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IL-24 is Expressed During Wound Repair and Inhibits TGF α induced Migration and Proliferation of Keratinocytes

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Abstract

Interleukin (IL)-24 is the protein product of melanoma differentiation-associated gene 7 (MDA-7). Originally identified as a tumor suppressor molecule, MDA-7 was renamed IL-24 and classified as a cytokine because of its chromosomal location in the IL-10 locus, its mRNA expression in leukocytes, and its secretory sequence elements. We previously reported that IL-24 is expressed by cytokine-activated monocytes and T lymphocytes. Here, we show that IL-24 is expressed in keratinocytes during wound repair. Paraffin-embedded tissues prepared from human skin sampled at days 2, 6, and 10 after wounding were examined by immunohistochemistry for expression of IL-24. Protein expression was detected in the keratinocyte population with maximum expression at days 2 and 6; and no expression by day 10 (4 of 4 subjects). In vitro studies showed that cytokines involved in wound repair, most notably TGF α , TGF β , IFN γ and IFN β , upregulated IL-24 protein expression in normal human epidermal keratinocytes (NHEK). Examination of the function of IL-24 in both in vitro wound repair and migration assays demonstrated that IL-24 inhibits TGF α induced proliferation and migration of NHEKs. These data support the hypothesis that IL-24 functions during an inflammatory response in the skin by inhibiting the proliferation and migration of keratinocytes.

Keywords

Wound repair; keratinocyte; Interleukin-24; cytokines

Introduction

At least five cellular genes have been found that encode secreted proteins with 20% to 33% homology to IL-10: IL-19 (1), IL-20 (2), IL-22 (3,4), IL-26 (5), and *mda-7*(6), which was renamed IL-24 with the approval of the HUGO Gene Nomenclature Committee (7,8). IL-10, IL-19, IL-20, IL-24 are encoded within a 195-kilobase cytokine cluster on chromosome 1q31/32. The relatively low amino acid sequence homology among the structurally related homodimeric IL-10 family members suggests that they have quite different biological functions, but those biological functions are still under investigation. All IL-10 family members use the class II family of receptors and signal through STAT1, STAT3, or both

(1,2,4,8–10). The class II receptor subunits have been shown to be involved in inflammatory conditions in the skin, including psoriasis and eczema (2,11). In general it is thought that the IL-10 family of cytokines is involved in the regulation of inflammatory and immune responses (10,12).

IL-24 protein is endogenously expressed in normal human skin melanocytes, implying that IL-24 is involved in skin biology (13). In addition, receptors for IL-24 have been detected on human keratinocytes (8). The relationship of IL-24 to IL-10 and IL-24's predicted cytokine features led us to study IL-24 expression in immune cells. We and others have reported that IL-24 protein is expressed by human T cells and peripheral blood monocytes in response to mitogen or antigen stimulation (7,14–16) which suggests that IL-24 is part of a cytokine network expressed during activation of the human cellular immune response.

IL-19, IL-20, IL-24 and IL-22 are all up-regulated in psoriatic skin lesions. Psoriasis is characterized by abnormal hyperproliferation and differentiation of keratinocytes. Both IL-20 and IL-22 promote these effects *in vivo* and *in vitro*, making them targets for therapy (2,17–19). IL-20 transgenic mice displayed abnormal epidermal proliferation and differentiation when IL-20 was expressed in the epidermis (2). IL-22 caused increased proliferation and differentiation of keratinocytes in cultured reconstituted human epidermis (19). The effects of IL-24 are still being examined. The work of Sa and others (20) suggested that IL-24 acts very much like IL-20 and IL-22, causing keratinocyte proliferation as well as acanthosis of reconstituted epidermis under certain conditions. In addition, it was reported by Soo et al that IL-24 was expressed in proliferating fibroblasts during wound healing in a rat model (21).

During wound repair inflammatory cytokines and other proteins are upregulated sequentially leading to the activation as well as inhibition of keratinocyte growth and migration (22, 23). The alpha and beta forms of IL-1 are the most common initiators of keratinocyte activation during wound healing. IL-1 is secreted by the injured keratinocytes as well as by fibroblasts, invading macrophages, and neutrophils. IL-1 in turn stimulates keratinocytes and macrophages in the surrounding dermis to produce and secrete TNF α , a cytokine that functions to maintain keratinocytes in their activated state (23). Once activated, keratinocytes produce other growth factors and cytokines, including TGF α , IL-3, IL-6, IL-8, granulocyte – colony stimulating factor (G-CSF), and granulocyte monocyte colony stimulating factor (GM-CSF). Activated keratinocytes are hyperproliferative, migratory, and show cytoskeletal changes with increasing levels of cell surface receptors. Together with fibroblasts, keratinocytes produce components of the basement membrane essential for re-epithelialization of the injured skin (23).

Since wound repair is accompanied by these rapid changes in cytokine expression we hypothesized that IL-24 would be expressed in cells involved in this process. Given our previous data showing that IL-24 is expressed in cytokine stimulated monocytes we hypothesized that during wound repair we would see expression of IL-24 in the infiltrating macrophages. Therefore, we examined human skin undergoing wound repair for expression of IL-24. Here, we report the results of these studies showing that IL-24 is expressed not only in macrophages but more importantly in keratinocytes during the process of wound repair. We present results of *in vitro* experiments demonstrating that IL-24 inhibits growth factor induced proliferation and migration of keratinocytes.

Material and methods

Cell lines and reagents

Normal human epidermal keratinocytes (NHEKs) were purchased from PromoCell (Heidelberg, Germany) and cultured in serum-free keratinocyte growth medium 2 (KGM-2; PromoCell) supplemented with 0.4% bovine pituitary extract; epidermal growth factor, 0.125 ng/ml; human insulin, 5 µg/ml; hydrocortisone, 0.33 µg/ml; human transferrin, 10 µg/ml; epinephrine, 0.38 µg/ml; and CaCl₂, 0.15 mM. For proliferation and migration experiments, keratinocyte basal medium (KBM; PromoCell) was used as the factor-free medium. All cells were used at low passage number (less than five) and were grown in humidified 5% CO₂ at 37°C. Human TNFα, IFNα, IFNβ, IFNγ, and IL-1β were purchased from eBiosciences (San Diego, CA), hepatocyte growth factor (HGF) was purchased from R&D Systems (Minneapolis, MN), and TGFα and TGFβ were purchased from BioVision (Mountain View, CA). Recombinant human IL-24 used in our initial in vitro wound repair assays (shown in Figure 3A and B) was purified in our laboratory from stably transfected HEK 293 cells by affinity chromatography following procedures described previously (24). IL-24 (used in all other experiments) was purchased from R&D Systems as were IL-20 and IL-22. All other chemicals and reagents were purchased from Sigma Aldrich (St. Louis, MO). Antibodies directed against IL-20R1 and IL-22R1 (goat IgG) were purchased from R&D Systems. The monoclonal antibody against IL-24 has been previously described (24).

Human wound repair and normal skin samples

Four normal adult volunteers, who gave informed consent under an institutional review board-approved protocol which followed the Declaration of Helsinki protocols, had three short scalpel incisions made on the upper arm that penetrated through the dermis and epidermis each long enough to allow for one subsequent punch biopsy. Wounds were then covered and allowed to heal. At days 2, 6, and 10 after wounding, biopsy samples were obtained using a 3.5-mm punch. These tissues were formalin-fixed and embedded in paraffin following standard procedures. Because day 0 skin biopsies were not obtained with this protocol, normal skin samples, from 5 individuals, left over after pathological examination were collected under the supervision of a dermatopathologist to serve as normal controls for these assays.

Immunohistochemistry

Sections, 3 µm thick, were cut from tissue blocks of the formalin-fixed, paraffin-embedded skin obtained from the four human volunteers. Immunocytochemical staining of deparaffinized, fixed slides was performed after antigen retrieval by heating (microwave) in citrate buffer. Slides were incubated with biotinylated goat anti-mouse IgG followed by avidin:biotinylated peroxidase complex (ABC reagent, Vector Laboratories, Burlingame, CA). The peroxidase was developed by 3-amino-9 ethylcarbazole 9 (red color, Vector Laboratories) and counterstained with Gill's hematoxylin (Vector Laboratories). Isotype-matched antibodies were used as negative control antibodies.

Keratinocytes, macrophages, and melanocytes were identified on the basis of morphology as well as staining with cell type-specific antibodies. Results included determinations of expression level. The following antibodies were used: anti-cytokeratin (1 µg/ml, clones AE1 and AE3, Millipore, Billerica, MA); anti-CD68 (0.4 µg/ml, clone PG-M1, DAKO, Carpinteria, CA); anti-gp100 (0.5 µg/ml, HMB45, BioGenex, San Ramon, CA); and anti-IL-24 (5 µg/ml, clone 7G11) (14). Optimal concentrations were determined empirically by staining cultured cell lines or PBMCs in cytopins. Four different fields were examined under high power (40X), with 100 cells counted in each field. Results were recorded in terms of the percentage of each cell type with positive immunostaining for each marker,

where 4+ indicated 75%–100% of cells positive in all fields read; 3+ indicated 50%–75% positive; 2+ indicated 25%–50% positive; and 1+ indicated 5%–25% positive. Results on immunostaining were considered negative when fewer than 5% of cells in all fields read were positive.

Detection of IL-24 protein

Intracellular IL-24 protein was detected by intracellular FACS analysis using a fluorescein isothiocyanate-conjugated monoclonal antibody directed against the amino-terminus of the IL-24 protein (14). Cells were treated with brefeldin A (Sigma-Aldrich) at 10 µg/ml 4 h before harvest to prohibit secretion of the cytokine. To detect intracellular IL-24, cells were fixed by treatment with 4% paraformaldehyde, permeabilized with the detergent n-octyl glucopyranoside at 7 mg/ml, and then stained with fluorescein isothiocyanate-anti-IL-24. As the controls for permeabilization, cells were also stained with monoclonal antibodies directed against the intracellular proteins vimentin or keratin (BioGenex). Standard protocols were followed for immunofluorescence staining. Immunofluorescence was analyzed on a FACSCalibur with Cell Quest software (BD Biosciences, Mountain View, CA). Secreted IL-24 was measured by ELISA (R&D Systems, Minneapolis, MN) following the manufacturer's protocol.

Immunoprecipitation and immunoblotting

NHEK cells were grown to 80% confluency in 6 well plates, growth factor starved for 48 h in keratinocyte base media (KBM), and then treated with IL-24 at 25 and 100 ng/ml for 1 h in KBM. NHEK were then treated with TGFα at 25 ng/ml for 5 min. Cells were then lysed in buffer containing 50mM Tris (pH7.9), 150mM NaCl, 1% NP40, 1mM EDTA, 10% glycerol, and protease inhibitor cocktail (Roche Pharmaceuticals, Nutley, NJ). Total protein was determined and 500 µg was immunoprecipitated with 2 µg/ml anti EGFR antibody (Neomarkers, clone Ab-13, Fremont, CA) overnight at 4°C followed by protein recovery using Protein A agarose beads following standard procedures. The immunoprecipitated proteins were separated by electrophoresis in a 6% SDS gel, and blotted to Hybond-ECL nitrocellulose membrane (GE Healthcare, Piscataway, NJ). Membranes were incubated with anti phosphotyrosine antibody, PY20, (Santa Cruz Biotechnology, Santa Cruz, CA) and developed using ECL detection reagent (GE Healthcare). Blots were stripped and incubated with anti EGFR (Santa Cruz Biotechnology) to detect total EGFR. The relative expression of phospho EGFR was determined by densitometry and normalized against total EGFR using Scion Image Software,

In vitro wound repair assay

To test cytokine effects on the migration of keratinocytes during wound healing, we used an in vitro model of wound healing. NHEKs were plated in 24-well plates and grown to 80% confluence, after which they were growth factor-starved for 18 h. The monolayers were then scratched with a pipette tip and then the monolayer was washed. At this point, depending on the experiment, monolayers were either incubated or not with mitomycin C at 25 µg/ml for 20 min and washed again. Cytokines were added, and the cells were incubated for 24 to 48 h. The cultures were examined microscopically (20X), and the scratch width was measured with an in-stage micrometer. Results are reported as the average of five measurements for each culture condition.

Migration assays

NHEKs, 1×10^5 cells in 0.5 ml, were plated in factor-free medium with or without IL-24 (25 ng/ml) in the top wells of Boyden chambers with uncoated inserts (BD Biocoat Invasion Chambers, BD Biosciences). TGFα (the chemoattractant) was placed in the bottom wells of

the chambers. After 24-h incubation, cells that had migrated to the other side of the filter were fixed and stained with Harleco Hemacolor (EMD Chemicals, Gibbstown, NJ). The number of migrating cells on each filter was counted under 10X magnification, and the results were reported as the mean of the counts from five independent fields.

Proliferation assays

Cells were seeded at 1×10^4 cells/well in 96-well plates in complete medium. After overnight incubation, the medium was replaced with factor-free medium, and cells were grown for an additional 12–18 h. IL-24, IL-22, and IL-20 were titrated in doses from 1.0 ng/ml up to 100 ng/ml with or without TGF α at 1 ng/ml up to 50 ng/ml or HGF at 50 and 10 ng/ml. All samples were plated in triplicate. After 20-h incubation, ^3H -thymidine (MP Biochemicals, Solon, OH) was added at 1 μCi /well and the incubation continued for 4 h. Cells were harvested, and ^3H -thymidine incorporation was measured by liquid scintillation counting using a Trilux MicroBeta (Perkin Elmer, Waltham, MA). Results were reported as the stimulation index which is equal to the experimental counts per minute/control counts per minute, where the control was the unstimulated or no-treatment wells.

Statistical analysis

Experiments were performed at least two times. The results are presented as the mean of $\pm$ standard deviation of replicate samples from a representative experiment. Comparisons were made using a Student's t-test and a Wilcoxon matched pair test. $P < 0.05$ was considered statistically significant.

Results

IL-24 is expressed by keratinocytes during wound repair

We examined human skin biopsy samples obtained 2, 6, or 10 days after wounding. In samples from all four subjects, the IL-24 expression in keratinocytes (confirmed by keratin staining) peaked between day 2 (Fig 1A) and day 6 (Fig 1B), and by day 10 (Fig 1C), keratinocytes no longer expressed IL-24 (shown in Fig. 1A–C and summarized in Table 1). Figure 1D is a composite image of the H&E stained wounded site. Immunohistochemical stains of day-2 biopsies from patient 2 are also shown in Figure 1. Sequential slides were stained with an IgG control antibody (Fig 1E), antibodies against CD68 to identify macrophages (Fig 1F), against gp100 to identify melanocytes (Fig 1G), against cytokeratin to identify keratinocytes (Fig 1H), and against IL-24 (Fig 1I). As expected, positive staining for IL-24 was seen in macrophages (Fig 1I insert). But the most striking immunostaining for IL-24 was seen in keratinocytes (Fig. 1I and insert). We also examined normal human skin from 5 different individuals for expression of IL-24. Positive staining for IL-24 was seen in melanocytes and low levels of staining in the macrophage (Fig 1J). The keratinocytes in normal skin stained negative for IL-24 (Fig 1J).

Cytokines stimulate the expression of IL-24 protein in keratinocytes

During wound healing, keratinocytes become activated and change from a differentiating cell to an activated cell that migrates, proliferates, and produces and secretes components of the extracellular matrix necessary for healing (25). Given our previous finding that IL-24 expression in macrophages was controlled by cytokines (14), we examined whether cytokines and growth factors involved in the healing process induced the expression of IL-24 protein in keratinocytes. We began these experiments using HaCaT cells, an immortalized keratinocyte cell line. Only TGF α consistently stimulated a significant increase in expression of IL-24 by HaCaT cells (data not shown). We repeated these experiments with NHEKs that had been cultured in medium containing growth factors. The

unique difference between HaCaT cells and NHEKs is that production of the autocrine growth factor TGF α is dramatically decreased in HaCaT cells compared to NHEKs (26). After starving NHEKs in factor-free medium for 24 h, we stimulated them with cytokines, including IL-1 β , TNF α , TGF α , IFN α , IFN β , and IFN γ . We found no difference between the expression of IL-24 in stimulated and unstimulated NHEK as measured by intracellular flow cytometry. Shown in Figure 2A is the expression of IL-24 in TGF α stimulated NHEK. We attributed this to the fact that NHEKs were grown in medium containing growth factors that lead to the production of significant amounts of TGF α (26). NHEKs grown in complete medium without additional cytokine stimulation already expressed significant levels of IL-24 (Fig 2A). We next examined the levels of secreted protein by ELISA in 24-h supernatants of cytokine stimulated NHEKs (Fig. 2B). TGF α , TGF β , IFN β and IFN γ stimulated significant levels of secreted IL-24 protein in NHEKs from 100 to 175 pg/ml ($p<0.05$ when compared to unstimulated NHEK).

IL-24 inhibits TGF α -induced keratinocyte migration in an in vitro wound assay

Next, to determine the effect of IL-24 on the migration of keratinocytes during wound healing, we used an in vitro model of wound healing. For these experiments, we used the procedure described by Koivisto et al. (27) to determine the effect of IL-24 on cell migration. Figure 3A shows a representative experiment with NHEKs. The medium-alone well (a) shows some migration of NHEKs. IL-24 inhibited this migration of NHEKs (Fig. 3A panel c). The addition of TGF α to increase migration resulted in rapid wound closure (Fig. 3A panel b), but this activity was inhibited in the presence of IL-24 by 30% ($P=0.03$; Fig. 3A panel d). To eliminate the possibility that cells were proliferating during the 24 h incubation, NHEKs were treated with mitomycin C. The results of this experiment are shown graphically in Figure 3B. Once again IL-24 inhibited wound closure by keratinocytes. Addition of IL-24 to TGF α -stimulated cells in the presence of mitomycin C caused 64% inhibition of wound closure (Fig. 3B; $*p<0.05$ for wells treated with medium only vs. those treated with IL-24 and/or TGF α).

To confirm that this inhibitory activity was due to IL-24, similar in vitro wounding experiments using NHEKs were performed. Prior to addition of TGF α we added antibodies directed against IL-24 or its receptor subunits IL-20R1 and IL-22R1. Addition of anti-IL-24 and anti-IL-20R1 blocked the inhibitory activity of IL-24 resulting in scratch closure in the TGF α stimulated NHEK while antibody against IL-22R1 did not inhibit the activity of IL-24 (Fig. 3C; $*p<0.05$ for wells treated with TGF α , IL-24, and antibody vs. those treated with TGF α alone). These results demonstrated that IL-24, signaling through IL-20R1/IL-20R2, inhibited TGF α induced migration.

IL-24 inhibits keratinocyte migration and proliferation

To further confirm that IL-24 affects keratinocyte migration we examined the effect of IL-24 on the migration of NHEKs in modified Boyden chambers. For these experiments we tested both TGF α (50 ng/ml and 10 ng/ml) and KGM-2 as chemoattractants in the bottom well of the chambers; only TGF α at 50 ng/ml and KGM-2 caused significant migration of NHEKs compared to unstimulated NHEKs. When NHEKs were cultured with increasing amounts of IL-24, from 1 ng/ml to 25 ng/ml, migration toward TGF α at 50 ng/ml was significantly inhibited in a dose dependent manner (Fig 4A). Shown in Figure 4 are the results from one lot of keratinocytes. To determine if the inhibition of TGF α migration caused by IL-24 varied between NHEK donors we repeated these experiments 5 times using NHEK from 3 different donors. The mean TGF α induced migration ($\pm$ standard deviation) was 46.7 $\pm$ 29.5. When IL-24 was added to these cultures at 25 ng/ml the mean number of migrating NHEK was 21.1 $\pm$ 14.3. Using a Wilcoxon matched pair test $p=0.0625$, when migration with IL-24 was compared to migration without IL-24. These experiments show that IL-24

inhibited TGF α induced migration although the level of inhibition varied between experiments and from donor to donor.

HGF has been reported as a strong chemoattractant for keratinocytes that is also produced by dermal fibroblasts and keratinocytes during wound repair (28,29). When NHEKs were cultured with increasing amounts of IL-24, migration toward HGF at 50 ng/ml was inhibited but this response was not statistically significant (Fig 4B).

IL-24, IL-22, and IL-20 all bind to the class II family of receptors that are expressed on keratinocytes. IL-24 and IL-20 share the same receptors, signaling through IL-20R1/IL-20R2 and IL-22R1/IL-20R2; while IL-22 signals through IL-22R1/IL-10R2. Therefore, we tested whether IL-22 and IL-20 would affect migration and proliferation of keratinocytes in a manner similar to that demonstrated for IL-24. We examined the effect of these three cytokines on TGF α -induced migration of NHEKs in modified Boyden chambers.

As shown in Figure 4C, when NHEKs were cultured with IL-24, migration toward the chemoattractant TGF α was significantly inhibited by 54% ($P=0.02$). In contrast, IL-22 significantly increased the number of migrating cells ($P=0.0003$), while IL-20 had no significant effect on TGF α -induced migration. KGM-2 was included as the positive control to demonstrate the migration ability of NHEK in complete media. Both IL-24 and IL-20 inhibited this response, ($P=0.09$ and $P=0.02$, respectively) and once again IL-22 significantly increased the number of migrating keratinocytes ($P<0.001$).

TGF α promotes not only the migration of keratinocytes but also their proliferation during wound repair. To examine the possibility that IL-24 may also affect the proliferation of TGF α -stimulated keratinocyte, we looked specifically at NHEK proliferation. We measured ^3H -thymidine incorporation in keratinocytes stimulated with TGF α in the presence of IL-24, IL-22, and IL-20. For these assays, cells were plated in complete medium and incubated overnight, and then medium was replaced with factor-free medium and cells were incubated for 24 h. Cytokines were then added in medium containing 20% of the normal concentration of growth factors. Under these conditions, all three cytokines (IL-24, IL-22, and IL-20) caused insignificant proliferation of NHEKs (Fig. 5). Shown for comparison is the proliferative response when cells were grown in complete KGM-2 medium (positive control) When TGF α was added to cultures that had been preincubated for 30min with IL-24 or IL-20 proliferation was inhibited ($P=0.001$ and $P=0.026$, respectively), whereas when NHEKs were preincubated with IL-22 and then cultured with TGF α there was a measurable but not significant increase in cell proliferation over cells stimulated with TGF α alone.

Shown in Figure 5 is a representative experiment using NHEK from a single donor. To determine whether keratinocytes from different donors respond differently to TGF α and IL-24 we repeated these experiments a total of five times using three different lots of NHEK. The mean stimulation index $\pm$ standard deviation for cultures incubated with TGF α was 2.1 ± 1.6 . When IL-24 was added at 25 ng/ml the mean stimulation index was 1.1 ± 0.4 . Using a Wilcoxon matched pair test, $p=0.0313$, when proliferation with IL-24 was compared to proliferation without IL-24. These experiments show that IL-24 significantly inhibits TGF α induced proliferation. In addition, the level of NHEK proliferation and inhibition of proliferation vary from donor to donor and between experiments.

The proliferative response of keratinocytes to HGF was also tested. While NHEKs did proliferate in response to this growth factor, albeit to a lesser extent, IL-24 did not significantly inhibit the HGF proliferation of NHEKs.

We have shown that IL-24 blocked both the proliferation and migration of TGF α treated NHEKs. To determine if this inhibition was at the level of ligand receptor interaction we examined the activation state of epidermal growth factor receptor (EGFR) after IL-24 treatment. As shown in Figure 5B treatment of NHEKs with TGF α resulted in EGFR phosphorylation that was inhibited by 25% and 53% when NHEKs were pre incubated with IL-24 at 25 ng/ml and 100 ng/ml.

Discussion

We have shown that IL-24 expression is up regulated in keratinocytes during human wound healing. IL-24 was first reported to be expressed during wound repair in a rat model (21). In these studies c49a, the rat homolog of IL-24, was shown to be associated with proliferating fibroblasts during wound repair in the rat skin. We have shown here that IL-24 is expressed in keratinocytes during human wound repair. Expression in keratinocytes (confirmed by keratin staining of sequential sections) peaked between day 2 and day 6, whereas by day 10, keratinocytes lost expression of IL-24. The location of IL-24 staining appears to be at the wounded site in the migrating keratinocytes rather than in the proliferating cells at the dermal-epidermal junction. This observation will need to be confirmed by further staining. Since our experiments lack a day 0 control we carried out immunohistochemical analysis of normal skin from 5 healthy individuals showing that keratinocytes do not express IL-24. In addition, our previously published results showed that keratinocytes in normal skin are uniformly negative for IL-24 (13). We conclude that IL-24 is induced in keratinocytes during the inflammatory process of wound repair. In vitro experiments with primary human keratinocytes, NHEKs, showed that cytokines involved in wound healing, including TGF α , TGF β , IFN β , and IFN γ , increased the secretion of IL-24 in keratinocytes, as measured by ELISA. Further experiments demonstrated that IL-24 inhibited TGF α -induced proliferation and migration of keratinocytes while IL-24 had little effect on HGF-induced proliferation and migration. These results suggest that IL-24, expressed by keratinocytes during wound repair, may be involved in the stage of wound healing when rapidly proliferating keratinocytes stop growing and revert to their normal, differentiated, nonproliferating state, the so-called resolution phase of wound repair.

The expression and function of IL-24 in the skin have been examined by numerous groups since it was reported that the receptors for IL-24, IL-20R1/IL-20R2 and IL-22R1/IL-20R2, are expressed on skin cells (2, 18, 20). Early on, we reported that IL-24 is expressed in epidermal melanocytes (13). This expression seemed to be constitutive; five of five normal tissues examined showed positive immunostaining for IL-24 in cells identified as melanocytes. In contrast, other groups have reported that melanocytes from uninvolved psoriatic skin do not express IL-24 protein (30). We previously found that keratinocytes were negative for IL-24 expression in human skin samples (13), but cultured keratinocytes, including the immortalized cell line HaCaT, have been reported to express IL-24 constitutively (19). In our study using a sensitive intracellular FACS assay we detected a low level of constitutive intracellular expression of IL-24 in HaCaT cells (data not shown) and significant levels of IL-24 expression in NHEKs. ELISAs were employed to measure the secreted IL-24 protein in the supernatants of cytokine stimulated NHEKs and our results indicate that TGF α significantly upregulated the levels of secreted IL-24 in these keratinocytes. Significant levels of secreted IL-24 were also measured in TGF β -, IFN β - and IFN γ -stimulated NHEKs. Therefore, in both primary and immortalized keratinocytes expression of IL-24 is upregulated by cytokines, specifically those involved in wound repair. Kunz et al. as well as Sa et al. have also reported that expression of IL-24 in keratinocytes is constitutive and upregulated by cytokines (18,20). In contrast, our own examination of normal skin did not reveal any IL-24-positive keratinocytes; therefore it is possible that the

“constitutive” expression seen in cultured keratinocytes was actually induced by culture conditions that include growth factors that would stimulate IL-24 expression in NHEKs.

Using a reconstituted human epidermal skin model Sa reported that IL-24 caused keratinocyte proliferation (20). We found that IL-24, IL-22, and IL-20 caused detectable but not statistically significant proliferation of NHEKs. Our different findings could be explained, as Sa suggested, by the expression levels of the IL-24 receptor subunits (IL-20R1 and IL-22R1) on keratinocytes which vary from donor to donor (20). We used three different lots of NHEKs, each from a different donor. All demonstrated low, but not statistically significant, levels of proliferation in response to IL-24. However, our most important novel finding is that IL-24 inhibits the migration and proliferation of TGF α -stimulated keratinocytes. While the level of proliferation and migration did vary among the three lots of NHEK used in our experiments, IL-24 consistently inhibited TGF α induced proliferation and migration ($p=0.0311$ and 0.0625 , respectively). These results, together with the expression of IL-24 measured over time in our human wound experiment, allow us to hypothesize that during wound repair, high levels of TGF α upregulate IL-24 in keratinocytes and that IL-24 functions through an autocrine pathway to inhibit the continued proliferation of keratinocytes in wounded skin.

Like IL-24, IL-20 also inhibited the proliferation of TGF α -stimulated keratinocytes. Work by numerous laboratories has shown that these cytokines bind to the same set of receptors, IL-20R1/IL-20R2 and IL-22R1/IL-20R2 (8,31,32). Furthermore it has been shown that these receptors are highly expressed in the skin (2,32,33,19). The results of the experiments presented here suggest that IL-24 and IL-20 signaling through IL-20R1/IL-20R2 may function in a similar manner in normal skin by directly inhibiting TGF α induced proliferation. This finding is contradictory to what is known about the function of IL-20 in psoriatic skin and in vitro skin reconstruct models where IL-20 has been shown to be upregulated in hyperproliferating keratinocytes. A recent publication by Wolk et al elegantly showed that IL-22, and to a lesser extent IL-20, are responsible for the impaired terminal differentiation of keratinocytes but they do not affect keratinocyte proliferation (34). In addition, Wang (35) et al recently reported that the source of IL-20 in psoriatic skin lesions is not keratinocytes but rather CD11c+CD68+ mononuclear cells and the expression of IL-20 in these cells is upregulated by TNF α . It is possible that the effect of the IL-20 family of cytokines on cells within the epidermis will be different depending on the inflammatory state of the surrounding tissue including normal skin, wounded skin, or psoriatic skin.

IL-24 was first identified as a tumor suppressor protein causing growth arrest in various tumor types, including melanoma. The molecular mechanism of this growth suppression has been shown to be dependent on the tumor cell type (12). In prostate cancer cells as well as non-small-cell lung cancer cells, it has been reported that IL-24 inhibits the phosphorylation of epidermal growth factor receptor (EGFR) (36,37). We have shown here that IL-24 inhibits the phosphorylation of EGFR in NHEKs. This may provide a possible mechanism by which IL-24 acts during wound repair. IL-24 may provide homeostatic control over keratinocytes, halting their rapid proliferation and causing them to return to their normal nonproliferating, differentiated state in normal skin. Studies to confirm this hypothesis are under way.

In conclusion, we have shown that IL-24 protein is expressed in keratinocytes during human wound repair. Our in vitro experiments provide evidence that IL-24 is secreted from cytokine stimulated keratinocytes and may function to inhibit the proliferation and migration of TGF α -stimulated keratinocytes through inhibition of EGFR phosphorylation. This report provides in vitro evidence that IL-24 is part of the cytokine cascade that regulates activated

keratinocytes. The exact role of IL-24 in wound repair awaits further in vivo experimentation.

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Abbreviations

NHEK	normal human epidermal keratinocytes
TGFα	transforming growth factor α
IL	interleukin

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FIGURE 1.

Expression of IL-24 in keratinocytes during wound repair. Paraffin-embedded tissue from punch biopsy specimens of wounded human skin stained with anti-IL-24 (A–C). Specimens from patient #1 were obtained 2 days (A), 6 days (B), or 10 days (C) after wounding; positively staining cells shown by arrows, magnification is 100x. H&E staining of a composite picture of the day 2 intact wound, note that the wound scab, as seen in panels A–C, is not present in this serial cross section of the wound tissue (D); arrows show the approximate site of the sections shown in A–C. Panels E–I show paraffin-embedded tissue from patient #2 obtained 2 days after wounding stained with isotype control IgG (E), anti-CD68 to identify macrophages (F), anti-gp100 to identify melanocytes (G), anti-cytokeratin to identify keratinocytes (H), and anti-IL-24 (I). Magnification is 200x. Arrows and boxes locate position of cells shown under higher magnification in inserts (400x). Panel I inserts identify keratinocytes (Ker) and macrophages (Mac) staining positive for IL-24. Panel J shows normal unwounded skin stained with anti IL-24, where melanocytes (Mel) and macrophages stain positive. Magnification is 200x with positively staining cells shown by arrow and in the inserts (400x).

FIGURE 2.

Cytokines induce the expression of IL-24 in keratinocytes. NHEKs were stimulated for 18 h with TGFa at 500 U/ml. IL-24 expression was quantified by intracellular flow cytometry (A). Shown are the histograms of unstimulated NHEKs stained with FITC IgG (black histogram) and FITC anti IL-24 (solid line) compared to the IL-24 expression in NHEKs stimulated overnight with TGFa (dashed line). IL-24 secreted from NHEKs (B) was also measured by ELISA. These results are reported as the mean $\pm$ standard deviation from triplicate samples. * $P < 0.05$ compared to cells without cytokine stimulation. These experiments were done two times. Shown are representative results.

FIGURE 3.

IL-24 inhibits TGF α -induced migration of keratinocytes in an in vitro model of wound repair. (A) Confluent monolayer of NHEKs that were growth factor starved in keratinocyte basal medium for 18 h were scratch-wounded and then pretreated with IL-24 at 25 ng/ml (c,d) or medium alone (a,b) for 30 min. TGF α at 100 ng/ml was added to specific cultures (b,d), and the incubation was continued for 24 h. Cells were then fixed and photographed. Outlines of the original scratch are marked with a dashed line. (B) Graphical presentation of results of a similar experiment in which NHEK cultures were treated or not treated with mitomycin C to block proliferation just prior to the addition of IL-24 at 25 ng/ml and/or TGF α at 10 ng/ml. These results are reported as the mean of 5 measurements of the scratch width $\pm$ standard deviation. These experiments were repeated two times. Shown are the results of one representative experiment. * $P < 0.05$ compared to wounded cultures without IL-24. (C) Confluent monolayer of NHEKs were grown and scratch-wounded as described above and then pretreated anti-IL-24, anti-IL-20R1 or anti-IL-22R1 for 30 min followed by a further pretreatment with IL-24 at 25 ng/ml for 30 min. TGF α at 10 ng/ml was added to specific cultures and the incubation was continued for 48 h. Mouse IgG and goat IgG were included as nonspecific control. All antibodies were used at 1 μ g/ml. These results are reported as the mean of 5 measurements of the scratch width $\pm$ standard deviation.

Experiments were repeated once; shown are the results of one representative experiment. * $P < 0.05$ compared to wounded cultures with TGF α alone. Experiments were done using our affinity purified IL-24 (A and B) and the commercial source of IL-24 (C).

FIGURE 4.

Inhibition of TGF α -induced migration of keratinocytes. NHEKs were seeded at 1×10^5 cells per upper well of BioCoat migration plates in KBM alone or KBM with increasing doses of IL-24 (A and B) or with IL-24, IL-22, or IL-20 at 25 ng/ml (C). The lower chambers contained either KBM alone, KBM with TGF α at 50 ng/ml or 10 ng/ml (A), KBM with HGF at 50 ng/ml or 10 ng/ml (B), or complete KGM-2 (A, C). Cells were allowed to migrate for 24 h, and then migrating cells were counted. Results were reported as the mean number from five fields counted $\pm$ standard deviation. Each experiment was done two times. Shown are the results of one representative experiment. * $P < 0.05$ compared to the number of TGF α -migrating cells in wells treated with IL-24 or IL-22 compared to those without. These experiments were done using the commercial source of IL-24.

FIGURE 5.

IL-24 inhibition of TGFα-induced proliferation through EGFR. For proliferation studies (A) NHEKs were seeded at 1×10^4 cells per well in 96-well plates in complete KGM-2 medium. After cells reached 50% confluence, they were growth factor starved overnight in KBM, and then cytokines were added for an additional 48 h. IL-24, IL-22, and IL-20 were used at 25 ng/ml, TGFα at 10 ng/ml and 2 ng/ml, and HGF at 50 and 10 ng/ml in medium containing 20% KGM-2. Proliferation was assessed in terms of ^3H -thymidine incorporation. All conditions were set up in triplicate, and results are reported as the mean stimulation index $\pm$ standard deviation, where the stimulation index is equal to the counts per minute of cytokine-treated cells divided by the counts per minute of untreated cells. $*P < 0.05$ comparing the TGFα-induced proliferation of cells treated with IL-24 or IL-20 compared to those treated with TGFα alone. The effect of IL-24 on EGFR phosphorylation in TGFα-stimulated NHEKs (B) was determined by immunoprecipitation (IP) with anti EGFR antibody followed by immunoblotting (IB) with anti phosphotyrosine antibody or anti EGFR antibody. The graph shows relative phospho EGFR expression levels determined by densitometry and normalized against total EGFR using Scion Image Software. These experiments were done twice using the commercial source of IL-24 with identical results. Shown are the results of one experiment.

Table 1

Expression of IL-24 in keratinocytes during wound repair*

<u>Subject</u>	<u>Time post wounding</u>		
	<u>Day 2</u>	<u>Day 6</u>	<u>Day 10</u>
1	3+ *	2+	0
2	2+	1+	0
3	2+	0	0
4	1+	2+	0

* Results of IL-24 staining of paraffin embedded punch biopsy tissue taken on days 2, 6, and 10 after wounding. Number of keratinocytes staining positive for IL-24 were graded where 4+ indicated 75%–100% of cells positive in all fields read; 3+ indicated 50%–75% positive; 2+ indicated 25%–50% positive; and 1+ indicated 5%–25% positive. Results on immunostaining were considered negative, 0, when fewer than 5% of cells in all fields read were positive.