Concerted action of TGF- β 1 and its type II receptor in control of epidermal homeostasis in transgenic mice

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Transforming growth factor- β 1 (TGF- β 1) is a modulator of cellular proliferation, differentiation, and extracellular matrix deposition. It is a potent epithelial growth inhibitor and can alter the differentiative properties of keratinocytes, *in vitro*, but little is known about its normal physiological function in the epidermis *in vivo*. Transgenic mice were generated using a keratin 10 (K10) gene promoter to drive constitutive expression of TGF- β 1 in the suprabasal keratinocyte compartment. Surprisingly, these mice showed a two- to threefold increase in epidermal DNA labeling index over control mice, in the absence of hyperplasia. The transgene, however, acted in the expected fashion, as a negative regulator of cell growth, when hyperplasia was induced by treatment by 12-tetradecanoyl-phorbol-13-acetate (TPA). Epidermal TGF- β type I and II receptor (T β RI and T β RII) levels were examined in control and transgenic mice during induction of hyperplasia by TPA. Whereas T β RI levels remained relatively constant, T β RII expression was strongly induced in TPA-treated skins, prior to the induction of the growth inhibitory response to TGF- β 1, and its level of expression correlated with growth sensitivity to TGF- β 1 *in vivo* and *in vitro*. These results suggest that TGF- β 1 and its type II receptor are part of the endogenous homeostatic regulatory machinery of the epidermis.

[**Key Words:** TGF- β 1; TGF- β receptor; epidermal homeostasis; transgenic; keratinocyte]

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Transforming growth factors- β (TGF β s) are a family of three multifunctional peptides that affect cellular proliferation and differentiation and that modulate the extracellular matrix (Roberts and Sporn 1990; Akhurst 1994). Biosynthesis of, and response to, TGF- β s are under complex control. All three TGF- β s are synthesized as biologically inactive latent complexes. Active TGF- β is processed from the carboxyl terminus of a larger prepropeptide. However, latency is conferred by the persistent noncovalent association of the proteolytically cleaved amino-terminal latency-associated peptide (LAP) with the bioactive mature form of TGF- β 1. The biological response thus requires activation of latent TGF- β , prior to the binding of the ligand to its receptor complex (Brunner et al. 1989; Roberts and Sporn 1990; Miyazono et al. 1993). Recently, increasing evidence has suggested that the three TGF- β s act via the same cell surface receptor complex, which consists of a hetero-oligomeric complex,

of type I and type II TGF- β receptors (T β RI and T β RII). However, T β RII is thought to be the primary receptor through which the ligand directly binds to this complex (Laiho et al. 1990; Geiser et al. 1992; Wrana et al. 1994).

The three TGF- β s have similar qualitative biological effects on different cell types *in vitro* (Graycar et al. 1989; ten Dijke et al. 1990). They are potent epithelial growth inhibitors (Tucker et al. 1984; Shipley et al. 1986; Coffey et al. 1988; Russell et al. 1988) and can alter the differentiative properties of keratinocytes (Reiss and Sartorelli 1987; Mansbridge and Hanawalt 1988). In a mouse skin model, we have shown that TGF- β 1 expression is rapidly induced in suprabasal keratinocytes *in vivo*, in response to hyperplasia (Akhurst et al. 1988; Fowles et al. 1992), thus implicating this growth factor in regulation of epidermal homeostasis. Furthermore, perturbations in the production of, or response to, TGF- β are thought to be involved in multistage carcinogenesis in several types of epithelia (Terzaghi-Howe 1989; Krieg et al. 1991; Glick et al. 1991, 1993; Manning et al. 1991; Missero et al. 1991; Fowles et al. 1992; Cui et al. 1994). Elucidation of the molecular mechanisms of control of epithelial homeostasis by TGF- β is therefore central to our under-

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standing of the molecular pathology of both neoplastic and nonneoplastic skin diseases.

Recent studies on TGF- β 1 transgenic mice have supported the concept that TGF- β 1 is primarily a keratinocyte growth inhibitor *in vivo* as well as *in vitro*. For example, attempts to make viable lines of transgenic mice with keratin 1 (K1)-targeted expression of TGF- β 1 to suprabasal keratinocytes failed because the founder mice died at birth as a result of inadequate epidermal proliferation (Sellheyer et al. 1993). Furthermore, mice with a homozygous genetic lesion in the TGF- β 1 gene have been reported to have a three- to fivefold elevated epidermal DNA labeling index over control mice (Glick et al. 1993).

In this study viable lines of transgenic mice with suprabasally targeted expression of bioactive TGF- β 1 were successfully generated. Surprisingly, contrasting with previous reports (Glick et al. 1993; Sellheyer et al. 1993), these mice showed a two- to threefold increase in epidermal DNA labeling index over control mice. This paper emphasizes the importance of the concerted action of both TGF- β 1 and T β RII in the regulation of homeostasis within the epidermis.

Results

Generation of transgenic mice with targeted expression of TGF- β 1 to suprabasal keratinocytes

A 7.25-kb K10-TGF- β 1^{act} gene construct was generated (Fig. 1C) to target TGF- β 1 expression to differentiated suprabasal keratinocytes of transgenic mice. A bovine cytokeratin VI gene promoter (Blessing et al. 1989), the homolog of human K10, was used that has been shown previously to effectively target gene expression to the suprabasal compartment of the epidermis (Bailleul et al. 1990). To ensure the expression of biologically active TGF- β 1, a simian TGF- β 1 cDNA was used, which had undergone site-directed mutagenesis of Cys-223 $\rightarrow$ Ser and Cys-225 $\rightarrow$ Ser. These mutations prevent dimerization of the TGF- β 1 LAP, by destruction of disulfide bridge formation, thus leading to the synthesis of only biologically activated TGF- β 1 (Brunner et al. 1989). Splicing and polyadenylation signals were provided by an SV40 small t-antigen cassette (Mulligan et al. 1979).

K10-TGF- β 1^{act} transgenic mice were generated on a CBA/Ca $\times$ C57/Bl6 genetic background. Three founder mice and their transgenic lines (A, G, and H) were obtained and analyzed by Southern blot analysis of *Bgl*III-digested genomic DNA, using the SV40 gene probe (Fig. 1A). Both the A- and H-line mice gave a 2.35-kb band on Southern analysis, as expected by integration of the full-length transgene, whereas the G line had undergone a transgene DNA rearrangement to give a *Bgl*III band of 3.5-kb.

Preliminary studies on the A and H lines, which involved induction of hyperplasia by topical application of TPA, revealed that the transgenic mice were abnormally hypersensitive to TPA, which manifested as a variable, severe skin inflammation. A similar variable response

Figure 1. Targeting bioactive TGF- β 1 expression to the suprabasal keratinocytes of transgenic mice. (A) Southern blot analysis of genomic DNA from the three transgenic lines, A, G, and H, using the SV40-specific hybridization probe (C) to detect the transgene and the mCFTR probe (Tata et al. 1991) as an internal DNA loading control. The SV40 probe hybridized to a 2.35-kb DNA fragment in both the A- and H-line mice, which was the correct size for the TGF- β 1 transgene. G-line DNA gave a 3.5-kb fragment, which indicates rearrangement of the transgene. Additional restriction digests confirmed the integrity of the A- and H-line transgenes and rearrangement of the G-line DNA. (He) H-line heterozygous genomic DNA; (A, G, and H) A, G, and H homozygous genomic DNA, respectively; (C) nontransgenic littermate DNA. (B) Northern blot analysis of recombinant TGF- β 1 mRNA expression in transgenic mice, using 15 μ g total epidermal RNA per lane, probed with the SV40 probe. Both H homozygous (H) and A heterozygous (A) mice showed recombinant transcripts of 2.5 and 1.9 kb, whereas G homozygous (G) mice showed no expression. Equal loading was verified by reprobing the membrane with a 7S RNA cDNA probe (Balmain et al. 1982). (C) Schematic diagram to show the structure of the 7.25-kb K10-TGF- β 1^{act} transgene. (B) *Bgl*III; (N) *Nru*I; (S) *Sal*I. The shaded region is the coding region of TGF- β 1. The location of the initiation codon (ATG) and the Cys $\rightarrow$ Ser mutations (amino acids 223 and 225) are indicated.

was seen in control CBA mice. The transgenes were therefore bred through four generations onto a high (94%) NIH/Ola inbred genetic background, the well-characterized mouse strain routinely used for chemical carcinogenesis studies in our laboratories. The mice were further bred to homozygosity, which was confirmed by outbreeding to control mice.

Expression of biologically active TGF- β 1 in the epidermis of K10-TGF- β 1^{act} transgenic mice

Transgene expression was confirmed by Northern blot analysis of total RNA extracted from the epidermis of heterozygous and homozygous mice (Fig. 1B). Only the A and H lines showed clear mRNA expression in both hemizygous and homozygous mice. G-line mice did not express the recombinant RNA, because of transgene rearrangement. Transgene expression levels in homozygous A- and H-line mice, as determined by Northern analysis, were approximately twice those observed in the heterozygous state (Fig. 1B; data not shown). The tissue specificity of gene expression was assessed by *in situ* hybridization, utilizing a transgene-specific probe. Recombinant TGF- β 1 mRNA expression was shown to be restricted to suprabasal keratinocytes of the epidermis in the A and H transgenic mice (Fig. 2A–F).

Despite confirmation of accurate transgene transcription, levels of immunohistochemically stainable TGF- β 1 protein were the same in skins of both transgenic and control mice, using two antibodies, either anti-LC or anti-CC, each of which have been well-characterized with respect to their histochemical staining patterns for TGF- β 1 (Flanders et al. 1989; Thompson et al. 1989).

This phenomenon was seen previously in K1-TGF- β 1^{act} transgenic founder mice that were presumed to express large quantities of recombinant bioactive TGF- β 1 in the skin, because they suffered neonatal mortality as a result of a lack of skin development (Sellheyer et al. 1993). It has been suggested that the inability to immunohistochemically detect the protein is the result of the exceptional instability of recombinant bioactive TGF- β 1 (Sellheyer et al. 1993).

Additional evidence that bioactive TGF- β 1 was expressed in the transgenic skins was provided by comparing expression of plasminogen activator inhibitor 1 (PAI-1) in transgenic versus control skins. PAI-1 is known to be positively regulated by TGF- β 1 (Lund et al. 1987) and reproducibly showed higher levels of expression in transgenic skins than in controls (Fig. 3A,B).

The epidermal proliferative response to TPA is attenuated in K10-TGF- β 1 transgenic mice

Further evidence that recombinant bioactive TGF- β 1 was expressed in the K10-TGF- β 1^{act} epidermis was provided by the fact that these mice did not exhibit the normal wave of basal keratinocyte DNA synthesis that occurs under conditions of perturbed homeostasis, following treatment with the tumor promoter, TPA (Krieg et al. 1974; Balmain and Hecker 1976). Bromodeoxyuridine (BrdU) labeling indices were measured in control and H-line transgenic mice at various time points (0, 6, 24, 36, 48, 72 hr, and 1 week) after topical application of TPA to the skin. Control mice exhibited the well-characterized increase of DNA synthesis in basal keratinocytes, which peaked 36 hr after TPA treatment (Figs.

Figure 2. Localization of TGF- β 1 transgene mRNA to the suprabasal keratinocytes of transgenic mice by *in situ* hybridization. Five-micron paraffin sections of mouse dorsal skin were subjected to *in situ* hybridization using ³⁵S-labeled SV40 antisense (A–C,E,F) or sense (D) riboprobe. Autoradiographic exposures were for 8–12 days. Bright-field (left) and dark-field (right) images are shown. (A–D) Adult H-line homozygous transgenic mouse skin; (E,F) H-line homozygous neonatal skin. Bars, 100 μ m (A,B,D,E,F) or 50 μ m (C).

Figure 3. Histochemical effects of expression of the K10-TGF- β 1^{act} transgene in the epidermis. (A,B) Immunohistochemical staining with the PAI-1 antibody in untreated, telogen epidermis of control (A) and transgenic (B) mice. There was a reproducible increase in PAI-1 staining intensity in the transgenic compared with nontransgenic epidermis. (C,D) Immunostaining with the anti-BrdU antibody in the skin of nontransgenic (C) and homozygous H-line transgenic (D) mice, 36 hr following TPA treatment. (E,F) Immunostaining with the anti-PCNA antibody in the skin of nontransgenic (E) and heterozygous H-line transgenic (F) mice, 36 hr following TPA treatment. Bar, 100 μ m.

3C and 4A). In contrast, incorporation of BrdU into the basal keratinocyte nuclei of the transgenic mice was substantially reduced at 36 hr post-TPA (Figs. 3D and 4A). Moreover, similar effects were shown on immunostaining for proliferating cell nuclear antigen (PCNA) (Fig. 3E,F), a marker of proliferative potential (Hall et al. 1990; Scott et al. 1991). Thus, it appears that the epidermal proliferative response to TPA is attenuated in the K10-TGF- β 1^{act} transgenic mice. Histologically, this was manifested as a slight reduction in the thickness of the transgenic mouse epidermis at 36 hr post-TPA (Fig. 3), which was more pronounced at later time points (data not shown).

K10-TGF- β 1 transgene expression increases the epidermal DNA labeling index in quiescent skin

Our expectation was that ectopic expression of bioactive TGF- β 1 in the epidermis would result in an inhibition of epidermal growth regardless of the state of homeostatic equilibrium. It has been reported previously by others that transgenic mice very similar to those generated in this study died at birth because of a complete inhibition of epidermal proliferation (Sellheyer et al. 1993). However, it was apparent that at early (up to 24 hr) and late (1

week) time points after TPA treatment, DNA labeling indices were increased in transgenic epidermis compared with controls (Fig. 4B). Examination of nuclear incorporation of BrdU into the epidermis of untreated mice showed that DNA synthetic indices were elevated two- to threefold in both the A- and H-line epidermis compared with controls. Despite this large increase in proliferative rate, there was no obvious histological evidence of hyperplasia or hyperkeratosis in the transgenic skins. Quiescent epidermal cells therefore appear to be refractile to negative growth inhibition by TGF- β 1 and are apparently growth stimulated, either directly or indirectly, by TGF- β 1 in the transgenic epidermis. Furthermore, overexpression of TGF- β 1 in the epidermis of the transgenic mice must increase cell turnover without disturbing physiological homeostasis.

T β RII, but not T β RI, is induced in response to epidermal hyperplasia

The negative growth response of basal keratinocytes to recombinant TGF- β 1 in K10-TGF- β 1^{act} transgenic mice did not appear until 36 hr post-TPA (Fig. 4A), by which time epidermal hyperplasia was apparent (Fig. 3C–F). Because T β RII has been shown to be critical for negative

Figure 4. Overexpression of TGF- β 1 in the suprabasal keratinocytes of transgenic mice alters DNA labeling indices in the basal cell compartment. (A) Incorporation of BrdU into basal keratinocyte nuclei following topical application of TPA was attenuated in the H-line homozygous transgenic mice (●) compared with the controls (○). Epidermal BrdU labeling indices were estimated at 0, 12, 24, 36, 48, and 72 hr, and 1 week after TPA treatment. The difference between transgenic and control skins was most pronounced at 36 hr post-TPA, when the control DNA labeling index peaked ($P=0.0025$, Student's *t*-test). The results were obtained from two independent experiments, assaying BrdU incorporation in three mice per time point in each experiment. (B) Increased DNA labeling index in keratinocytes of untreated adult skin from K10-TGF- β 1^{act} transgenic compared with control mice. The histogram shows the extent of anti-BrdU labeling in the epidermis of adult control and H-line homozygous mice. Each bar represents one mouse. The data indicate a significant increase in the number of keratinocyte nuclei stained for BrdU in transgenic mice compared with controls ($P<0.0001$, Student's *t*-test).

growth regulation of epithelial cells (Laiho et al. 1990; Geiser et al. 1992; Wrana et al. 1994), we tested the possibility that the onset of epidermal negative growth regulation in vivo might correlate with changes in T β RII receptor levels.

A murine T β RII cDNA probe was cloned by reverse transcription-polymerase chain reaction (RT-PCR) using RNA extracted from a keratinocyte cell line. The fidelity of the PCR product was verified by bidirectional DNA sequencing. Expression levels of T β RII mRNA, in

nontransgenic epidermis with or without TPA treatment and in quiescent transgenic epidermis, were assessed by Northern blot analysis with the mouse T β RII cDNA probe and by semiquantitative RT-PCR, using total RNA extracted from epidermal scrapes of mouse dorsal skin at various time points (0, 6, 12, 24, and 36 hr) after TPA treatment. For the semiquantitative RT-PCR, glyceraldehyde phosphate dehydrogenase (GAPDH) cDNA was coamplified as an internal control. The overall levels of expression of T β RII were low in both untreated and TPA-treated epidermis. Nevertheless, both RT-PCR and Northern analysis showed that T β RII RNA was strongly induced between 6 and 12 hr after TPA treatment and remained at an elevated level for at least 36 hr (Fig. 5A,B).

The specificity of induction of T β RII was shown by the fact that T β RI levels were largely unaffected by TPA treatment (Fig. 5D). The same series of RNA samples shown in Figure 5B were amplified using RT-PCR with primers derived from the murine T β RI cDNA sequence (Tomoda et al. 1993; Fig. 5D, top), and the corresponding Southern blot was probed using a human T β RI probe (Franzen et al. 1993; Fig. 5D, bottom). T β RI levels remained relatively constant throughout, suggesting that only the type II receptor is subject to regulation by the proliferative status of the epidermal cells.

T β RII induction following TPA treatment was also examined by immunohistochemistry in control and H-line homozygous mice, using a polyclonal antibody against human T β RII (Moustakas et al. 1993; Henis et al. 1994). Both control and H-line transgenic skins showed similar spatial distributions and kinetics of induction of T β RII protein. In quiescent skin, T β RII polypeptide was immunohistochemically undetectable (Fig. 6A). By 12 hr post-TPA, before appearance of hyperplasia, T β RII immunostaining could be seen in both basal and suprabasal keratinocytes (Fig. 6B), which was still apparent at 24 hr post-TPA (Fig. 6C). By 48 hr, when epidermal hyperplasia was apparent, T β RII was predominantly localized in the suprabasal keratinocytes (Fig. 6D). The lack of growth inhibition of basal keratinocytes in homeostatic equilibrium may thus be the result of low levels of expression of T β RII, whereas negative growth regulation by TGF- β may be acquired as a consequence of the induction of T β RII expression, during TPA-induced hyperplasia.

As keratinocytes are known to be potently growth inhibited by TGF- β in vitro (Tucker et al. 1984; Shipley et al. 1986; Coffey et al. 1988), we investigated the possibility that this might also be because of elevated expression of T β RII, when cells are placed in culture. Figure 5C shows that this is the case. T β RII RNA expression levels were barely detectable in adult (Fig. 5C) and neonatal keratinocytes in vivo (data not shown). There was a massive induction of T β RII RNA expression when neonatal keratinocytes were placed in culture (Fig. 5C), to levels much higher than those seen even in the TPA-treated skin in vivo (data not shown). T β RII RNA expression levels were also examined, by RT-PCR, in the untreated epidermis of control versus H-line mice. Although the levels of T β RII RNA were very low in all the samples,

the H line reproducibly showed reduced expression levels of T β RII compared with controls (Fig. 5B, lanes N,H).

Discussion

Coordinate induction of TGF- β 1, T β RII, and TGF- β 1 growth sensitivity in response to hyperplasia

We have shown previously that TGF- β 1 expression is rapidly and transiently induced in suprabasal keratinocytes in response to TPA-stimulated hyperplasia [Akhurst et al. 1988; Fowles et al. 1992]. This molecule might be partially responsible for mediating the early wave of keratinocyte differentiation induced by TPA [Hawley-Nelson et al. 1982; Parkinson and Emmerson 1982; Reiners and Slaga 1983; Reiss and Sartorelli 1987; Mansbridge and Hanawalt 1988] and/or for modulating the epidermal hyperplastic response to TPA [Tucker et al. 1984; Shipley et al. 1986; Coffey et al. 1988; Russell et al. 1988]. Here, we show that in quiescent mouse epidermis T β RII expression levels are very low but, like TGF- β 1, they are induced in response to TPA-stimulated hyperplasia. This induction of the receptor correlates with acquisition of negative growth sensitivity of keratinocytes to recombinant TGF- β 1 in K10-TGF- β 1^{act} transgenic mice. Thus, keratinocyte growth responsiveness to TGF- β 1 in vivo appears to be determined by the levels of expression of T β RII. In contrast, the type I receptor levels are not substantially altered by perturba-

tion of epidermal homeostasis. It has been shown similarly that modulation of epithelial cell growth in vitro is associated with changes in type II, but not type I, receptor expression [Okamoto et al. 1994].

In this study we have also demonstrated that T β RII expression levels are massively induced when keratinocytes are placed in culture. Thus, the extreme growth sensitivity of cultured epithelial cells to TGF- β [Tucker et al. 1984; Shipley et al. 1986; Coffey et al. 1988; Russell et al. 1988], might be the consequence of elevated T β RII expression which occurs in response to culture (hyperplasia). In vitro studies on the production of, and responsiveness to, TGF- β by epithelial cells cannot therefore be extrapolated to the in vivo situation. In particular, induction of T β RII expression and consequent increased sensitivity to TGF- β in vitro should be taken into account when assessing responsiveness of human squamous tumor cell lines to TGF- β [Suardet et al. 1992].

Transgenic mice expressing TGF- β 1^{act} in suprabasal keratinocytes have an increased basal keratinocyte proliferative index

In this study viable lines of transgenic mice were generated with targeted suprabasal keratinocyte expression of bioactive TGF- β 1. Unexpectedly, and in contrast to similar [Sellheyer et al. 1993] and complementary studies [Glick et al. 1993], these mice had an increased epider-

Figure 6. Induction of T β RII protein in response to TPA-induced hyperplasia. Immunohistochemical staining of H-line homozygous transgenic skins with an anti-T β RII antibody at various time points after TPA treatment. (A) Untreated mouse skin; (B) 12 hr post-TPA; (C) 24 hr post-TPA; (D) 48 hr post-TPA; (E) 48 hr post-TPA with antibody preincubated with the T β RII blocking peptide. Identical staining patterns were obtained in nontransgenic and transgenic skins alike. Bar, 50 μ m.

mal DNA labeling index suggesting that, in vivo, quiescent basal keratinocytes are refractile to negative growth regulation by TGF- β 1.

Sellheyer et al. (1993) produced transgenic mice using the K1 gene promoter to drive expression in suprabasal keratinocytes. They showed a complete inhibition of DNA synthesis in the transgenic founders, which resulted in neonatal lethality. The differences between that study and the current one could be attributed to the use of different gene promoters, mouse strain-specific differences in neonatal expression levels of T β RII, or, more likely, to differences in recombinant TGF- β 1 expression levels. In this study the K10 promoter was transcribed exclusively in suprabasal keratinocytes, whereas the K1 promoter used by Sellheyer et al. (1993) was expressed additionally in a subset of basal keratinocytes (Greenhalgh et al. 1993). More importantly, it must be assumed that the transgenic mice generated by Sellheyer et al. (1993) expressed much higher levels of TGF- β 1 protein. The K1-TGF- β 1^{act} gene construct was designed to generate a more stable mRNA by incorporating the endogenous K1 gene 3'-noncoding region and to have more effective translation, by modification of the translation start site. At pharmacological levels of TGF- β 1 expression, any effects as a physiological regulator of epidermal homeostasis might be masked by other biological activities. Studies of the effects of transgenic expression of TGF- β 1 in the mammary gland have also shown substantial differences in phenotype as a consequence of the level and/or cell specificity of transgene expression. The whey acidic protein gene promoter induced high-level expression in mammary epithelium and completely inhibited growth of the mammary gland (Jhappan et al.

1993). In contrast, mouse mammary tumor virus (MMTV)-controlled expression of the same transgene inhibited only ductal but not lobuloalveolar development (Pierce et al. 1993).

Our results also contrast with the observation that homozygous TGF- β 1^{null} mice are reported to have a three- to fivefold increased epidermal DNA labeling index (Glick et al. 1993). However, the TGF- β 1 "knockout" model is very complex, because cells of all tissues completely lack TGF- β 1. Perturbation in the expression of many cytokines and growth regulatory molecules is likely to have occurred in these mice, and paracrine and secondary effects are thus more likely to prevail. Interestingly, in our hands, when the TGF- β 1^{null} allele was bred onto a 50% or 93% NIH/Ola genetic background, there was no difference in epidermal DNA labeling indices between homozygous TGF- β 1^{null} mice and their wild-type littermates (P.J. Kerr and R.J. Akhurst, unpubl.).

Molecular mechanisms leading to increased proliferation in K10-TGF- β 1^{act} transgenic epidermis

The increased DNA labeling index seen in K10-TGF- β 1^{act} transgenic mice was an unexpected observation, which could be the result of a number of phenomena. It is clear from the current observations that the keratinocyte growth inhibitory response to TGF- β 1 in vivo is directly correlated with expression levels of T β RII. Our data might suggest that in the transgenic mice, ectopic TGF- β 1 expression directly or indirectly down-regulates already low levels of T β RII expression, with a resultant further reduction in TGF- β sensitivity. This would only

explain an increased DNA labeling index in the transgenic mice if normal epidermis was usually kept in a state of growth suppression by TGF- β .

Another possibility is that the increased epidermal DNA synthesis in K10-TGF- β 1^{act} transgenic mice is the result of the induction, by recombinant TGF- β 1, of positive growth regulatory molecules, such as TGF- α (Coffey et al. 1987; Derynck 1988), keratinocyte growth factor (Finch et al. 1989), or growth-stimulatory extracellular matrix components (Chiquet-Ehrismann et al. 1989), either in the epidermis or dermis. Epidermally synthesized TGF- β 1 protein is certainly thought to reach the dermis (Fowles et al. 1992) and could be responsible for such paracrine effects.

Finally, TGF- β 1 is known to be a potent inducer of epithelial differentiation (Jetten et al. 1986; Masui et al. 1986; Choi and Fuchs 1990). In vitro, it can alter the profile of differentiation-specific keratinocytes (Reiss and Sartorelli 1987; Mansbridge and Hanawalt 1988), though it remains controversial whether this growth factor is an endogenous keratinocyte differentiation-inducing agent, because current in vitro culture systems do not accommodate the full epidermal differentiation program. TGF- β 1 has also been implicated as an inducer of apoptosis of a number of epithelial cell types (Rotello et al. 1991; Lin and Chou 1992; Bursch et al. 1993; Oberhammer et al. 1993). It is therefore possible that recombinant TGF- β 1, constitutively synthesized in suprabasal keratinocytes, may act in a predominantly autocrine fashion, to stimulate terminal differentiation and/or apoptosis. As cells differentiate and consequently withdraw from the proliferative compartment, basal cell contact inhibition would be relieved and would indirectly result in increased basal cell proliferation and an overall increase in epidermal turnover, in the absence of hyperplasia.

It is interesting that the increased DNA labeling index in the K10-TGF- β 1^{act} transgenic mice does not lead to epidermal hyperplasia. A similar effect has been observed previously in mouse epidermis in response to gentle massage, as opposed to treatment with tumor promoters that induce overt hyperplasia (Bertsch et al. 1976). Therefore, whatever the mechanisms for this elevated basal cell proliferation, one must also infer that there is increased keratinocyte turnover (differentiation and/or apoptosis) in the transgenic epidermis. A role for TGF- β 1 in keratinocyte turnover would be supported by the predominant immunolocalization of T β RII to suprabasal cells seen during the differentiative phase following TPA treatment. Thus, in vivo, TGF- β 1 might have more complex actions than those revealed from in vitro studies. It could act as a regulator of both keratinocyte proliferation and turnover, to maintain homeostatic equilibrium within the epidermis.

Materials and methods

Generation of K10-TGF- β 1^{act} gene construct

A 5-kb HindIII-EcoRI fragment of the bovine cytokeratin VI (K10) promoter (Blessing et al. 1989; Bailleul et al. 1990) was

inserted into the plasmid pIC20H, followed by insertion into the XhoI site of a 0.85-kb BamHI-BglII fragment, containing the SV40 small t intron and polyadenylation signal (Mulligan and Berg 1980). Finally, a 1.4-kb HindIII-XbaI fragment that encodes activated simian TGF- β 1^{act} was excised from pTGF- β 1 (Brunner et al. 1989) and blunt end-ligated into the EcoRV site of the K10-SV40 poly(A) cassette. The fidelity of subcloning was checked by bidirectional DNA sequencing over the coding region of the transgene.

Generation and identification of transgenic mice

The 7.25-kb K10-TGF- β 1^{act} transgene was excised with *SalI* and *NruI*, purified, and microinjected into the pronuclei of fertilized eggs obtained by mating superovulated C57BL/6 $\times$ CBA F₁ females with F₁ males (Allen et al. 1987). Genomic integration of the transgene was assessed by Southern blot hybridization of BglII-digested genomic DNA isolated from tail biopsies. The presence of the transgene was detected by hybridization with the SV40 probe. The transgenic founder mice were bred to homozygosity, which was estimated by cohybridization of the SV40 probe with a probe for the mouse cystic fibrosis transmembrane regulator (CFTR) (Tata et al. 1991) to check quantitative DNA loading. Homozygosity was confirmed by breeding.

Cloning of mouse T β RII cDNA probe

A murine T β RII cDNA probe was cloned by RT-PCR using RNA from a mouse keratinocyte cell line, carB (Fowles et al. 1992). The oligonucleotide primers were designed to generate a fragment encoding the extracellular and transmembrane domains (amino acids 1–192): primer A, 5'-CGAGCGTCGACG-GCTGCCATGGGTC-3' and primer B, 5'-GCCGGTCGACAC-GCTAACAGTAGA-3'. The PCR fragment was then cloned into pBluescript SKII(+) (Stratagene), and its correct identity was confirmed by bidirectional DNA sequencing.

RNA preparation and analysis

Total RNA was isolated from mouse epidermis or cell culture as described (Fowles et al. 1992). Fifteen micrograms of total RNA was used for Northern blot hybridization with ³²P-labeled SV40, mT β RII, or 7S RNA probes. Hybridizations were carried out at 42°C overnight and washed for 10 min in 2 $\times$ SSC, 0.1% SDS, at 42°C and twice for 15 min in 0.2 $\times$ SSC, 0.1% SDS, at 65°C. The membranes were exposed to Kodak X-OMAT film at -70°C (1-hr exposure for 7S RNA and 2–6 days for other probes). Each Northern was repeated at least once.

In situ hybridization

Mouse dorsal skins were fixed overnight at 4°C in fresh 4% paraformaldehyde, dehydrated, and embedded in paraffin wax. Five-micron paraffin sections were subjected to in situ hybridization using ³⁵S-labeled SV40 antisense or sense riboprobes (Akhurst 1993). Autoradiographic exposures were for 8–12 days. Photomicrography was performed on an Olympus BH2 microscope using PANF film (Ilford).

TPA treatment of skin

The dorsal skins of 8- to 10-week-old female mice were shaved 2 days prior to treatment. Two hundred microliters of 10⁻⁴ M TPA solution in acetone was applied topically to the skin of those mice shown to be in the telogen phase of the hair cycle, as

assessed by lack of hair regrowth. At specified times after treatment, 0, 6, 12, 24, 36, 48, 72 hr, the skins were harvested, either fixed in 4% paraformaldehyde or snap-frozen in liquid nitrogen, for further analysis.

BrdU labeling and immunodetection

One hour prior to sacrifice, mice were labeled by intraperitoneal injection of BrdU (50 μ g/gram body weight) in saline. Dorsal skins were collected, fixed in 4% paraformaldehyde, and embedded in paraffin. Five-micron sections were cut and subjected to immunostaining with anti-BrdU antibody (Amersham RPN202) and horseradish peroxidase-labeled second antibody. The number of BrdU-labeled nuclei per 1 mm of skin was counted. BrdU labeling was done in triplicate, at least, and for each section, independent counts from at least 10 fields of 1 mm were taken.

Culture of primary keratinocytes from neonatal mouse

Dorsal skins from 2-day-old neonates were collected and washed in phosphate-buffered saline (PBS) containing antibiotics, before overnight incubation at 4°C in 0.125% trypsin. The skins were transferred into ice-cold Dulbecco's modified Eagle medium (DMEM) containing 15% fetal calf serum, and the epidermis was carefully separated from the dermis, chopped finely, and disrupted using an 18-gauge needle. After washing twice in PBS, the keratinocytes were seeded at 2×10^6 cells per 25-cm² flask in Clonetics keratinocyte medium at 37°C. The total RNA was extracted from the keratinocytes after 48 hr in culture.

Analysis of T β RII and T β RI expression by RT-PCR

Five micrograms of total RNA, isolated from epidermal scrapes of mouse dorsal skin, was reverse-transcribed using 200 units of Moloney murine leukemia virus (Mo-MLV) reverse transcriptase and 0.2 μ g of oligo(dT) for 1 hr at 37°C. One-fifth of the resulting cDNA was coamplified with two pairs of primers: mouse T β RII primer F (5'-ATGAAGGAAAAGAAAAGGGCG-GCG-3') and primer G (5'-GTATCTTGCCGTTCACCTCCCG-3'); rat GAPDH primer p1 (5'-TGAGTATATCGTGGAGTCTAC-3') and primer p2 (5'-GGCCATGTAGGC-CTAGAGGTC-3'), using 2 units of *Taq* polymerase. The amplification was carried out for 25 cycles. Each cycle included denaturation at 94°C, annealing at 60°C, and extension at 72°C. The PCR products were resolved on 1% agarose gels, and the specificity of the T β RII fragment was confirmed by hybridization with the mT β RII cDNA probe. Each set of semiquantitative RT-PCR reactions was repeated three to four times, using two independent RNA preparations. The primers used for RT-PCR analysis of T β RI expression were P3 (5'-GC-TCCTCATCGTGTGGTGGCGGC-3') and P4 (5'-TTGTCTT-TGTTGTCTGCTGCTATA-3') (Tomoda et al. 1993). The PCR conditions were identical to those described for T β RII mRNA, and the probe for hybridization of the Southern blots of PCR products was a 1.2-kb *Eco*RI fragment from the 5' end of human T β RI (Franzen et al. 1993).

Immunohistochemical staining

The T β RII antibody was a polyclonal antibody (Moustakas et al. 1993; Henis et al. 1994), raised against the carboxy-terminal peptide sequence (NH₂) Cys-Ser-Glu-Glu-Lys-Ile-Pro-Glu-Asp-Gly-Ser-Leu-Asn-Thr-Thr-Lys (COOH). Five-micron paraffin sections were deparaffinized, and endogenous peroxidase was blocked for 20 min in 1.2% hydrogen peroxide in methanol. Sections were preincubated at room temperature for 30 min in

0.1% bovine serum albumin (BSA), 3% donkey serum, and 3% mouse serum and then overnight at 4°C in the same mixture containing T β RII antibody (5 μ g/ml). After washing in PBS, the primary antibody was detected using biotinylated donkey anti-rabbit IgG, followed by avidin-biotin complex (DAKO). Peroxidase was detected using diaminobenzidine as the chromogen. To check antibody specificity, the T β RII antibody was preblocked with a 100 M excess of the peptide for 30 min at room temperature, prior to application to the sections.

The anti-PCNA staining was similar to that of T β RII, except that mouse serum was omitted because the primary antibody was a mouse monoclonal antibody (Boehringer Mannheim).

Immunohistochemical staining for PAI-1 was performed on cryostat sections as described previously (Romer et al. 1991).

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