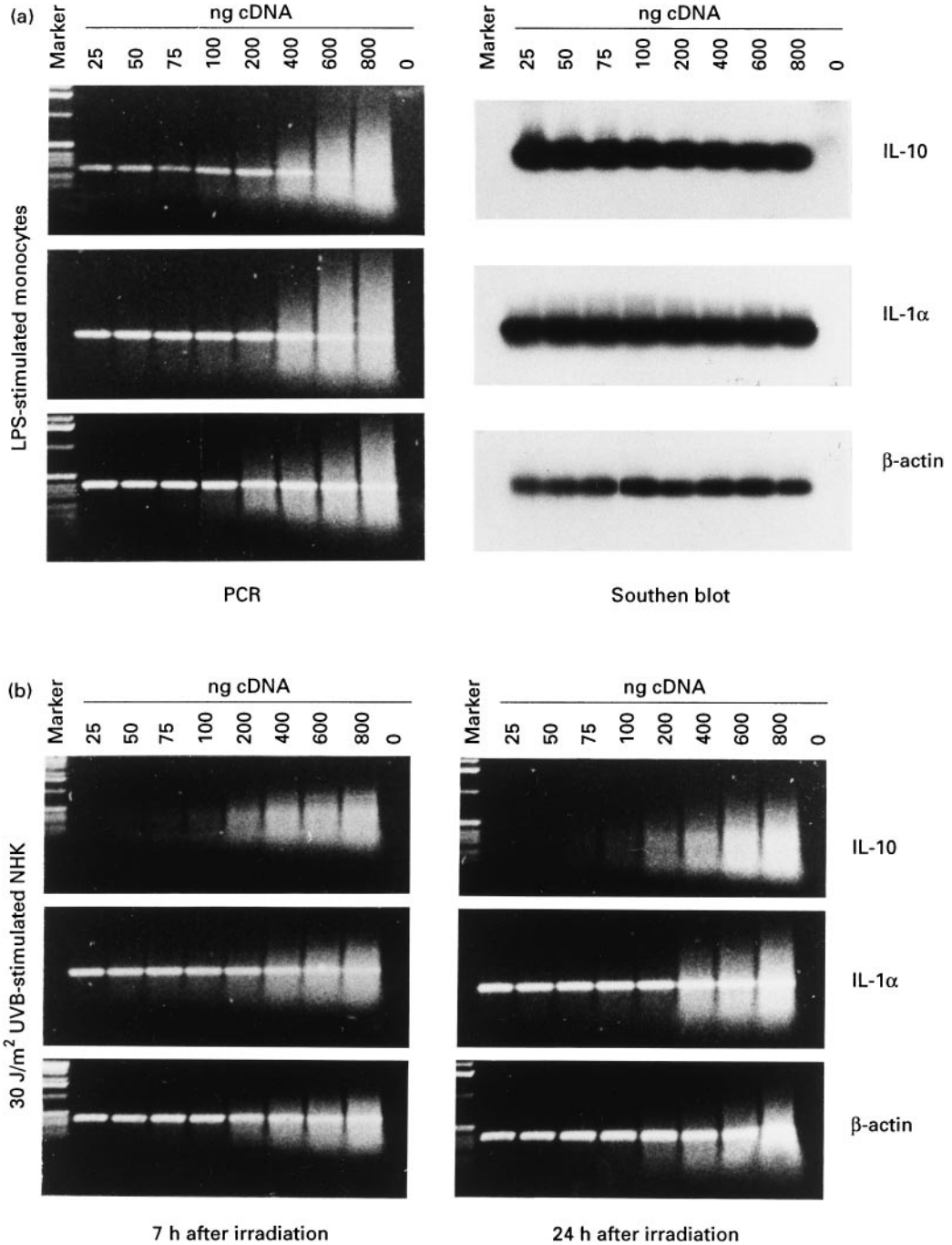

Fig. 1. IL-10 mRNA is present in human peripheral blood monocytes but absent in normal human keratinocytes (NHK). Monocytes (a), used as positive control, were stimulated for 16 h with 1 μg/ml lipopolysaccharide (LPS) before total RNA was isolated. (b) NHK were stimulated with 30 J/m^2 UVB and total RNA was extracted 7 h or 24 h post-irradiation. RNA was reverse transcribed to cDNA and indicated nanograms of cDNA were subjected to polymerase chain reaction (PCR) amplification, applying IL-10, IL-1α or β-actin-specific primers. The PCR reaction products were loaded on a 1·4% agarose gel, and after electrophoresis PCR products were visualized by ethidium bromide staining. Southern blot analysis of the PCR products with specific probes confirmed the specificity of the PCR products.

IL-10 ELISA

The production of human IL-10 protein was determined by four different specific ELISAs, each of which was composed of different catching and detecting antibodies. We used commercially available ELISA kits from Medgenix Diagnostics (Fleurus, Belgium) and Bender MedSystems (Vienna, Austria) and two home-made ELISAs as described in detail by Abrams *et al.* [29] (optimized by Van der Poll *et al.* [30]) and by Llorente *et al.* [31]. The minimal detectable levels of the former two are 1 pg/ml and of the latter three 10–50 pg/ml. Presence of murine IL-10 was determined by an ELISA purchased from Bender MedSystems, having a detection level of 10 pg/ml.

IL-10 bioassay

IL-10-specific bioactivity in supernatants or cell lysates from both human and murine keratinocyte cultures (24 h and 48 h) was determined by the IL-10-dependent mouse mast cell line D36 [32]. IL-10 can be measured in a concentration range from ≈ 10 U/ml to 0·05 U/ml: 1 U/ml corresponds to 100–200 pg/ml rmIL-10 and to 3–6 ng/ml rhIL-10.

RESULTS

Absence of IL-10 mRNA in human keratinocytes and epidermoid cell lines

The initial series of experiments was designed to detect IL-10-specific mRNA in total RNA derived from NHK and A431 by means of Northern blot analysis. No IL-10 mRNA could consistently be found in NHK irrespective of their passage number, the presence of any stimulus, or at any time point after stimulation, ranging from 1 h to 72 h (data not shown). Therefore, experiments were repeated applying the much more sensitive RT-PCR technique which was optimized for the detection of IL-10 mRNA. As shown in Fig. 1a, total RNA from human LPS-stimulated monocytes, one of the two positive controls used in these RT-PCR studies, showed a clear IL-10 PCR product of the appropriate size which hybridized with an IL-10 probe. Consistently, no IL-10 signal was observed after RT-PCR analysis of RNA extracts from NHK (Fig. 1b) and human keratinocyte cell lines A431 and HaCaT, even when the cDNA input was increased up to 800 ng or the PCR performance was extended to 50 cycles (data not shown). The smears shown in the lanes containing 200–800 ng cDNA were caused by cDNA input itself and not by amplification during PCR. Because these smears could mask any possible IL-10 PCR product, Southern blot analysis of the PCR-amplified samples of NHK was carried out, demonstrating that IL-10 was absent (data not shown). To exclude the possibility that absence of a RT-PCR signal was due to a particular primer set, four different IL-10-specific primer pairs were used. No IL-10 mRNA could be detected in any of the NHK-derived RNA extracts tested, although these four primers were successfully applied to detect IL-10 mRNA in Th0 and Th2 clone cells and monocytes. This lack of IL-10 mRNA was certainly not due to bad quality of the RNA derived from the NHK or cell lines, as indicated by the clear IL-1α and β-actin signals in these RNA extracts (Fig. 1b).

To investigate whether NHK were actually stimulated, total RNA extracts from stimulated NHK were screened for IL-1α transcripts by means of Northern analysis. NHK showed a significant constitutive expression of IL-1α, and the production of this cytokine was markedly increased after stimulation (Fig. 2), indicating that the cells were properly stimulated. Despite the fact that UVB is harmful, limited doses of radiation appeared to be a good stimulus for IL-10 production in murine keratinocytes [14]. To evaluate the phototoxic effect of UVB on NHK, viability and proliferation were determined 3 days after exposure to graded single doses of UVB. As shown in Fig. 3, doses up to 64 J/m^2 UVB hardly affected the viability, whereas a high dose of 512 J/m^2 killed only 50% of the NHK. UVB appeared to affect the proliferative capabilities of NHK more significantly. Proliferation was unaffected at doses up to 32 J/m^2, but higher doses clearly inhibited and even blocked multiplication of still viable NHK. In addition, UVB doses higher than 32 J/m^2 resulted in a reduction of the total RNA yield 24 h post-irradiation. In most of our experiments we applied the relatively safe, though stimulating, dose of 32 J/m^2, thus excluding deleterious effects of UVB.

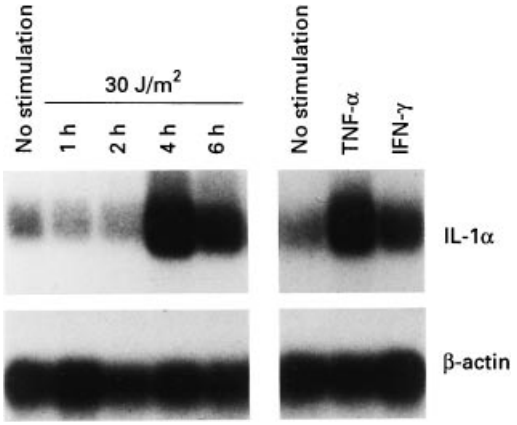

Fig. 2. Stimulation of cytokine production of normal human keratinocytes (NHK) by UVB irradiation. One to 6 h after exposure to 30 J/m^2 UVB radiation, or 16 h after incubation with 0·5 ng/ml tumour necrosis factor-alpha (TNF-α) or 1000 U/ml IFN-γ, total RNA was extracted from the stimulated NHK. The RNA was subjected to Northern blot analysis using specific probes for IL-1α and β-actin.

Comparison of human and murine keratinocytes

Because other investigators clearly showed IL-10 mRNA production by murine keratinocytes or PAM212 cells [13,14] and we failed to detect IL-10 mRNA in human NHK and A431 and HaCaT cell lines, we felt it necessary to compare both murine and human keratinocytes, applying similar culture and RT-PCR conditions within one experiment. As shown in Fig. 4a, IL-10 mRNA was not present in freshly isolated murine keratinocytes (lanes 1 and 7), but a clear message for IL-10 became detectable after overnight culture of these cells (lanes 2–4 and 8–11). It appeared that even in absence of any stimulus the murine keratinocytes were triggered to synthesize IL-10 mRNA, perhaps as a result of the stress resulting from taking these cells into culture. Titration of cDNA from

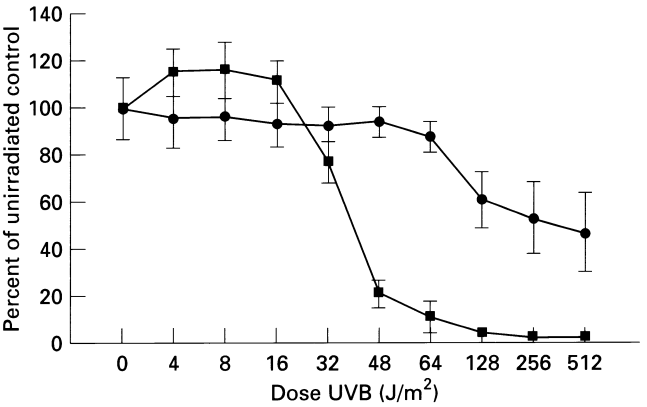

Fig. 3. Sensitivity of normal human keratinocytes (NHK) to UVB radiation. NHK were irradiated with graded doses of UVB and viability (●) and proliferation (■) were determined 3 days post-irradiation. Viability was assessed by eosin dye exclusion and proliferation by means of liquid scintillation counting of incorporated ^{3}H-thymidine. Data are mean results (± s.d.) of three experiments and are expressed as percentage of unirradiated control.

murine keratinocytes in a time-course study indicated that expression of IL-10 mRNA was easily detectable at all time points (2–24 h) after stimulation, using as little as 1·5 ng cDNA for PCR amplification (data not shown). In sharp contrast, no IL-10 mRNA could be detected in NHK (Fig. 4b), despite murine and human keratinocytes being tested under comparable conditions.

Attempts to detect IL-10 mRNA in NHK via different approaches

In the next series of experiments several strategies were used to enhance possible production or to improve detection of IL-10 in NHK. To increase the sensitivity of our RT-PCR system, we purified mRNA out of the total RNA fraction derived from NHK, but nevertheless IL-10 mRNA consistently remained undetectable in NHK (Fig. 5). Although all four primer sets were screened in GenBank for exclusive homology with IL-10 mRNA, we next analysed the possibility that RNA from NHK contained particular unknown sequences which are (partially) complementary to the IL-10-specific primers and consequently would inhibit the PCR amplification of IL-10 mRNA. To test for this, serial dilutions of cDNA from Th2 were mixed with a constant amount of 100 ng cDNA from NHK before amplification by PCR. This experiment revealed that when as little as 0·1 ng of cDNA from Th2 cells was amplified in the presence of 100 ng cDNA from NHK, a positive signal for IL-10 could still be observed (Fig. 6). Because IL-10 has strong down-regulatory effects on its own production [24] and we found that NHK constitutively express IL-10R (data not shown), we added neutralizing anti-IL-10 MoAb 19F1 to NHK cultures, to prevent this autocrine negative feedback. This approach was not successful (data not shown). It has been reported [33,34] that IL-10 mRNA expression could be greatly enhanced and stabilized by addition of cycloheximide, a protein synthesis inhibitor. However, no IL-10 mRNA could be detected in the RNA from NHK, maintained in the presence of this agent (data not shown). Hydrocortisone, one of the constituents of KGM, has been demonstrated to suppress IL-6 production by NHK [35]. Analysis of RNA from NHK, which were maintained in hydrocortisone-free medium, did not result in the detection of IL-10 mRNA (Fig. 7). Because the state of confluency might be important for the capacity of NHK to produce cytokines and the responsiveness of NHK to UVB [36], we tested subconfluent (60–90%) and confluent NHK cultures, but no IL-10 mRNA was found (Fig. 7). Although in most of our experiments we used the low but effective dose of 32 J/m^2, we also applied higher doses (up to 200 J/m^2), because the dose of UVB might be critical. Using higher doses of UVB ($n = 9$) did not lead to induction of IL-10 mRNA in NHK

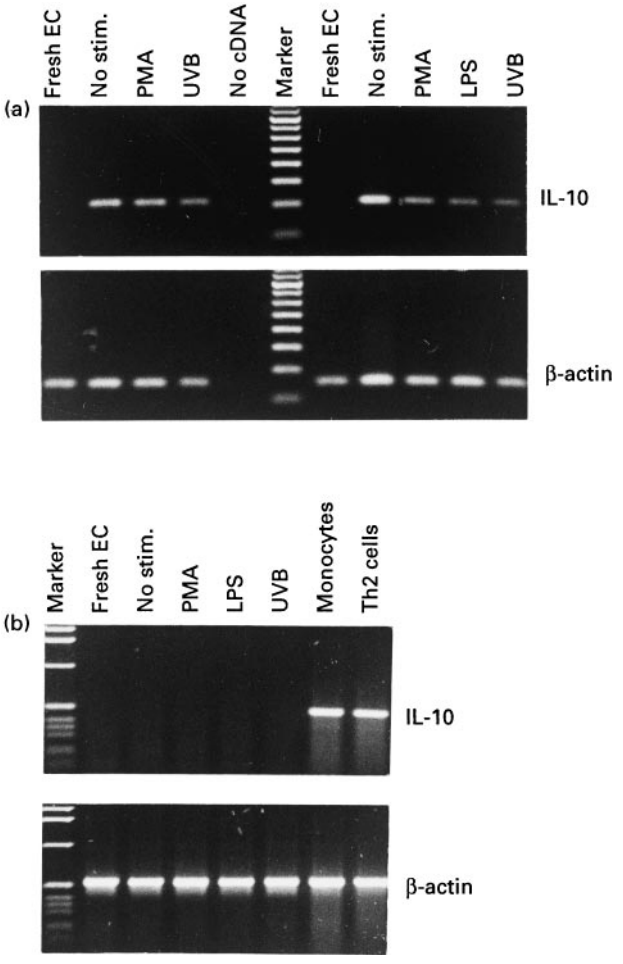

Fig. 4. IL-10 mRNA is present in murine keratinocytes but absent in human keratinocytes when tested under comparable conditions. (a) Murine keratinocytes were isolated from ears of BALB/c (lanes 1–4) or Swiss (lanes 7–11) mice. (b) Human keratinocytes were isolated from breast skin. Total RNA was extracted directly after isolation of the cells or after overnight culture upon indicated stimulation. RNA from stimulated human monocytes and Th2 cells served as positive controls for the IL-10 signal. RNA was reverse transcribed to cDNA and amplified with specific murine or human primer sets for IL-10 and β-actin. Following electrophoresis the amplification products were visualized by ethidium bromide staining. PMA, Phorbol myristate acetate; LPS, lipopolysaccharide.

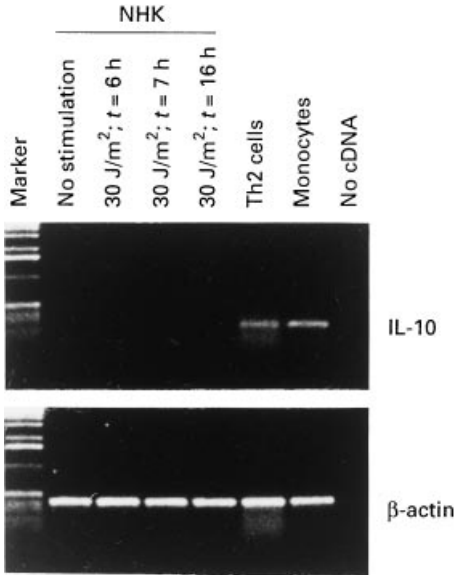

Fig. 5. IL-10 signal absent in purified mRNA from human keratinocytes. Normal human keratinocytes (NHK) from foreskin were exposed to 30 J/m^2 UVB radiation and total RNA was extracted 6, 7 or 16 h later. By means of oligo(dT) conjugated magnetic beads mRNA was enriched from this total RNA. Purified mRNA was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) using IL-10 and β-actin-specific primers. Total RNA from Th2 cells and monocytes served as positive controls. Amplification products were visualized by ethidium bromide staining following electrophoresis.

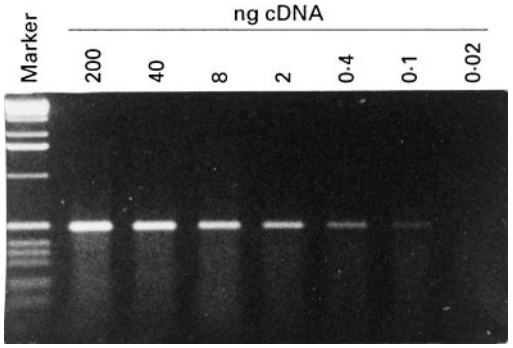

Fig. 6. Detection of IL-10 polymerase chain reaction (PCR) product in normal human keratinocyte (NHK)-derived cDNA mixed with T cell-derived cDNA. Total RNA from Th2 cells and from UVB-exposed NHK (7 h post-irradiation with 60 J/m^2 UVB) were reverse transcribed to cDNA. The T cell-derived cDNA was serially diluted (as indicated) in a constant amount of 100 ng cDNA from NHK. These mixed samples were subjected to PCR amplification and PCR products specific for IL-10 were detected in agarose gels by ethidium bromide staining.

(Fig. 7). Our results thus far indicated that if mRNA for IL-10 is present in NHK, it is present at a very low concentration. Especially for the detection of low copy number targets, a so-called Hot Start PCR was developed, providing improved sensitivity/specificity [37]. However, this method also did not lead to IL-10 mRNA detection in NHK (data not shown). In a very recent publication, it was suggested that UVA1 or UVB in combination with high Ca^{++} concentration would be the strongest stimulus for IL-10 production by NHK [38]. Unfortunately, we were not able to demonstrate this (data not shown). In summary, all our attempts to detect IL-10 mRNA in NHK failed, irrespective of the approach used and having excluded interindividual differences (40 donors), sex differences (male or female), topographic differences (skin from breast, abdomen, scalp, foreskin, leg) and having applied a variety of stimuli. There was one exception: when the NHK-derived cDNA was amplified using an IL-10-specific RT-PCR of 35 cycles and subsequently subjected to a second amplification round of 35 cycles, a weak IL-10 signal could be observed. Assuming that NHK were the origin of this IL-10 mRNA, this finding would suggest that the expression is extremely low.

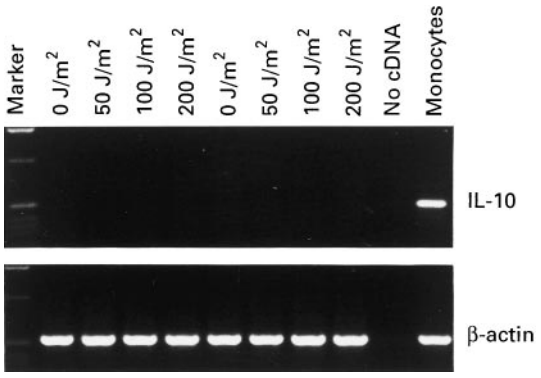

Fig. 7. IL-10 mRNA is not present in subconfluent and confluent normal human keratinocytes (NHK) cultured in hydrocortisone-free medium. NHK from breast skin were maintained under hydrocortisone-free conditions for at least two passages. At a confluent stage of 60% (lanes 2–5) or 100% (lanes 6–9), NHK were irradiated with 0, 50, 100, or 200 J/m^2 UVB. The RNA was extracted 16 h later and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) using IL-10 and β-actin primer sets. Monocyte-derived RNA (lane 11) served as positive control. Amplified products were analysed by electrophoresis and ethidium bromide staining. Identical results were obtained when NHK were maintained in keratinocyte growth medium (KGM), which contains hydrocortisone (data not shown).

Absence of IL-10 protein in human keratinocyte-derived supernatants and cell lysates

Concurrently with the RT-PCR analysis of keratinocyte-derived RNA, we tested culture supernatants and cell lysates of NHK, A431 and HaCaT, for the presence of IL-10 protein by four different IL-10-specific ELISAs. In order to determine whether NHK-derived samples possess IL-10 bioactivity, supernatants and lysates were also tested in a recently developed IL-10-specific bioassay [32]. Keratinocytes, either freshly isolated or cultured for up to four passages, were stimulated with a variety of chemicals (see Materials and Methods) or exposed to UVB (up to 200 J/m^2), UVA or UVA1, and 24, 48 and 72 h later, supernatants and lysates were collected. We were consistently unable to detect IL-10 protein in any NHK-derived culture supernatant or cell lysate, irrespective of culture conditions, the stimulus used, the harvesting time-point or the site NHK originated from (data not shown). The same held true for A431 and HaCaT cells, which were repeatedly tested. In addition, no IL-10 bioactivity was observed in supernatants from NHK and from both the epidermoid cell lines (data not shown). Cell lysates could not be tested in the bioassay because they strongly inhibited the proliferation of the detector cells of the assay. The positive controls, human monocytes and Th0-type T cells produced clearly detectable amounts of IL-10 (9·64 ng/ml per 10^6 cells and 45·57 ng/ml per 10^6 cells, respectively). Preliminary experiments ($n = 3$) to detect IL-10 protein by means of biosynthetic labelling of NHK with radioactive-tagged methionine and cysteine followed by IL-10-specific immunoprecipitation also failed to demonstrate the presence of IL-10 protein in NHK, whereas monocytes showed a clear signal (data not shown). In normal murine keratinocytes (BALB/c, C57Bl/6 and Swiss), IL-10 protein could easily be detected in 24-h culture supernatants by ELISA ($\approx$ 300 pg/ml per 10^6 cells). The murine PAM212 cells constitutively produced IL-10, and upon stimulation were able to secrete up to 128 U/ml per 10^6 cells. Absence of IL-10 protein in NHK supernatants and significant presence of this cytokine in supernatants of murine keratinocytes are in agreement with the RT-PCR analysis.

IL-10 serum levels following total-body UVB exposure

Because strongly increased levels of IL-10 (a peak of 3 ng/ml at 36 h post-irradiation) have been demonstrated in murine serum after UVB exposure [39], we investigated whether a similar phenomenon could be seen after UVB exposure of humans. Four volunteers were total-body irradiated with 1·5 MED and the IL-10 concentration in serum was tested by ELISA at various time points. Circadian rhythmometry of serum IL-10 in man revealed that the amount of IL-10 in the serum of normal subjects exhibits a biphasic temporal pattern during a 24-h period [40], having an average of 74 pg/ml (range 8–266 pg/ml). Considering this, no significant increase of IL-10 could be demonstrated in humans upon UVB irradiation (Table 1).

Table 1. Serum IL-10 levels following total-body UVB irradiation

	IL-10 (pg/ml)†									
Donor*	−24 h	−12 h	0 h	6 h	12 h	24 h	32 h	48 h	72 h	96 h
JDB	b.d.	b.d.	b.d.	b.d.	15·58	b.d.	b.d.	b.d.	b.d.	92·97
JJ	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	b.d.	43.03	b.d.
CWK	18.36	24.44	36.17	b.d.	15.14	17.55	72.89	38.88	52.49	63.98
MBMT	b.d.	85.4	79.98	44.58	74.82	72	55.76	69.45	103.5	176.9

* Human volunteers were total-body exposed to 1–1·5 minimal erythemal dose (MED) UVB irradiation.
† IL-10 concentration was detected by using a specific ELISA.
b.d., Below the detection limit of 10 pg/ml.

Identification of the origin of IL-10 mRNA in human epidermal cell suspensions

Just prior to submission of this study, four different research groups stated that NHK are able to produce significant amounts of IL-10 mRNA and protein [38,41–44]. These findings seem to contradict our own, and this prompted us to attempt to resolve this conflict. Our observation that the IL-10 signal could only be detected in NHK-derived RNA after a second round of PCR may suggest that not the NHK but rather a contaminating trace cell population was the source of this signal. Fresh preparations of NHK contain Langerhans cells, melanocytes and, occasionally, fibroblasts. Long-term cultures of NHK maintained in special commercially available KGM always contain small contaminations of melanocytes that co-proliferate at a low rate. To determine the origin of the mRNA signal in NHK suspensions, we tested pure populations of NHK, melanocytes, Langerhans cells and fibroblasts. Equal amounts of RNA from the individual populations were subjected to our routine RT-PCR analysis of 35 cycles. As shown in Fig. 8, only melanocytes were able to express detectable amounts of IL-10 mRNA, whereas neither in NHK (purified mRNA) nor in Langerhans cells (fresh and cultured) and fibroblasts could such a signal be demonstrated. Even after application of a second amplification round of 35 cycles, the IL-10 signal in the latter three cell types remained absent. Next, we investigated whether melanocytes can produce significant amounts of IL-10 protein. It appeared that no IL-10 could be detected by ELISA (detection limit 10 pg/ml) in 24 h or 48 h supernatants from melanocytes (10^6/ml) that were cultured for 24 h or 48 h and were unstimulated or stimulated by PMA, IFN-γ or UVB ($n = 2$; data not shown).

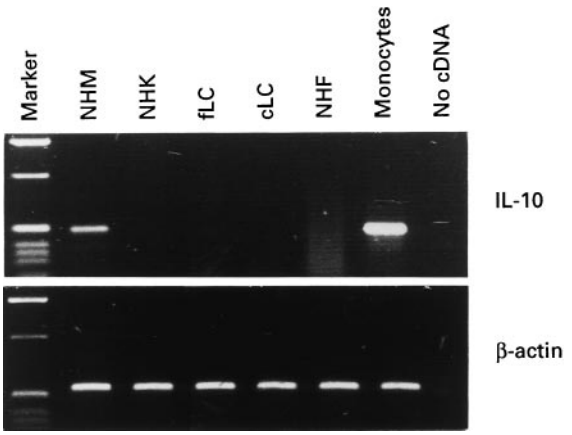

Fig. 8. Human melanocytes are the only source of IL-10 mRNA in epidermal cell suspensions. RNA was extracted from pure suspensions of normal human melanocytes (NHM), normal human keratinocytes (NHK), freshly isolated or cultured Langerhans cells (fLC and cLC, respectively) or normal human fibroblasts (NHF). Monocyte-derived RNA served as positive control. Total RNA (purified mRNA in case of NHK) was reverse transcribed to cDNA and amplified by 35 cycles using IL-10 and β-actin primer sets. Polymerase chain reaction (PCR) products were loaded on an agarose gel and visualized by ethidium bromide following electrophoresis.

DISCUSSION

In this study we addressed the question of whether human keratinocytes and keratinocyte cell lines A431 and HaCaT are able to produce IL-10 and, if so, whether keratinocyte-derived IL-10 in humans is involved in UVB-induced immunosuppression, comparable to that demonstrated in mice. NHK, derived from 40 different donors, were maintained/stimulated under many different conditions and analysed for their capability to express IL-10 mRNA or protein by means of routine RT-PCR and ELISA or bioassay. No IL-10 mRNA or protein could be observed in any of the NHK-, A431-, or HaCaT-derived RNA and supernatants/lysates, whereas in all cases the positive controls (monocytes or T cell clones) showed a clear signal. Despite our many efforts to induce or enhance production or to improve detection of IL-10, no IL-10 signal could be consistently demonstrated in NHK. There was one exception, however. When the amplified cDNA from NHK was subjected to a second round of PCR amplification a weak signal could be observed. We reasoned that either the expression of IL-10 mRNA must be extremely low in NHK, that only a very small subpopulation of NHK was able to express IL-10, or that a trace number of contaminating cells in the NHK suspension was the source of the observed IL-10 mRNA signal. Freshly prepared suspensions of NHK are always contaminated with melanocytes and Langerhans cells and occasionally with fibroblasts, whereas long-term cultures of NHK maintained in special commercially available KGM always contain a small number of co-proliferating melanocytes. RT-PCR analysis of pure NHK, melanocytes, Langerhans cells and fibroblast suspensions provided conclusive proof that within normal epidermis only melanocytes were able to synthesize IL-10 mRNA. Our finding that melanocytes express IL-10 mRNA is in agreement with the observation of other investigators [45,46]. We and others [45] could not detect IL-10 protein in the supernatants of normal human melanocytes. As yet it

is unknown whether the melanocytes were not appropriately stimulated, whether the melanocyte-derived IL-10 protein was below the detection limit of our ELISA, or whether cells could not secrete this protein at all.

Our extensive search clearly proved that IL-10 is not expressed by normal keratinocytes and by keratinocyte cell lines in the human system. In marked contrast, a clear expression of IL-10 mRNA was found in murine keratinocytes, when cultured under comparable conditions and tested by the same RT-PCR technique. Enk & Katz [13] depleted contaminating cells in murine epidermal cell suspensions by means of cell type-specific MoAb plus complement, and found that murine keratinocytes were the major source of IL-10 in the epidermis. Comparison of our data on mice and man revealed that murine IL-10 mRNA could be demonstrated at all time points after stimulation (from 2 h to 24 h) using as little as 0·1 ng/ml cDNA, whereas the application of even 10^5 times more cDNA from NHK as PCR input did not lead to the appearance of an IL-10 mRNA signal at any time point tested (from 1 h to 72 h). The detection of IL-10 protein in crude 24-h supernatants from murine keratinocytes and in the murine epidermoid cell line PAM212 is in line with our PCR results and confirms earlier observations [13].

Our results on IL-10 strongly point to a species difference between human and murine keratinocytes. Recently, a similar discrepancy was found for the production of IL-3. Murine keratinocytes were demonstrated to produce biologically active IL-3 [47], whereas this cytokine was not detectable in human keratinocytes, as detected by RT-PCR, ELISA and bioassay [48]. We have to conclude that human and murine keratinocytes have a different pattern of cytokine production. Consequently, results on cytokine production by murine keratinocytes, as well as interpretations of the function of the murine epidermal cytokine network in the skin immune system, can not directly be extrapolated to the human system, but should be evaluated with caution.

To our surprise, four independent research groups recently reported that NHK were able to express IL-10 mRNA and protein [38,41–44]. Although at first sight these reports appear to disagree with our findings, we can nevertheless explain how the results in these reports and our data fit together. Nickoloff *et al.* demonstrated that after repeated tape stripping of human skin, IL-10 could be detected in the epidermis by means of RT-PCR [41] and immunohistochemistry [42]. However, they showed that mRNA for IFN-γ was present in the epidermal RNA extract as well. This means that the epidermis used contained significant amounts of T cells, which are notorious producers of IL-10. We have found that NHK express IL-10R (unpublished observation), so a positive staining for IL-10 in epidermal keratinocytes does not necessarily mean that the detected IL-10 originated from keratinocytes. It is not unlikely that the keratinocytes collected this cytokine out of their surrounding micromilieu by their IL-10R. Kang *et al.* [43] found IL-10 mRNA in NHK enriched from skin that had been UVB-exposed *in vivo* 48 or 72 h before. Coincidently, the same research group earlier demonstrated that UVB radiation induces the influx of $CD1a^-DR^+CD36^+CD11b^+$ macrophages into the epidermis, starting 24 h post-irradiation and comprising more than 5% of epidermal cells at 72 h [49–51]. Given the facts that (i) the increase and peak of the IL-10 mRNA signal correlates with the appearance of the macrophages in the epidermis, (ii) these macrophages are potent producers of IL-10, and (iii) the purity of the magnetic bead-selected NHK suspension was stated to be $>90\%$ (not 100%), it is tempting to believe that the IL-10 mRNA detected by Kang *et al.* [43] must be macrophage-derived. In contrast to our negative results, Grewe *et al.* [38] and Enk *et al.* [44] detected IL-10 mRNA in long-term cultures of NHK and in five out of nine blister roofs from non-irradiated skin by RT-PCR analysis. Comparison of their experimental set-up with ours revealed that in essence we all used the same culture conditions, but that the RT-PCR performance and visualization of the PCR products were different. We used ethidium bromide to stain these products in agarose gels, whereas both other groups used other techniques: endonuclease digestion assay followed by high performance liquid chromatography [38] and liquid hybridization with a ^{32}P-labelled internal probe followed by autoradiography [44]. Because Grewe *et al.* and Enk *et al.* were able to detect IL-10 mRNA in all their NHK cultures after a single round of PCR amplification and we needed two rounds to pick up the IL-10 signal, it might be concluded that they used a more sensitive technique. However, they did not use pure NHK suspensions and did not exclude the possibility that contaminating cells are the source of the detected IL-10. Therefore, it is not inconceivable that they detected a melanocyte-derived IL-10 signal. This notion is supported by our finding that an IL-10 mRNA signal is present in routine NHK cultures (containing melanocytes), but absent in pure NHK cultures (deprived of melanocytes) after two successive rounds of PCR amplification.

As outlined in the Introduction, in mice, keratinocyte-derived IL-10 is presumably involved in the mechanism of UVB-induced local and systemic immunosuppression. Our results indicate that in man keratinocytes can not fulfil such a role via IL-10, but perhaps another, as yet unidentified, cytokine might mediate this role. It is not likely that human melanocyte-derived IL-10 has any significant function in UVB-induced immunosuppression. Macrophages that infiltrate human epidermis upon UVB exposure display a potent production and secretion of IL-10, and therefore may account for a local immunosuppressive environment [43]. Because we could not detect any increase of IL-10 serum levels after total-body exposure to UVB it is not likely that these macrophages could account for systemic suppression. Taken together, the data in this study give rise to the hypothesis that, compared with mice, UVB-induced immunosuppression in man is probably exerted by a different network of keratinocyte-derived cytokines.

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