Cachexia and graft-vs.-host-disease-type skin changes in keratin promoter-driven TNFα transgenic mice

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Tumor necrosis factor α (TNFα) orchestrates a wide range of effects that combat severe infections in animals. At lower levels, TNFα plays an important protective role in stimulating chemotaxis and antimicrobial activity of neutrophils, macrophages, and eosinophils. During chronic illness, TNFα secretion can be elevated markedly, giving rise to cachexia, hemorrhage, necrosis and, ultimately, death. Although TNFα may mediate many of its effects through macrophages, 30% of TNFα injected into animals concentrates in the skin. In recent years, it has been shown that keratinocytes can be induced to synthesize TNFα. To explore the role of TNFα synthesis in keratinocytes, we used a keratin-14 (K14) promoter to target human TNFα expression in the epidermis and other stratified squamous epithelia of transgenic mice. Most mice expressing the K14–TNFα transgene stopped gaining weight within 1 week postbirth, and exhibited retarded hair growth. In the skin, adipose production was profoundly inhibited, whereas signs of fibrosis and immune infiltration were evident in the dermis. Over time, the epidermis exhibited an increased stratum corneum, as signs of necrosis began to appear in the skin. Within 3–5 weeks, the mice displayed features characteristic of cachexia and necrosis. Our results suggest that TNFα expression by keratinocytes not only plays a role in inflammatory and graft-versus-host-disease-like responses in the skin, but also in other tissues, apparently by virtue of stratified squamous epithelial-derived TNFα entering the bloodstream. Our results have enabled the first evaluation of many of the effects of TNFα in transgenic animals.

[Key Words: TNFα; keratin-14 promoter; epidermal expression; adipose suppression; transgenic mice]

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Tumor necrosis factor α (TNFα) is a polypeptide [17.3 kD] that mediates a wide range of inflammatory and immune responses involving cells of epithelial and myeloid origin [for review, see Beutler and Cerami 1989; Schreiber et al. 1990; Cerami 1992]. TNFα was originally discovered as a cytotoxic factor for tumor cells and as a factor involved in cachexia induced by chronic illnesses such as cancer and acquired immunodeficiency syndrome (AIDS) [Carswell et al. 1975; Oliff et al. 1987; Tracey et al. 1988]. Elevation of serum/sinovial fluid levels of TNFα has also been associated with endotoxic shock, rheumatoid arthritis, and autoimmunity in graft-vs.-host disease (GVHD) [Beutler et al. 1985, 1986; Sale et al. 1985; Tracey et al. 1986; Piquet et al. 1987; Saxne et al. 1988; Yocum et al. 1989].

Most, if not all, cells possess TNFα receptors. TNFα, however, seems to be synthesized by a much smaller repertoire of nontransformed cell types, including activated macrophages, T cells, natural killer cells, mast cells, and keratinocytes [Urban et al. 1986; Djeu et al. 1988; Gordon and Galli 1990; Kock et al. 1990]. Much attention has been given to the roles that stimulated induction of TNFs by inflammatory cells might play in TNF-induced responses. Therefore, it is surprising that nearly 30% of the TNFα intravenously injected into mice concentrates in the skin [Beutler et al. 1985]. That skin is susceptible to TNFα has been suspected from the typical cutaneous lesions that occur during the acute phase of GVHD, where serum TNFα levels are elevated [Sale et al. 1985]. Indeed, intravenous administration of anti-TNFα antibodies blocked GVHD-induced skin necrosis [Piquet et al. 1987, 1990], further implicating TNFα in immune-related responses of the skin. More recently, culture studies have shown that keratinocyte growth is profoundly, but reversibly, inhibited by nanomolar amounts of recombinant TNFα [Pillai et al. 1989; Symington 1989]. The in vivo effects of constitutive and abnormal TNFα production by keratinocytes have not yet been evaluated, however, nor is it known whether keratinocyte-derived TNFα can act directly or indirectly to influence other TNFα-related biological activities.
The roles of different cell types in manifesting the pleiotropic effects of TNFα remain a major issue in TNFα biology. One approach to investigating this question is to target TNFα overexpression in transgenic mice. Recently, a gene encoding a human TNFα mRNA with a modified 3'-noncoding sequence was used to generate transgenic mice, which exhibited features of chronic inflammatory polyarthritis (Keffer et al. 1991). In this case, however, transgene TNFα was neither present in the bloodstream nor detected in most cells that normally express TNFα. Thus, perhaps not surprisingly, these animals did not develop cachexia, GVHD, necrosis, or most other aberrancies typically associated with TNFα overproduction, and the role of different TNFα-producing cells in generating these responses could not be evaluated.

In the present study, we have examined the role of TNFα produced by keratinocytes. Using a human keratin-14 (K14) promoter, we have overexpressed TNFα in the stratified squamous epithelia of transgenic mice. Surprisingly, the serum levels of TNFα were also greatly elevated in these mice, and the mice displayed numerous morphologic aberrancies, including cachexia, growth inhibition, GVHD-like changes in the skin and, in subsequent phases, intestinal and liver necrosis and death.

Results

**K14-TNFα transgenic mice exhibit cachexia and growth inhibition**

Figure 1A illustrates the keratin promoter–TNFα gene construct used in the generation of transgenic mice. The human K14 promoter has been shown previously to drive expression of reporter transgenes in keratinocytes (Vassar et al. 1989; Vassar and Fuchs 1991; Turksen et al. 1992). The strategy of removing much of the TNFα 3'-noncoding sequence was based on the knowledge that these sequences are involved in a tightly controlled post-transcriptional regulation of TNFα mRNA (Beutler et al. 1986; Han et al. 1990; Keffer et al. 1991). The strategy of introducing human growth hormone (hGH) sequences as an intron-containing 3'-noncoding segment–polyadenylation signal was used previously as a necessary measure to generate appreciable levels of TGFeα mRNAs in transgenic mice (Sandgren et al. 1990). Ten founder mice tested positive for the transgene, as judged by polymerase chain reaction (PCR) analysis of their ear DNAs. Of founders harboring the K14–TNFα transgene, all showed morphologic differences distinguishing them from normal mice [Fig. 1B]. Similar to rats injected subcutaneously with TNFα (0.5 μg/g of body weight per day) [Fong et al. 1989], these transgenic mice were markedly underweight, becoming very frail, exhibiting signs of impaired movement, and wasting within 2–3 weeks after birth. In addition, transgenic mice displayed marked skin abnormalities, with notably thinner skin, revealing the underlying internal organs [Fig. 1C]. Through the skin, the intestine of the TNFα 2 mouse appeared blackened, suggestive of necrosis [see arrow]. This was confirmed after sacrifice and dissection.

Transgenic animals began exhibiting abnormalities by ~4–6 days of age. Deviations in weight between transgenic mice and their control littermates were observed at this time and increased markedly during neonatal develop-
opment (Fig. 2). By 20 days of age, some animals were as much as threefold underweight. Most of these mice became chronically ill and either died or were sacrificed by 3–4 weeks of age.

The slender physique of our TNFα transgenic animals was suggestive that these mice may have an adipose deficiency. No intrascapular, perirenal, or subcutaneous fat was seen in any of these mice (data not shown). Collectively, the macroscopic evidence from visual inspection and dissection suggested that K14–TNFα mice had developed cachexia as well as cutaneous and intestinal derangements.

Transgenic human TNFα is present in stratified squamous epithelia and in serum

To examine how the macroscopic abnormalities correlated with TNFα transgene expression, we isolated organ mRNAs from 6-day-old transgenic and control mice and tested for the presence of transgene mRNAs by PCR analysis with oligonucleotide primers specific for human TNFα (hTNFα) (Fig. 3A). Relative to mouse β-actin mRNA expression, brain, heart, lung, liver, kidney, spleen, muscle, and intestine were judged negative for hTNFα mRNA expression. In contrast, transgenic tissues that contain keratinocytes, such as skin, thymus, forestomach, and tongue, were positive for the diagnostic transgene band. In all cases, control tissue mRNAs from a nontransgenic littermate were negative [not shown]. In addition, identical results were obtained when tissue mRNAs from a second transgenic mouse and control littermate were analyzed. The pattern of expression of the transgene was in agreement with the known pattern of expression of both the human K14 promoter and the endogenous mouse K14 in these tissues [Nelson and Sun 1983; Vassar et al. 1989; S. Zinkel and E. Fuchs, unpubl.]. To verify that stable hTNFα protein

Figure 2. Growth rates of TNFα transgenic mice. TNFα and control littermates were weighed daily, beginning at day 5 when size differences first appeared, and ending at the day they were sacrificed for analyses. Data on four founder mice are shown. Body weights are given in grams. To verify the accuracy of the values for the nontransgenic control, four additional mice were weighed, and growth plots similar to the one shown were achieved.

Figure 3. PCR and immunohistochemistry analysis of human TNFα expression in transgenic tissues. (A) PCR analysis. PCR products from transgenic and control organ mRNAs were resolved by electrophoresis and visualized by staining with ethidium bromide [see Materials and methods]. Shown here are data from a set of RNAs from one of the two transgenic animals analyzed. Both animals gave identical results with respect to tissue specificity of transgene expression. As expected, none of the control littermate samples produced any PCR products with the primers for hTNFα, and all samples gave signals of comparable intensity with the primers for mouse β-actin. (B) Immunohistochemistry. To verify transgene expression, skin samples of transgenic and control mice were fixed in 2% paraformaldehyde, embedded in paraffin, sectioned (5 μm), and stained with an antiserum specific for hTNFα (Genzyme, Boston, MA). The antibody-staining procedure was immunogold enhancement, described previously [Vassar et al. 1989]. (Left) TNFα transgenic mouse skin; (right) skin from a control littermate. Broken lines denote demarcation between epidermis and dermis. Bars, 15 μm.
was produced in keratinocytes, we used an anti-hTNFα antibody to stain tissue sections. As expected, anti-hTNFα staining was detected in the epidermis and hair follicles of transgenic skin, but not control skin (Fig. 3B).

The absence of fat and the presence of intestinal necrosis and cachexia in our TNFα transgenic animals were consistent with the known effects of elevated serum levels of TNFα, but they were not consistent with a keratinocyte-restricted expression pattern of the transgene. Therefore, we tested the possibility that TNFα was present in the bloodstream of our transgenic mice. The amount of TNFα was quantified by adding serial dilutions of serum samples to TNFα-sensitive 1591-RE3.5 cells and analyzing the effect on viable cell numbers with a colorimetric assay (Teng et al. 1991). As illustrated in Figure 4 (left), transgenic but not control serum contained high levels of TNFα. The majority of this could be neutralized with a mouse monoclonal antibody specific for human TNFα (Fig. 4, right). In contrast, little or no neutralization was obtained with an anti-mouse TNFα antisera (not shown). That proteins produced and secreted by epidermal cells can enter into the bloodstream has been demonstrated previously (Fenjves et al. 1989; Teumer et al. 1990), but this was not observed when the same K14 promoter–enhancer was used to drive the expression of TGFα or interleukin 6 (IL-6) mRNAs tagged with the hGH gene segment as 3'-non-coding sequences (Vassar and Fuchs 1991; Turksen et al. 1992). It seems unlikely that these differences arose solely from differences in sensitivities of the serum assays used to detect different factors, because TNFα was the only one of these three factors to show systemic effects on nonkeratinocyte-containing tissues. Furthermore, it is unlikely that the blood TNFα was derived from hitherto unrecognized regulatory sequences downstream from the transcription initiation site of the hTNFα gene, as a different promoter was recently used to express this same gene in transgenic mice, and yet these mice did not have appreciable levels of TNFα in their blood (Keffer et al. 1991). Collectively, these observations suggest that the presence of TNFα in the bloodstream of our transgenic animals came from the tissue specificity provided by the K14 promoter.

**Figure 4.** Detection of biologically active hTNFα in the serum of transgenic mice. (Left) Sera from transgenic and control littermates were assayed as outlined in Materials and methods. Serum levels of biologically active TNFα were measured by examining serum-induced cytotoxicity of an extremely TNFα-sensitive murine skin tumor cell line, 1591-RE3.5. Cytotoxicity–cytostasis was measured by dye uptake of degenerating cells (see Materials and methods). Test runs were made with serial dilutions of serum from a TNFα transgenic mouse (TNFα 1) and a control littermate. Serial dilutions of recombinant hTNFα (5 U/ml; 100 pg/ml) were used as standards. (Right) The experiment shown at left was repeated, this time using serum from TNFα mouse 1, either in combination with or without added mouse monoclonal antibody specific for hTNFα. Note that the anti-hTNFα antibody neutralized the TNFα activity, indicating that the majority of the serum TNFα was human (i.e., transgene), rather than mouse (i.e., endogenous).
Systemic effects of TNFα in transgenic mice

hair follicle numbers are fixed by birth and diluted naturally during neonatal development. It is notable, however, that TNFα has been found previously to elicit effects on hair growth, particularly when produced in the brain (Cerami 1992).

To further explore differences in transgenic and control skin, we examined their ultrastructure [Fig. 5E–H]. Although many transgenic epidermal cells appeared indistinguishable from control cells, there was evidence of isolated degeneration in some basal cells [Fig. 5E, asterisk]. Cell debris was also quite prominent in the dermis, where there was an infiltration of macrophages, polymorphonuclear neutrophils, and eosinophils, as well as an increased number of lymphocytes [Fig. 5F,G; lymphocytes and macrophage engulfing a degenerated cell; polymorphonuclear neutrophil]. Although our light microscopy studies indicated that the leukocytic infiltration was not massive, the ultrastructural studies clearly revealed an increase in leukocytes in transgenic versus control skin. Furthermore, our results are in agreement with a number of recombinant TNFα studies demonstrating that this factor, directly, indirectly, or synergistically, promotes chemotaxis and antimicrobial activity of neutrophils, macrophages, and eosinophils [Rothstein and Schreiber 1988; Sharpe et al. 1988; Tracey et al. 1988; Munro et al. 1989; Rampart et al. 1989].

In addition to the changes in dermal leukocyte populations, dermal fibroblasts were also increased in number. A localized increase in fibroblast numbers was also observed previously, when recombinant TNFα was subcutaneously injected into mice [Vilecek et al. 1986; Sharpe et al. 1988; Piguet et al. 1990; Postlethwaite and Seyer 1990]. The fibroblasts appeared unusually active, with many undergoing mitosis, and the majority exhibiting an enriched endoplasmic reticulum, suggestive of collagen synthesis [Fig. 5H, top: fibroblast at right undergoing mitosis; fibroblast at left with abundant endoplasmic reticulum; Fig. 5H, bottom: portion of a fibroblast cytoplasm at higher magnification to show greater details of rich endoplasmic reticulum].

Despite the general stimulation of fibroblast growth in the dermis of transgenic skin, some signs of fibroblast degeneration were evident that were not seen in control skin [Fig. 5F,G]. These observations suggested that TNFα overexpression by epidermal cells caused both positive and negative effects on fibroblast growth and differentiation. In this regard, it is interesting that although recombinant TNFα injections have revealed signs of elevated collagen levels [Piguet et al. 1990], the effects of recombinant TNFα on collagen synthesis in vitro have been negative [Brenner et al. 1989]. It remains to be investigated whether the ability of TNFα to elicit both positive and negative effects on fibroblasts is reflective of (1) differences between primary versus secondary responses to TNFα and the factors it induces, (2) differences in overall TNFα levels, or (3) differences between the direct effects of TNFα on fibroblasts versus indirect effects on fibroblasts exerted by other cell types within the skin that are influenced by TNFα.

The TNFα-mediated skin changes described above were even more pronounced in mice as they grew older. By 2–4 weeks of age, transgenic epidermis was notably thinner than the control, and cells showed signs of hypotrophy [Fig. 6A, transgenic; Fig. 6B, control]. Conversely, the stratum corneum was markedly thickened. Whether stratum corneum thickening was the result of a factor-mediated enhancement of the terminal differentiation program or, alternatively, an increase in squame adhesiveness remains to be evaluated. It was interesting, however, that the stratum corneum was also increased in the epidermis of transgenic animals overexpressing IL-6 driven off the same K14 promoter [Turksen et al. 1992].

By 14 days, signs of fat production were nearly absent in TNFα skin [Fig. 6, cf. C and D], and the distance between the epidermis and the muscle had declined by approximately five- to sixfold, leaving the hair follicles significantly shortened. Surprisingly, surface hair length was not markedly reduced from control animals. However, the hair coat still appeared to be less dense than normal, suggesting that the growth of some follicles may have been retarded. Presumably, the deeper follicles were the ones affected, because their structures were perturbed by the shortened distance between the epidermis and the underlying muscle.

Additional skin differences were evident at the ultrastructural level. In some regions, the epidermis was remarkably thin, with only a single layer of spinous cells...
Figure 6. Histopathology and electron microscopy of skin changes in 2- to 4-week-old TNFα transgenic mice. Backskins from 23-day-old (A, B, E, F) or 14-day-old (C, D) transgenic and control mice were fixed and processed as outlined in the legend in Fig. 5. (A, B) Hematoxylin- and eosin-stained skin sections from TNFα and control mice, respectively. Note the presence of a thinner spinous layer and thicker stratum corneum in A. Note also that epidermal cells in A are somewhat smaller and less columnar than those in B. (C, D) Toluidine blue-stained semithin skin sections of TNFα and control mice, respectively. (E, F) Electron micrographs of negatively stained ultra-thin transgenic skin sections. (E) Basal epidermal cell (BC), illustrating flattened cell shape, and region of spinous layer (SP) that is only one cell thick. (SC) stratum corneum; (N) nucleus; (bm) basement membrane; (Fc) fibrocyte. (F) Dermis revealing necrosis of fibroblasts (encased by arrowheads) and degranulation of mast cells (MC). Necrosis of other leukocytes was also seen in the dermis. (E, F) Note the presence of dense collagen fibrils in dermis. Bars, (A, B) 15 μm; (C, D) 35 μm; (E, F) 1 μm.
As seen to a lesser extent in younger transgenic animals, basal cells often appeared flattened, and occasional epidermal necroses were seen (not shown). The density of collagen in the dermis was even more pronounced than observed at the younger ages. Although this was also seen at the light microscopy level (Fig. 6, cf. A and B), it was striking at the ultrastructural level (Fig. 6E). In addition, although the cellularity of the dermis was not as prominent as before, dermal necroses were significantly more prevalent than seen previously. This extended to most types of leukocytes as well as fibroblasts. Interestingly, whereas mast cells in the skin of transgenic animals at day 6 exhibited many granules, these cells contained predominantly empty granules at later ages (Fig. 6F). Necrosis of macrophages and lymphocytes provided additional signs that the immune system was compromised in the older TNFα-expressing mice.

The extensive degeneration in the skin of older TNFα-expressing mice was similar to the response elicited during GVHD, where TNFα levels are known to be elevated dramatically in the skin (Sale et al. 1985; Symington 1989; J. Cheng and E. Fuchs, unpubl.). The thinness of the epidermis in some of our older TNFα transgenic animals was consistent with the notion that TNFα might also be growth inhibitory to keratinocytes in vivo. To assess whether TNFα expression may also influence epidermal growth in vivo at earlier ages, we incubated samples of transgenic and control skin in medium containing 40 μM bromodeoxyuridine (BrdU). Anti-BrdU staining of paraformaldehyde-fixed skin sections revealed only a slight decrease in the total number of DNA-synthesizing cells in the epidermis (Fig. 7A, left). Labeling in control skin, however, was more uniform than in transgenic skin (anti-BrdU-stained sections, Fig. 7B, left and right, respectively). Thus, most regions of transgenic skin exhibited only a few stained basal cells,

**Figure 7.** Changes in the number of DNA synthesizing epidermal and dermal cells in TNFα transgenic mice. Samples of skin [1 x 2 mm] from 8-day-old TNFα transgenic and control mice were placed for 4 hr in medium containing 40 μM BrdU. Samples were washed, fixed in 2% paraformaldehyde, and sectioned, followed by staining with an anti-BrdU antibody, immunogold labeling, and silver enhancement (Vassar and Fuchs 1991). [A] The percentages of labeled cells per unit area of the basal epidermal layer [left] and the dermis [right] were estimated by counting labeled and unlabeled cells in photographs from 10 random fields. Levels of significance for comparisons between samples were determined using the two-tailed Student’s t-test. Statview software was used to calculate mean and standard deviations. [B] Representative examples of the BrdU labeling of skin from control (left) and transgenic (right) mice are also shown. Arrowheads denote labeled cells. Bar, 15 μm.
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with the majority of labeling concentrated in a few isolated regions. These patches of labeling might have been caused by local proliferation induced by the observed patchiness in necroses that occurred within the epidermis. If so, this effect might have tempered somewhat the degree to which TNFα might have actually inhibited keratinocyte growth. This would also explain why Piguet et al. (1990) observed a localized hyperplastic response when mice were injected subcutaneously with TNFα at sufficiently high concentrations to cause substantial epidermal necroses. The patchiness in necrosis may be a further indication that necrosis arises from the synergistic effects of TNFα with other factors, including those that are bacterially derived (Rothstein and Schreiber 1988, Schreiber et al. 1990).

In contrast to its inhibitory effects on epidermal keratinocytes, TNFα has both proliferative (Vilcek et al. 1986, Piguet et al. 1990) and chemoattractive (Postlethwaite and Seyer 1990) effects on fibroblasts. Transgenic dermis showed an increase in the density of BrdU-labeled cells (Fig. 7A, right; cf. labeling in the dermis of anti-BrdU-stained sections in Fig. 7B). Most of the DNA-synthesizing cells in the dermis were fibroblasts (arrowheads denote labeled cells), and this increase in labeling was consistent with the proliferative and chemoattractive effects of TNFα and with the enhanced cellularity within the dermis.

**Induction of mouse TNFα, IL-6, and IL-1 mRNAs in hTNFα transgenic skin**

TNFα is known to activate a wide variety of genes in a multitude of target cells (Fong et al. 1989, Akira et al. 1990, Cerami 1992). This includes an autoinduction process as well as an induction of IL-6 and interleukin 1 (IL-1). To determine whether these factors are induced in our mice as a consequence of transgenic TNFα expression, we isolated skin mRNAs from transgenic and control mice and subjected 500-ng RNA aliquots to PCR analyses using oligonucleotide primers specific for mouse TNFα, IL-6, and IL-1α mRNAs (Fig. 8). As expected, control oligonucleotide primers specific for mouse β-actin and mouse K14 gave rise to PCR cDNAs of the expected sizes for both transgenic and control skin mRNAs. In contrast, oligonucleotide primers specific for mouse TNFα mRNA generated an intense PCR band of the expected size in the transgenic, but not the control, skin sample. That mouse TNFα mRNA was induced in keratinocytes was confirmed by selectively removing the epidermis from the skin and repeating the mRNA extraction and PCR analyses (not shown). Despite the ability of endogenous TNFα mRNA to be induced in hTNFα-expressing transgenic keratinocytes, most of the active TNFα produced by these mice appeared to be hTNFα (transgene), as distinguished by the species-specific antibodies used in the blood serum assays [see Fig. 4].

We were struck by the morphologic similarities in the epidermis of 3- to 4-week-old transgenic animals expressing TNFα [this paper] and IL-6 (Turksen et al. 1992) driven off the K14 promoter–enhancer. To assess whether IL-6 might be induced in the skin of transgenic mice, we repeated the PCR analyses, this time with oligonucleotide primers specific for mouse IL-6. As illustrated in Figure 8, an IL-6 PCR band of the appropriate size was present in the transgenic, but not the control, skin sample. Again, IL-6 mRNAs were induced in the keratinocyte fraction of the skin, as judged by epidermal fractionation (not shown). Thus, our findings are consistent with the notion that some of the changes in the epidermis may be the result of induction of IL-6 gene expression by TNFα.

Another factor known to be up-regulated with treatment of epidermal cells with TNFα is IL-1α (for review, see Akira et al. 1990). IL-1 was discovered as a pyrogen, and it is the most potent inflammatory cytokine that can generate prostaglandin E2 synthesis and become chemotactic and mitogenic for thymocytes, lymphocytes, and
other immune cells. A strong PCR band was seen in the keratinocyte mRNA fraction of transgenic, but not control, skin, suggesting the possibility that IL-1α may play a role in mediating a part of the inflammatory response seen in our TNFα mice. IL-1β mRNA was also induced in TNFα mice, but this appeared to be largely in the dermal component of the fractionated skin. Additional studies will be necessary to determine the extent to which other cytokines and factors, including interleukin 8 (IL-8), transforming growth factor-β (TGF-β), and granulocyte macrophage–colony-stimulating factor (GM-CSF), might be relevant to the wide range of pleiotropic effects observed in our mice, both in the skin and in other organs.

Discussion

Keratinocyte-expressed TNFα appears to play a major role in eliciting the pleiotropic effects of TNFα

The TNFα-expressing transgenic mice that we have generated using a keratinocyte promoter and an altered 3’-noncoding segment had a markedly different phenotype from those generated previously by use of the natural TNFα promoter and an altered 3’-noncoding segment [Keffer et al. 1991]. Most notably, although our mice developed weight loss, cachexia, inflammation, keratinization, fibrosis, necrosis, and intravascular hemorrhaging, the previous TNFα transgenic mice only developed one feature typically associated with high levels of TNFα, namely chronic inflammatory polyarthritis. The obvious explanation for these differences is the targeting of deregulated TNFα expression to different tissues. Keffer et al. [1991] detected transgene mRNAs primarily in thymus, lung, spleen, kidney, brain, joints and possibly, skin, but these mice did not produce appreciable serum levels of transgenic TNFα. In contrast, we targeted TNFα transgene expression to tissues containing keratinocytes, and these mice readily produced detectable serum levels of transgenic TNFα.

Our findings have important implications for the role of TNFα-expressing keratinocytes in inflammatory and immune responses in mammals. Prior to our studies, it had been widely assumed that macrophages were the major mediators of TNFα action. Although macrophages may still play a seminal role in exerting the TNFα-induced immune responses, our studies suggest that other TNFα-producing cells, such as keratinocytes, can also mediate a chemotactic recruitment of macrophages, polymorphonuclear neutrophils, and eosinophils to the skin. Our mice exhibited most, if not all, of the effects known to be characteristic of TNFα. We have not unequivocally ruled out the possibility that expression of the transgene by nonkeratinocyte cells could have escaped our detection. The major source of transgene TNFα in our mice was the keratinocyte, however, and specifically, the epidermal keratinocyte. Thus, it seems most likely that keratinocyte-derived TNFα was able to find its way into the bloodstream, thereby activating a global response to the immune system of the animal.

Grafting transgenic epidermal keratinocytes to the skin of nude mice, analogous to procedures used by Teumer et al. [1990], should be able to test this hypothesis directly.

Transgene hTNFα leads to up-regulation of endogenous mouse TNFα mRNA levels

An interesting consequence of our transgene hTNFα expression was the up-regulation of endogenous mouse TNFα (mTNFα) mRNA. Although we presented data only for up-regulation of skin mTNFα mRNAs, we noticed that many other transgenic tissues also showed elevated levels of endogenous TNFα mRNAs (data not shown). Because this up-regulation in mTNFα mRNA levels occurred irrespective of whether the tissue contained keratinocytes, much of the induction may have been a consequence of circulating hTNFα. In this regard, it was curious that the TNFα present in the blood was mostly human, as judged by neutralization of the activity with antibodies that were highly specific for hTNFα. It is well-known, however, that TNFα has intricate regulatory mechanisms at post-transcriptional levels. Thus, it was shown previously that after activation of TNF mRNA translation by lipopolysaccharide [a major inducer of inflammation], transcription from the TNFα gene is augmented three times, whereas steady-state TNF mRNA levels are increased ≥100 times [Beutler et al. 1986; Han et al. 1990]. Moreover, when the hTNFα transgene was recently introduced into the germ line of mice, no apparent phenotype was detected, although hTNFα mRNAs were produced in a number of different tissues [Keffer et al. 1991]. In contrast, a TNFα mRNA with a modified 3’-noncoding sequence was deregulated, and biologically active TNFα was produced [Keffer et al. 1991], perhaps in a fashion similar to our TNFα mRNA, which has extended hGH sequences in place of the natural 3’-noncoding sequence. These differences could readily account for our failure to detect appreciable mTNFα, although the endogenous TNFα mRNA was up-regulated quite dramatically. Given the potentially life-threatening effects of TNFα, intricate regulation is not surprising.

TNFα action: direct vs. indirect effects

TNFα secretion can be elevated to dangerous levels during severe infections, leading to such deleterious effects as fever, hypotension, acidosis, diffuse intravascular coagulation and, ultimately, death [for review, see Tracey et al. 1986; Schreiber et al. 1990]. At lower levels, TNFα plays an important protective role in infectious diseases, stimulating chemotaxis and the antimicrobial activity of neutrophils, macrophages, and eosinophils [Sharpe et al. 1988; Tracey et al. 1988; Munro et al. 1989; Rampart et al. 1989]. That TNFα is the mediator of these pleiotropic responses has been demonstrated by neutralizing the effects with anti-TNFα antibodies [Beutler et al. 1985; Piguet et al. 1987; Tracey et al. 1987; Keffer et al. 1991]. In addition, administration of sublethal doses of recom-
binant TNFα to rodents has been shown to cause cachexia, anemia, neutrophil-, eosinophil-, and macrophage-mediated inflammation, septic shock during lethal bacterial, epidermal and dermal necroses similar to GVHD, and hemorrhagic necrosis in certain tumors (Sale et al. 1985; Tracey et al. 1986, 1987, 1988; Oliff et al. 1987; Piguet et al. 1987; Djeu et al. 1988; Rothstein and Schreiber 1988; Gordon and Galli 1990; Teng et al. 1991).

Given the myriad regulatory factors that are influenced by TNFα expression, it is highly likely that TNFα acts in concert with these other cytokines, factors, and inflammatory cells to elicit the varied and extensive macroscopic changes in animal physiology. In this regard, it has been shown that at least in part, the effects of TNF on severe bacterial infections may be the result of a strong synergy between TNF and bacterial substances and other factors which, in turn, may induce other synergistic cytokines (Rothstein and Schreiber 1988). IL-6 is one cytokine that is thought to be a major player in mediating some of the responses of TNFα (for review, see Akira et al. 1990; Cerami 1992). We have recently prepared transgenic mice overexpressing keratinocyte IL-6 (Turksen et al. 1992). The effects on the stratum corneum and hair growth that were elicited by TNFα overexpression seemed similar to those elicited by IL-6 overexpression. Thus, the effects that we have observed on the epidermis in our TNFα mice might be indirect, arising from IL-6 induction. In contrast, we did not detect an appreciable inflammatory response in our IL-6 mice, nor did we detect the other major changes in the skin and other organs that were seen in our TNFα mice. Therefore, it seems that these additional features must arise either from other cytokines and factors, or from IL-6 acting synergistically either with TNFα itself or with other induced factors.

The inflammatory responses in the skin could have been mediated by several of the cytokines known to be activated by TNFα. Of these, IL-1 was discovered as a pyrogen, and it is the most potent inflammatory cytokine that can generate prostaglandin E₂ synthesis and become chemotactic and mitogenic for thymocytes, lymphocytes, and other immune cells (Fong et al. 1989; Akira et al. 1990; Cerami 1992). This factor is expressed in keratinocytes and dermal endothelial cells in response to TNFα, and it provides adhesion sites for neutrophils and lymphocytes (Griffiths et al. 1989). In our studies, IL-1α was found preferentially in the epidermis, whereas IL-1β was found mostly in the dermal fraction of TNFα transgenic skin. That IL-1 acts synergistically with TNFα has been strongly suggested from previous studies (Rothstein and Schreiber 1988, Schreiber et al. 1990).

A number of other cytokines have been implicated in mediating the effects of TNFα, including IL-8, GM-CSF, M-CSF, G-CSF, and TGF-β (for review, see Akira et al. 1990; Cerami 1992). Although we have not yet examined whether these other factors are induced in the skin of our TNFα mice, many of these factors are clearly candidates for producing some of the effects that we have observed in the skin. As future transgenic studies are conducted, the extent to which these factors contribute either linearly or synergistically to the wide range of effects observed here should become more apparent.

Materials and methods

Generation of transgenic mice

The vector pK14–TNFα–bGH was constructed as outlined in the legend to Figure 1. Transgenic mice were generated as described (Vassar et al. 1989) and were identified by PCR analysis of ear DNAs.

Assay for biological activity of TNFα in serum

Serum TNFα levels were quantified as described previously (see Teng et al. 1991 and references therein). Serum samples from transgenic and control animals and recombinant hTNFα (Genentech, South San Francisco, CA) standards were serially diluted in culture medium in 96-well plates containing the TNFα-sensitive cell line, 1591–RE3.5, isolated from a UV-induced murine skin tumor. After a 2-day incubation, 100 μl of medium was removed from each well and was replaced with 20 μl of a 5-mg/ml solution of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO) in PBS. Plates were incubated for an additional 4 h, after which the dye was solubilized overnight with 100 μl of 10% SDS, 0.01 N HCl. The absorbance from 570 to 650 nm was read on a Molecular Devices Vmax plate reader. Percent cytotoxicity–cytostasis was determined as follows: % cytotoxicity/cytostasis = [1 – OD of experimental well/average OD of control wells] × 100.

Each point shown is the average of duplicate wells. A mouse anti-recombinant hTNF monoclonal antibody (TNF-E; 1,2) was added at a final concentration of 1 μg/ml to a duplicate round of test sera.

PCR analyses

Organs from two 3- to 4-week-old transgenic and control littersmates were taken for analyses. In addition, skins from the backs of 6-day-old transgenic and control mice were removed and either used directly or subjected to a 15-min incubation with 2 mM sodium bromide at 37°C to selectively remove the epidermis. Tissues were then snap-frozen in liquid nitrogen, ground to a frozen powder, and processed for total RNA isolation (Vassar et al. 1989). To synthesize single-stranded cDNA, 5 μg of RNase-free, DNase-treated total RNA was mixed with 1 μg of oligo(dT) primer, 30 units of reverse transcriptase (Seikagaku America, Inc.), and 40 units of RNasin (Promega Biotec, Madison, WI) in reaction buffer [100 mM Tris hydrochloride (pH 8.0), 30 mM KCl, 10 mM MgCl₂, 1 mM deoxynucleoside triphosphates] and incubated for 90 min at 42°C. RNA was then hydrolyzed in 0.5 N NaOH for 30 min at 70°C, and single-stranded cDNA was precipitated with ethanol. The cDNA pellet was washed with 70% ethanol and dissolved in TE buffer. cDNAs were amplified under conditions suggested by Perkin-Elmer Cetus, and reactions were analyzed by agarose gel electrophoresis. To ensure reliability, total RNA preparations were prepared from two different transgenic animals, and two rounds of PCR amplifications were conducted on each RNA sample. PCR bands were only generated from mRNA samples to which reverse transcriptase was added in the initial reaction, demonstrating that the PCR bands were from mRNA, rather than from genomic DNA.

The PCR primers used are listed below, with the predicted size of the amplified product given in parentheses (see also Murray et al. 1990). Primers were for (1) mouse β-actin [348 bp],
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