Inflammatory Skin Disease in K14/p40 Transgenic Mice: Evidence for Interleukin-12-Like Activities of p40

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The proinflammatory cytokine interleukin-12, a p35/p40 heterodimer, is produced by resident cells in skin and has been implicated as a pathogenetic factor in T-cell-mediated skin diseases. Secretion of heterodimeric interleukin-12 is always accompanied by production of p40 monomer and p40/p40 homodimer. To investigate the possible in vivo role of p40, we generated mice that constitutively express inducible in resident skin cells including Langerhans cells and keratinocytes. These mice spontaneously developed an eczematous skin disease that was characterized by hyperkeratosis, focal epidermal spongiosis, and a mixed inflammatory infiltrate composed of T cells (CD4+), macrophages, eosinophils, mast cells, and few neutrophils. Fluorescence-activated cell sorter analysis of transgenic epidermal cell suspensions revealed induction of major histocompatibility complex class II molecules on keratinocytes and a 2–3-fold increase in the content of Langerhans cells.

Interleukin-12 (IL-12) is an immunoregulatory cytokine that promotes cell-mediated immunity. Besides its capacity to augment CTL and natural killer cell activity, IL-12 commits differentiation of naive CD4 and CD8 T cells to cells that preferentially produce interferon-γ (IFN-γ) and interleukin-2 (IL-2) (Trinchieri, 1995; Gately et al., 1998). The importance of IL-12 in vivo has been demonstrated using IL-12 antagonists, which suppress Th1-associated autoimmune responses such as experimental allergic encephalomyelitis, insulin-dependent diabetes mellitus, and inflammatory bowel disease (Leonard et al., 1995; Neurath et al., 1995; Trembleau et al., 1997), and recombinant IL-12, which inhibits allergen-induced airway hyperreactivity and eosinophil accumulation in the lung in Th2-mediated murine models of allergic asthma (Gavett et al., 1995; Kips et al., 1996).

IL-12 p35 and p40 mRNA as well as p40 and p70 protein are inducible in resident skin cells including Langerhans cells and human keratinocytes (Müller et al., 1994; Heufer et al., 1996; Yawalkar et al., 1996). Participation of endogenous IL-12 p70 and p40 in pathophysiologic processes relevant to cutaneous inflammation has been suggested by increased IL-12 p40 mRNA expression in atopy patch test sites as well as IL-12 p40 mRNA and IL-12 p70 protein expression in psoriatic skin (Junghans et al., 1998; Yawalkar et al., 1998). In addition, a pivotal role for IL-12 p70 in the induction of contact hypersensitivity was demonstrated in blocking experiments using an anti-p40 antibody as well as an anti-IL-12 antiserum. This resulted in inhibition of sensitization and induction of hapten-specific tolerance (Müller et al., 1995b; Riemann et al., 1996).

IL-12 p70 is a heterodimeric protein consisting of two covalently linked chains, p35 and p40 (Wolf et al., 1994; Trinchieri, 1995). Both subunits expressed by one cell are required for IL-12 bioactivity. The biologic activities of IL-12 are mediated by the high affinity IL-12 receptor (IL-12R), which is composed of the IL-12R β1 and the IL-12R β2 chains (Presky et al., 1996). Studies investigating the tissue distribution of the high affinity IL-12R have shown its presence primarily on natural killer cells and T cells (Thibodeaux et al., 1999; Wu et al., 2000). There also exist reports demonstrating IL-12R β expression on B cells and melanoma cells, however (Thibodeaux et al., 1999; Yue et al., 1999).

In contrast to p35, which is constitutively expressed on many cell types, p40 is inducible in dendritic cells, macrophages, and keratinocytes by a variety of stimuli (Müller et al., 1995a; Heufer et al., 1996). Cells that are able to secrete heterodimeric IL-12 always produce an excess of the free p40 subunit (D’Andrea et al., 1992; Podlaski et al., 1992). Whereas the biologic effects of IL-12 p70 are well studied, the physiologic significance of IL-12 p40...
remains controversial. For example, it has been demonstrated that p40 homodimer and, to a lesser degree, P40 monomer antagonize IL-12 bioactivity by binding to the IL-12Rβ1 without delivering a signal (Ling et al., 1995; Gately et al., 1996). Despite its potent IL-12 antagonistic function in many systems, endogenous p40 was reported to act as an IL-12 agonist in certain situations (Decken et al., 1998; Piccotti et al., 1998).

Although it is attractive to speculate about the role of IL-12 p40 in skin inflammation, experimental systems designed to test this hypothesis are missing. In this study we evaluate whether the local presence of endogenous IL-12 p40 in skin is a cause or a consequence of cutaneous inflammation. Using a keratinocyte-specific keratin promoter (K14), we have made mice that constitutively express IL-12 p40 in the basal layer of the epidermis but, at the same time, do not produce detectable amounts of biologically active epidermal IL-12. Our results show that p40 single transgenic mice exhibit skin changes identical to those seen in mice injected with recombinant IL-12. To our knowledge, these results are the first that demonstrate that IL-12 p40, produced in vivo by basal keratinocytes, results in a spontaneous inflammatory skin phenotype.

**MATERIALS AND METHODS**

**DNA construct and transgenic mice** A cDNA comprising the entire coding region of murine p40 (1035 bp) (the kind gift of Dr. M. Gately, Hoffman LaRoche, Nutley, NJ) was inserted into the Bam HI site of the K14/hGH expression vector by blunt end ligation. (Fig 1) was used for microinjection of fertilized ova of FVB/N mice (Taconic, Germantown, NY). Transgenic founders were identified by polymerase chain reaction (PCR) and by Southern blot analysis and bred to establish lines. All animals were bred under pathogen-free conditions and used for experiments at 6–12 wk of age.

**RNA analysis** For reverse transcriptase PCR (RT-PCR) total RNA was prepared from cultured basal transgenic and control keratinocytes and from phorbol 12-myristate 13-acetate (PMA) stimulated spleen cells using the RNEASY Minikit from Qiagen (Valencia, CA). One microgram of total RNA was reverse transcribed using the Superscript First Strand Synthesis System for RT-PCR from GibcoBRL (Life Technologies, Gaithersburg, MD). Two microliters of the reverse transcriptase reaction was amplified using primers specific for murine p35 and β-actin. For both primers published sequences were used (Tokunaga...
et al, 1986; Piccotti et al, 1996; Khiri et al, 1996). For PCR amplification, 50 µl of reaction containing 1 µl cDNA, 200 mM dNTP (each), 20 pmol of each primer, and the standard buffer supplemented with Platinum Taq polymerase (2.5 U per reaction; GibcoBRL) and 1.5 mM MgCl₂ were used. Primer sequences and product sizes were as follows: P35 sense, 5'-ACCAGCACATTGAAGACCTG-3'; P35 antisense, 5'-GACTGCATCAGCTCATCGAT-3', 272 bp; β-actin sense, 5'-GTCGTACCACAGGCATTGTGATTG-3'; β-actin antisense, 5'-GCAATGCCTGGGTACATGGTGG-3', 490 bp. Samples were amplified in a thermal cycler (Perkin Elmer, Foster City, CA) with an initial 3 min denaturation step followed by 35 cycles of 94°C, 56°C, and 72°C, all for 1 min. PCR products were separated at 120 V in a 1.5% agarose gel. PMA-stimulated splenocytes and the K14/p35/hGH vector served as a positive control for p35. Amplification of β-actin verified the presence of cDNA.

For northern blot analysis total RNA was isolated from various tissues of transgenic and littermate mice by TRI reagent (Molecular Research Center, Cincinnati, OH). Ten micrograms of total RNA were size fractionated by gel electrophoresis on a 1% formaldehyde/agarose gel. RNA was transferred to a nylon membrane (Hybond N + Amersham Pharmacia Biotech, Piscataway, NJ) and hybridized with P³²-labeled cDNA for murine p40. After hybridization blots were washed twice at 65°C in 1× standard saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS), and twice in 0.1× SSC/0.1% SDS. Blots were then exposed to X-ray film at −70°C.

**Table I. p40 and IL-12 ELISA**

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<th>n</th>
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*Transgenic keratinocytes secrete p40 and p40/p40 but no heterodimeric IL-12. Immunoreactive p40 and p35/p40 protein in media conditioned by transgenic and control keratinocytes and in plasma from transgenic and control mice were measured by ELISA. The relative content of p40 and p40/p40 in total p40 immunoreactivity was determined by fractionating the samples by size exclusion chromatography followed by measuring individual fractions by ELISA.

**Figure 2. P40 transgenic mice exhibit a spontaneous macroscopic and microscopic phenotype.** (A) 5-d-old littermate and p40 transgenic mice. Note the scales on the back of the transgenic mice. (D) 10-wk-old littermate mouse. (H) Inflammatory skin lesions located on the ears, the back, and the face of a 10-wk-old homozygous mouse. (F) Less affected heterozygous p40 transgenic mouse. (B, C, E, G, I) Histologic evaluation of sections from the back skin of transgenic and littermate mice. A subtle epidermal thickening is visible in 5-d-old transgenic mice (B) compared to littermate controls (C) (magnification, ×60). Epidermal thickening, focal intraepithelial edema, and inflammatory infiltrate in skin sections of heterozygous (G) and homozygous (I) mice. (E) Back skin from a littermate mouse. (E, G, I; magnification, ×140).
and p40/40 in media conditioned by transgenic keratinocytes and plasma. To determine the relative content of p40, Column chromatography, Genzyme (Woburn and Cambridge, MA). of transgenic mice, the samples were fractionated by size exclusion chromatography, and the p40 immunoreactivity of individual fractions was assessed. A calibrated size exclusion column (83 cm) of Sephadex G100 (Amersham Pharmacia), eluted with phosphate-buffered saline (PBS), was employed.

Cytokines, cytokine assay systems, and epidermal cell culture Epidermal cell suspensions were obtained by sequential dispase and trypsin digestion of split mouse ears as previously described (Rauschmayr et al., 1997). Keratinocytes thus obtained were cultured on collagen-coated plates, using non-differentiating conditions, with medium containing 0.05 mM Ca$^{2+}$ to allow maintenance of K14 expression. After 48 h of culture immunoreactive cytokines secreted into the supernatant were quantitated by enzyme-linked immunosorbent assay (ELISA).

Murine IL-12 p40 was assayed using the p40 ELISA according to the manufacturer’s instructions (Pharmingen, San Diego, CA). ELISA reagents for detection of IL-12 p70 were a kind gift of Dr. Presky, Hoffman LaRoche, Nutley, NJ. In contrast to the p40 ELISA, which is reactive with all IL-12 proteins (p40, p40/p40, and p35/p40), the p70 ELISA exclusively detects heterodimeric IL-12 (p35/p40). The IL-12 p70 assay was performed as originally described (Wilkinson et al., 1996). Brieﬂy, flat bottom 96-well plates (Costar, Cambridge, MA) were coated with rat anti-mouse IL-12 p70 monoclonal antibody (MoAb) (9A5). Immunoreactive IL-12 p70 was detected using a peroxidase-labeled rat antimouse IL-12 p40 MoAb (SC3) and the appropriate substrate system. Samples were also analyzed with the highly sensitive Quantikine M murine IL-12 p70 immunoassay from R&D (Minneapolis, MN). IL-1α and tumor necrosis factor α (TNF-α) secreted by transgenic and control keratinocytes were measured using ELISA kits from Endogen and Genzyme (Woburn and Cambridge, MA).

Column chromatography To determine the relative content of p40 and p40/40 in media conditioned by transgenic keratinocytes and plasma of transgenic mice, the samples were fractionated by size exclusion chromatography, and the p40 immunoreactivity of individual fractions was assessed. A calibrated size exclusion column (63 x 1.5 cm) of Sephadex G100 (Amersham Pharmacia), eluted with phosphate-buffered saline (PBS), was employed.

Histology and immunostaining Hematoxylin and eosin stainings were performed by standard methods. Eosinophils were stained with Biebrich red according to Luna’s method and mast cells were visualized using a conventional chloroacetate esterase stain (Leder, 1979).

Antibodies for immunostaining Monoclonal antibodies purified from hybridoma supernatants were purchased from ATCC [M5-114.15.2 (anti-l-Aβ4.4 and 1-Eβ4.4, TIB 120)], GK1.5 (anti CD4, TIB 207), F4/80 (macrophage) or from Serotec [Oxford, U.K.: NLDC-145 (anti-DEC-205)]. The MoAb RB6-8C5 (Gr-1) and fluorescein isothiocyanate (FITC) 30F11.1 (anti-CD45) were obtained from Pharmingen and the phycoerythrin-conjugated MoAb M5-114.15.2 (anti-l-Aβ4.4 and 1-Eβ4.4) from Boehringer Mannheim (Vienna, Austria). Secondary antibodies, the biotinylated sheep anti-rat Ig (polyclonal) and the biotinylated goat anti-hamster Ig (polyclonal), were purchased from Amersham (Arlington Heights, IL) and Medac (Hamburg, Germany), respectively. FITC-labeled goat anti-rat Ig (polyclonal) was obtained from Pharmingen. Control antibodies used included irrelevant isotype-matched MoAb, rat or hamster IgG, from Pharmingen.

Immunofluorescence staining Ear skin and back skin biopsies from transgenic and control mice were embedded in Tissue-Tec II (Miles, Elkhart, IN) and frozen in isopentane precooled in liquid nitrogen. Cryostat sections (5 μm) were fixed for 10 min in acetone at 4°C and subjected to immunostaining. Briefly, sections were incubated overnight at 4°C with the indicated primary antibodies appropriately diluted in PBS. Following washing, antibody binding was visualized by further incubation with the indicated biotinylated secondary antibody and, subsequently, with Texas-Red-labeled streptavidin (Amersham) for 1 h at room temperature.

Immunoperoxidase staining Cryostat sections were fixed in acetone (4°C) for 10 min. After washing with PBS, sections were incubated overnight with the respective MoAb appropriately diluted in PBS. For detection of p40, cryostat sections were fixed in 4% paraformaldehyde in PBS followed by washing with PBS containing 0.1% saponin, and immunostaining was performed as previously described (Gorak et al., 1998). Briefly, sections were incubated at room temperature with rat anti-mouse p40 antibody C17.8 (Pharmingen) appropriately diluted in

Figure 3. Identification of the inflammatory infiltrate in the skin of K14/p40 transgenic mice by immunophenotyping. Frozen ear skin sections from littermate (right panel) and heterozygous p40 transgenic mice (left panel) were stained for CD4 (A, B), DEC 205 (C, D), F4/80 (E, F), MHC class II (G, H), and Gr-1 (i, f) as indicated. Chloroacetate esterase (K, L) and Biebrich (M, N) staining. (A–L, N: magnification, ×70; M: magnification, ×250).
Generation of K14/p40 transgenic mice Transgenic mice were generated using the construct shown in Fig 1(A). The mouse cDNA for p40 was cloned into the K14/hGH vector described previously. Two transgenic founders, which transmitted the transgene to their offspring, were identified by PCR and Southern blot analysis. Thus, we obtained two heterozygous lines designated K14/p40.1 and K14/p40.10 with an estimated copy number of integrated transgene of 40 and 5, respectively. Northern blot analysis of ear skin from K14/p40.1 and K14/p40.10 mice demonstrated tissue-specific transgene mRNA expression (data not shown). We also obtained homozygous mice, but due to their reduced breeding behavior we could not establish lines from these mice. All experiments were performed with age- and sex-matched heterozygous K14/p40.1 and littermate mice.

Keratinocytes from K14/p40 mice secrete p40 and p40/40 but no heterodimeric (p35/p40) IL-12 High level transgene expression in basal keratinocytes was demonstrated by immunohistochemical analysis of ear skin sections stained with the anti-mu IL-12 p40 MoAb (C17.8) (Fig 1B). No immunoreactivity was observed in control animals (data not shown). A specific ELISA for mouse p40 established transgenic protein secretion by K14/p40 keratinocytes. Medium conditioned by transgenic keratinocytes and plasma from transgenic mice contained 36.2 ng per ml and 32 ng per ml of total immunoreactive p40 in K14/p40.1, and 14.04 ng per ml and 8.04 ng per ml in K14/p40.10 mice, respectively. As p40 exists in two forms, i.e., as a monomer and as a homodimer, we investigated whether K14/p40 transgenic keratinocytes can secrete either of these. Medium and plasma proteins were fractionated by size and the amount of p40 in individual fractions was measured by ELISA. Specific total immunoreactivity in medium comprised 57% p40 and 43% p40/40 in the p40.1 line and 72% p40 and 28% p40/40 in the p40.10 line. Plasma from K14/p40.1 mice contained 52% p40 and 48% p40/40 and plasma from K14/p40.10 mice 69% p40 and 31% p40/40. No heterodimeric IL-12 could be detected by ELISA in either line (Table I). To investigate whether the lack of heterodimeric IL-12 was a result of missing p35 mRNA expression, RT-PCR for p35 was performed in littermate and transgenic keratinocytes. In contrast to PMA-stimulated splenocytes, which served as a positive control, no p35 mRNA was detectable in either littermate or transgenic basal keratinocytes (Fig 1C). Thus, the absence of heterodimeric IL-12 protein measured by ELISA can be explained by the complete lack of p35 mRNA.

K14/p40 mice spontaneously develop an inflammatory skin disease Contrary to what we had expected, mice from both K14/p40 lines consistently displayed a striking inflammatory skin disease. At birth, K14/p40 mice were indistinguishable from nontransgenic littermates. At the age of 5 d, just before the first hair growth could be detected, K14/p40 mice displayed a pronounced inflammatory skin disease with skin lesions most prominent on and behind the ears, on the face, and on the back (Fig 2A). The skin disease progressed spontaneously. Adult mice exhibited an inflammatory skin disease with skin lesions most prominent on and behind the ears, on the face, and on the back (Fig 2F, H). The most severely affected animals were essentially erythrodermic and some of them developed a wasting syndrome. Clinically, the skin phenotype was characterized by erythema, erosions, crusts, hair loss, and skin thickening. We also observed frequent scratchmarks in K14/p40 mice suggesting that transgenic mice suffer from pruritus. Homozygous mice (Fig 2H) were more severely affected than heterozygous mice (Fig 2F).

Histologic evaluation and immunophenotyping of skin from K14/p40 mice In order to identify early histologic changes, sections were taken from transgenic and littermate back skin of 5-d-old mice (Fig 2A). Histologic evaluation revealed subtle thickening of the transgenic epidermis (Fig 2B) compared to age-matched littermate control skin (Fig 2C). In both transgenic and littermate mice, the dermis was dominated by long hair follicles.
Subcutaneously injected recombinant IL-12, p40/p40, or vehicle periods of two consecutive weeks. We found that injections of IL-12 resulted in a skin phenotype comparable to that of K14/p40 mice. Similar to the spontaneous ear thickening in K14/p40 mice, we observed an ear swelling response in littermate mice upon repeated injections of recombinant IL-12 (Fig. 5A). In addition, cellular characteristics such as MHC class II surface expression on keratinocytes and a higher number of epidermal Langerhans cells in K14/p40 mice were identical to our findings in K14/p40 mice (Fig. 5B,A). In contrast, injections of p40/40 or vehicle alone did not alter ear thickness (Fig. 5A) and the FACS profile (Fig. 5B,C and 5B,E) of epidermal cells compared to un.injected littermates (Fig. 4A, B). In K14/p40 mice, the extent of IL-12-induced ear swelling was comparable to that in IL-12-injected littermates (Fig. 5A). Injections of p40/40 had no influence on ear thickness and on the epidermal phenotype of K14/p40 mice. These results speak for an IL-12-like activity of transgenic p40 in K14/p40 mice. The reason for the discrepancy between the effect of exogenously injected p40/40 and endogenous p40 expression in transgenic mice has yet to be clarified. Assembly of an as yet unidentified endogenous protein (a substitute for p35 that mediates biologic effects similar to IL-12) with transgenic p40 provides a possible explanation.

IL-1α and TNF-α are involved in mediating inflammatory skin lesions In the next series of experiments, we wanted to determine whether transgenic p40 stimulates the synthesis or release of proinflammatory cytokines such as IL-1α and TNF-α in vivo. Analyzing secretion of these cytokines from cultured keratinocytes, we found a low baseline secretion of IL-1α and TNF-α from nontransgenic keratinocytes. In contrast, transgenic keratinocytes spontaneously secreted higher quantities of both cytokines, indicating their capability to sustain the ongoing inflammation in K14/p40 mice (Fig. 6).
Transgenic p40 does not induce IFN-γ production by splenocytes

One of the major biologic activities of IL-12 is the induction of IFN-γ from T and natural killer cells. To determine whether induction of IFN-γ secretion is part of the IL-12-like activity of transgenic p40, we investigated the ability of transgenic p40 to induce IFN-γ secretion by splenocytes and compared it to IL-12-dependent IFN-γ secretion. Whereas stimulation of splenocytes with IL-12 resulted in a dose-dependent induction of IFN-γ secretion, a rise of IFN-γ production was not seen when K14/p40 keratinocyte-conditioned medium (containing 50 ng per ml transgenic p40) was added (Fig 7). To also study a potential antagonistic activity of transgenic p40, we investigated whether IL-12-induced IFN-γ secretion can be diminished by the addition of transgenic p40. As determined in titration experiments (data not shown), we found that recombinant p40/p40, when added at a concentration between 12.5 and 200 ng per ml, completely blocked IL-12 (10, 40, and 200 pg per ml)-mediated IFN-γ induction. In contrast to recombinant p40/p40, no inhibitory effect was exerted by the transgenic product (Fig 7). These results suggest that IFN-γ is not a major factor contributing to the proinflammatory activities of transgenic p40. It thus appears that IL-12 and transgenic p40, despite exhibiting similar in vivo effects, differ in their in vitro functions, at least with regard to IFN-γ induction.

DISCUSSION

Both IL-12 p70 and p40 have been shown to participate in pathophysiologic processes relevant to cutaneous inflammation (Junghans et al, 1998; Hong et al, 1999). Whereas IL-12 p70 was assumed to act as an immunoregulatory cytokine directing T-cell-specific cutaneous immune responses towards a Th1-type response, the role of p40 is less well defined (Trinchieri, 1995; Gately et al, 1998).

We constructed K14/p40 transgenic mice that constitutively express high levels of IL-12 p40 in basal keratinocytes to investigate the in vivo role of transgenic p40 secreted in the cutaneous environment. Transgene mRNA expression of keratinocytes was confirmed by northern blotting. Both forms of p40 (p40 and p40/p40) were detected at high levels in media conditioned by transgenic keratinocytes and in plasma from transgenic mice, suggesting that transgenic protein is able to cross the basal lamina and enter the blood system. An important point is that p40 transgenic keratinocytes do not secrete heterodimeric IL-12. The absence of this form of IL-12 was confirmed with an ELISA specific for heterodimeric IL-12 and with a bioassay that measures IL-12-dependent IFN-γ secretion of cultured splenocytes. Recombinant murine p40/p40 (200 ng per ml) and Sheep7 (1.5 μg per ml), a blocking antibody to IL-12, completely inhibit IL-12-induced IFN-γ secretion. Data are expressed as mean ± SD of six replicates per group.

Transgenic p40 does not induce IFN-γ production by splenocytes

Confocal microscopy of transgenic p40 keratinocytes stained with non-blocking anti-p40 antibody C15.1 and either recombinant mu IL-12, mu p40/p40, or medium conditioned by K14/p40 and littermate keratinocytes. Immunoreactive IFN-γ secretion was measured after 48 h by ELISA. A potential antagonistic activity of transgenic p40 was tested by its addition to IL-12 (10 pg per ml) stimulated cultured splenocytes. Recombinant murine p40/p40 (200 ng per ml) and Sheep7 (1.5 μg per ml), a blocking antibody to IL-12, completely inhibit IL-12-induced IFN-γ secretion. Data are expressed as mean ± SD of six replicates per group.

Figure 6. Spontaneous secretion of IL-1α and TNF-α by cultured p40 transgenic keratinocytes is enhanced compared to littermate control keratinocytes. Media conditioned by transgenic and littermate control keratinocytes were quantitated after 48 h of culture for immunoreactivity with IL-1α and TNF-α by ELISA. Values are presented as the mean amount of cytokine (pg per ml) ± SD of three keratinocyte cultures.

Figure 7. Transgenic p40 neither induces nor inhibits IFN-γ secretion by splenocytes. Before addition of splenocytes (5 × 10⁵), 96-well culture plates were coated with the non-blocking anti-p40 antibody C15.1 and either recombinant mu IL-12, mu p40/p40, or medium conditioned by K14/p40 and littermate keratinocytes. Immunoreactive IFN-γ secretion was measured after 48 h by ELISA. A potential antagonistic activity of transgenic p40 was tested by its addition to IL-12 (10 pg per ml) stimulated cultured splenocytes. Recombinant murine p40/p40 (200 ng per ml) and Sheep7 (1.5 μg per ml), a blocking antibody to IL-12, completely inhibit IL-12-induced IFN-γ secretion. Data are expressed as mean ± SD of six replicates per group.
nature of the proinflammatory activity of transgenic p40 is yet unclear, three possibilities can be entertained to explain this phenomenon. First, the inflammatory response is mediated by heterodimeric IL-12, but our methods were not sensitive enough to measure it. Second, p40 and p40/40 have a proinflammatory potential. Third, transgenic p40 combines with an endogenous protein that substitutes for p35. Although it is conceivable that transgenic p40 assembles with minute amounts of endogenous p35 to form functional IL-12, our data point against a causative role for heterodimeric IL-12 in initiating the inflammatory response. Using two methods, an ELISA specific for heterodimeric IL-12 and a bioassay that measures IL-12 function in vitro, we were unable to detect biologically relevant levels of IL-12 secreted by transgenic keratinocytes. Because of the extraordinary sensitivity of the IL-12 ELISA and the bioassay, which both go down to 2.5 pg per ml, and the lack of p35 mRNA expression in murine keratinocytes, it is very unlikely that biologically relevant amounts of heterodimeric IL-12 are released in vivo. Our data are in accordance with previous studies showing that, in contrast to human keratinocytes, murine keratinocytes do not express p35 mRNA (Müller et al, 1994; Heufler et al 1996). In addition, the theoretical possibility of extracellular pairing of transgenic p40 with dimeric p35 is unlikely to occur as the p35 chain is only secreted as a heterodimer in association with the p40 chain (Trinchieri, 1995). Secretion of isolated p35 protein has not been detected in amounts (D’Andrea et al, 1992). We cannot exclude the possibility, however, that p35 derived from non-keratinocytes, i.e., neighboring apoptotic Langerhans cells, combines with transgenic p40 in the extracellular compartment to form heterodimeric IL-12.

The second possible explanation, that p40 or p40/40 are responsible for the inflammatory response in p40 transgenic mice, is unlikely because these proteins failed to do this in vivo when used in recombinant form. Although the in vitro functional activity of recombinant p40 monomer and homodimer was confirmed by measuring p40-induced inhibition of IL-12-dependent IFN-γ secretion by splenocytes, we cannot definitively exclude the possibility that, in the injection experiments, we missed the proinflammatory effect by not achieving a sufficiently high in vivo concentration. Nevertheless our data are consistent with the observation that p40 homodimers can function as IL-12 antagonists, leading to the hypothesis that p40/40 is produced to limit IL-12-dependent immunologic reactions (Gately et al, 1996; Trembleau et al, 1997; Yoshimoto et al, 1998). The reason for the difference in function between exogenously applied p40 homodimer and monomer and endogenously secreted transgenic p40 is unclear and merits further investigation. It is possible that a post-translational modification by keratinocytes, which is not present in recombinant p40, is necessary to provide IL-12 p40 with proinflammatory potential.

Although we have no definitive proof, we favor the third possible explanation that transgenic p40 assembles with an endogenous protein that substitutes for p35. A promising candidate is the novel protein p19, which has recently been described to pair with IL-12 p40 to form IL-23, a T-lymphocyte-activating cytokine (Oppmann et al, 2000). In transgenic mice, a situation may therefore exist where agonistic forms of p40, such as IL-23, predominate over antagonistic variants (P40, p40/p40). Such a constellation would lead to inflammation. The discrepancy between the agonistic predominance of the transgenic product in vivo and the complete lack of both IL-12 agonistic and antagonistic in vitro bioactivities is evident. Potentially, the in vivo and in vitro generated transgenic product differs in its contents. A situation with a balanced coexistence of competing IL-12 activities provides a possible explanation for the results of the in vitro experiments.

Transgenic p40, whatever it is, has remarkable clinical effects resulting in a cutaneous skin phenotype that corresponds in many ways to what is seen in eczema, e.g., atopic dermatitis (Cooper, 1994; Leung et al, 1999). The pattern of the inflammatory response, as evidenced by infiltration of lymphocytes, monocytes, dendritic cells, mast cells, and eosinophils, as well as focal spongiosis and epidermal thickening, was similar to cutaneous inflammation induced by injection of recombinant IL-12.

At the present time the expression of IL-12R on resident and passenger skin cells has not yet been thoroughly investigated. It is also not clear whether the transgenic product acts in an autocrine or paracrine fashion. The recent discovery of IL-12R expression and responsiveness on human melanoma cells and a melanoma cell line in vitro raises the possibility that, similar to IL-12, the transgenic product mediates a direct effect on nonhematopoietic cells such as melanocytes, keratinocytes, and fibroblasts (Yue et al, 1999). In view of reports about the existence of a membrane-bound form of heterodimeric IL-12 on macrophages and renal tubular cells, it will be interesting to see whether a membrane-bound form of transgenic p40 exists and whether it is responsible for the phenotype of p40 transgenic mice (Fan et al, 1996, 1997).

P40 transgenic keratinocytes are in an activated state, which results in surface expression of MHC class II and intercellular adhesion molecule 1 (ICAM-1) and spontaneous secretion of the proinflammatory cytokines IL-1α and TNF-α. There is abundant evidence suggesting an important role for IL-1α and TNF-α in initiating skin inflammation (Enk and Katz, 1992; Cheng et al, 1992; Groves et al, 1995; Kupper and Groves, 1995). In this context, it remains to be elucidated whether in p40 transgenic mice the injection of MHC class II and ICAM-1 is mediated directly by transgenic p40 or indirectly by other proinflammatory cytokines induced by the transgenic product. The fact that both IL-1α and TNF-α are spontaneously released by transgenic keratinocytes in vivo supports the involvement of indirect effects in triggering the inflammatory cascade.

P40 transgenic mice have increased numbers of epidermal Langerhans cells and dermal dendritic cells. Whether this results from an increased influx of precursor cells, decreased eflux to lymph nodes, or reduced apoptosis is currently under investigation. Interestingly, IL-4 transgenic mice also exhibit a bigger compartment of epidermal Langerhans cells (Elbe-Bürger et al, 1999). In contrast to p40 and IL-4 transgenic mice, there is a depletion of epidermal Langerhans cells in IFN-γ-expressing mice, probably because of induction of their migration into the lymph nodes (Carroll et al, 1996). The increased number of Langerhans cells and dermal dendritic cells in p40 transgenic mice can be either a direct effect of p40 on dendritic cells or induced by the altered cytokine milieu (Kelleher and Knight, 1998). Elevated IL-1α levels alone are unlikely to mediate this effect as the number of Langerhans cells is unaltered in IL-1α transgenic mice (Groves et al, 1995). Similarly, we do not believe that TNF-α plays an important role. In fact, this cytokine increases the migratory potency of Langerhans cells, resulting in their accumulation in lymph nodes (Kimber and Cumberbatch, 1992; Wang et al, 1996; Cumberbatch et al, 1997).

In contrast to IL-12, IFN-γ does not appear to be an important mediator of the proinflammatory activities of transgenic p40. From our in vitro experiments it is clear that direct induction of IFN-γ by the transgenic product does not occur. A possible indirect induction of IFN-γ in vivo, however, cannot be excluded by these experiments. A powerful way to examine this would be the crossing of IFN-γ−/− with p40 transgenic mice.

In summary, our data provide first evidence that keratinocyte-derived transgenic p40 is able to initiate cutaneous inflammation. The fact that skin inflammation in p40 transgenic mice strongly resembles the skin phenotype induced by injections of recombinant IL-12 supports the idea that heterodimeric IL-12 and transgenic p40 exert similar in vivo functions. Studies are in progress to establish the contribution of transgenic p40 to immunity. Should it turn out that transgenic p40 is able to polarize T helper immune responses similar to IL-12, it will be a promising new target to modulate immune responses.

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