Targeted deletion of the TGF- β 1 gene causes rapid progression to squamous cell carcinoma

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To study the contribution of autocrine and paracrine TGF- β 1 to tumor progression in a well-defined system of multistage carcinogenesis, keratinocytes with a targeted deletion of the TGF- β 1 gene were initiated in vitro with the v-*ras*^{Ha} oncogene and their in vivo tumorigenic properties were determined by skin grafting initiated cells onto athymic mice in combination with either wild-type or null dermal fibroblasts. Grafts of v-*ras*^{Ha}-initiated null keratinocytes progressed rapidly to multifocal squamous cell carcinomas within dysplastic papillomas irrespective of the fibroblast genotype, whereas the initiated control genotypes formed well-differentiated papillomas. Malignant progression was not associated with mutations in the c-*ras*^{Ha} gene, alterations in p53 protein, or loss of responsiveness to TGF- β 1. The tumor cell labeling index was elevated in grafts of initiated null keratinocytes with wild-type fibroblasts compared to tumors of other genotypes. However, labeling index in all tumors was reduced when TGF- β 1 null fibroblasts formed the stroma. The null tumor cells could not accumulate TGF- β 1 from the host, but grafts of uninitiated null keratinocytes, which formed a normal epidermis, became TGF- β 1 positive even though they did not express TGF- β 1 mRNA. These results demonstrate that autocrine TGF- β 1 suppresses the frequency and rate of malignant progression, and that autocrine and paracrine TGF- β 1 can have opposing effects on tumor cell proliferation. The lack of paracrine inhibition of tumor cell progression appears to result from the inability of tumor cells to localize host-derived TGF- β 1 by a mechanism that operates in normal cells.

[**Key Words:** TGF- β 1 gene; targeted deletion; keratinocytes; fibroblasts; squamous cell carcinoma; tumor cell proliferation]

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Studies of human tumors and experimentally induced tumors in animals indicate that cancers arise through a multistep process (Farber 1984; Yuspa and Poirier 1988; Fearon and Vogelstein 1990). In multistage carcinogenesis of the mouse epidermis, carcinogens, such as dimethylbenz[*a*]anthracene (DMBA), cause mutation of the c-*ras*^{Ha} gene at codon 61 (Balmain et al. 1984). Benign squamous tumors or papillomas result from repeated topical application of tumor promoters, which induce clonal expansion of the initiated cells (Yuspa 1994). Progression of these benign lesions to malignancy is a low frequency event characterized by increasing growth autonomy, reduced or aberrant differentiation, aneuploidy, and invasion (Yuspa 1994). Although a number of genetic changes, such as trisomy of chromosomes 6 and 7 (Aldaz et al. 1989), amplification of the mutated c-*ras* allele (Husain et al. 1990; Quintanilla et al. 1986), and allelic loss (Bremner and Balmain 1990; Buchmann et al. 1991;

Burns et al. 1991), have been associated with squamous carcinomas, little is known about the earliest stages of premalignant progression.

A number of studies indicate that populations of benign papillomas have different rates of premalignant progression and conversion (Hennings et al. 1985). Initiation with DMBA and promotion with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) produces a large number of papillomas with a low rate of premalignant progression and a malignant conversion frequency of 3–5% (Hennings et al. 1985). Minor subpopulation of papillomas have been identified with much higher rates of premalignant progression and conversion. These “high-risk” benign tumors arise rapidly after limited TPA treatment or promotion with the weak promoter mezerein, become promoter independent once established (Hennings et al. 1985), express markers of premalignant progression at early time points (Tennenbaum et al. 1993), and have a three- to fivefold higher frequency of malignant conversion (Hennings et al. 1985).

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Recently, we have shown that the loss of transforming growth factor- β 1 (TGF- β 1) protein expression is one of the earliest changes of premalignant progression specifically associated with the high-risk papilloma phenotype (Glick et al. 1993). TGF- β 1 is a member of a multigene family of multifunctional polypeptides (Roberts and Sporn 1990) that act as potent *in vitro* inhibitors of keratinocyte proliferation (Coffey et al. 1988; Glick et al. 1991). TGF- β 1 protein is expressed by the basal cells of the normal epidermis (Pelton et al. 1991; Whitby and Ferguson 1991; Glick et al. 1993) and by cultured keratinocytes under conditions promoting a basal cell phenotype (Glick et al. 1990, 1991). Recent studies using transgenic mice with a targeted overexpression of TGF- β 1 to the epidermis (Sellheyer et al. 1993) and mice with a targeted deletion of the TGF- β 1 gene (Glick et al. 1993) indicate that TGF- β 1 acts as an endogenous negative regulator of basal cell proliferation. Because TGF- β 1 is also absent from squamous cell carcinomas (Fowles et al. 1992; Glick et al. 1993), and resistance to the growth inhibitory effects of TGF- β 1 appears to be associated only with late stages of tumor progression (Haddow et al. 1991), a causal role for loss of this growth factor in the early phase of tumor progression is possible.

In contrast to the loss of tumor cell TGF- β 1 during premalignant progression, the growth factor is highly expressed in the stroma of mouse and human skin carcinomas (Fowles et al. 1992; Stamp et al. 1993), in squamous tumors of the mouse oral cavity (Zenklusen et al. 1994), and in the dermis and epidermis after treatment of mouse skin with tumor promoters (Fowles et al. 1992; Escherich et al. 1993). Furthermore, wounding, a tumor-promoting stimulus, elevates TGF- β 1 in the skin (Kane et al. 1991), and subcutaneous injection of TGF- β 1 enhances skin tumor promotion (Furstenberger et al. 1989), suggesting that stromal TGF- β 1 could have a significantly different effect on tumor cell growth than autocrine TGF- β 1.

To assess directly the role of autocrine and paracrine TGF- β 1 in premalignant progression of squamous tumors, we have reconstituted papillomas in a skin grafting model system using combinations of *v-ras*^{Hs} retrovirus-initiated keratinocytes and dermal fibroblasts from mice with a targeted deletion of the TGF- β 1 gene (Kulkarni et al. 1992) or the wild-type littermates. Skin grafts of *v-ras*^{Hs} retrovirus-initiated BALB/c keratinocytes produce benign squamous papillomas that recapitulate accurately premalignant progression of chemically induced papillomas (Sutter et al. 1991; Weinberg et al. 1991; Tennenbaum et al. 1992). Our results show that genetic deletion of autocrine TGF- β 1 in the tumor cells results in an accelerated, multifocal progression to squamous carcinoma, whereas autocrine TGF- β 1 expression suppresses malignant progression. We also find that fibroblast TGF- β 1 enhances tumor cell proliferation but does not influence malignant conversion of control or null keratinocytes. In addition, squamous tumor cells do not sequester paracrine TGF- β 1 from the host or stroma, whereas normal TGF- β 1 null keratinocytes not only sequester paracrine TGF- β 1 but are growth suppressed.

Results

Skin grafts of v-ras^{Hs}-initiated TGF- β 1 null keratinocytes produce squamous carcinomas

Previous studies have shown that reconstituted skin grafts on athymic mice composed of *v-ras*^{Hs} transduced normal BALB/c keratinocytes, together with cultured dermal fibroblasts as the stromal component, produce benign papillomas (Roop et al. 1986) with a malignant conversion frequency of 20–30% after 8 weeks (Weinberg et al. 1991). Primary keratinocyte cultures established from the epidermis of newborn mice that were wild type, heterozygous, or null for the TGF- β 1 allele were initiated by infection with the replication-defective *v-ras*^{Hs} virus and combined, after 5 days, with cultured wild-type primary dermal fibroblasts for grafting onto the dorsum of athymic mice. In three separate experiments terminated between 18 and 26 days post graft, 60% of the grafts of *v-ras*^{Hs}-initiated TGF- β 1 homozygous null keratinocytes were squamous carcinomas, whereas the remainder were dysplastic papillomas (Table 1). Many of the tumors were ulcerated with obvious invasive growth properties, and histological examination showed that they were composed of moderate to poorly differentiated foci of squamous carcinoma within dysplastic papillomas (Fig. 1C). The squamous carcinomas showed clear invasion of the dermis, and in some cases little stroma separated the tumor cells from the dorsal musculature. Additional analysis showed that the malignant TGF- β 1 null tumors expressed markers such as keratin 8 (Fig. 1F), elevated expression of the matrix metalloproteinase stromelysin-1 (Fig. 1G), suprabasal α 6 integrin, γ -glutamyl transpeptidase activity, and loss of keratin 1 (data not shown). Thus, these tumors are biochemically similar to chemically induced epidermal squamous carcinomas (Klein-Szanto et al. 1983; Matriasian et al. 1986; Larcher et al. 1992; Tennenbaum et al. 1993).

A significant characteristic of the null tumors was the multifocal nature of malignant conversion. Several tumors had more than one histologically evident focus of squamous carcinoma. In addition, keratin 8, a marker expressed in very dysplastic papillomas and squamous carcinomas (Larcher et al. 1992; Tennenbaum et al.

Table 1. Transduced TGF- β 1 null keratinocytes form squamous carcinomas when grafted with wild-type fibroblasts onto the dorsum of athymic mice

TGF- β 1 genotype	Tumors (number)		
	benign papilloma	dysplastic papilloma	squamous carcinoma
Wild type	17	0	0
Heterozygous	13	4	0
Null	0	8	12

Tumors were isolated 18–26 days postgrafting and fixed in 70% ethanol or Carnoy's fixative. Data are from three independent primary keratinocyte preparations and grafting experiments.

Figure 1. Grafted *v-ras*^{Hs}-initiated TGF- β 1 null keratinocytes give rise to squamous carcinomas while control genotypes produce benign papillomas. Neonatal mouse epidermal keratinocyte cultures from wild-type, heterozygous, and null mice were infected with the *v-ras*^{Hs}-defective retrovirus and skin grafted after 1 week together with wild-type dermal fibroblasts onto athymic mice. Tumors were isolated 18 days after skin grafting, and sections were stained with hematoxylin and eosin. Tumor histology is from wild-type keratinocytes (A), heterozygous keratinocytes (B), and null keratinocytes (C). (D–F) Keratin 8 immunostaining of frozen sections from tumors shown in A–C. Magnification, 200 $\times$ (A–C); 400 $\times$ (D–F). (G) Northern analysis of stromelysin-1 mRNA expression in total RNA isolated from tumors; each lane represents RNA from an individual tumor. (ST-1) Stromelysin-1; (GAPDH) glyceraldehyde-3-phosphate dehydrogenase. Normalization of densitometer-scanned ST-1 signal to GAPDH showed, on average, threefold higher levels of ST-1 transcript in null tumors relative to controls. TGF- β 1 genotype: (+/+) wild-type; (+/-), heterozygous; (-/-) null.

1992) was present in multiple foci throughout each null tumor section, with as many as 15–30 in some tumors. This suggests that malignant conversion was occurring independently in multiple cells throughout the tumor.

In striking contrast, none of the grafts of *v-ras*^{Hs}-initiated control genotypes produced squamous carcinomas within this short time. Histological examination showed that 100% of the tumors from TGF- β 1 wild-type and 75% from TGF- β 1 heterozygous keratinocytes were benign well-differentiated papillomas, whereas the remaining 25% of the latter were dysplastic papillomas (Table 1, Fig. 1A,B). Analysis of biochemical markers of progression confirmed the histological assessment of these tumors as benign papillomas (Fig. 1D,E). These results demonstrate that loss of autocrine TGF- β 1 accelerates the rate and frequency of premalignant progression and malignant conversion.

TGF- β 1 protein and mRNA are absent in null tumors

To verify that the tumor phenotype was associated with the absence of autocrine TGF- β 1, the tumors were analyzed for both TGF- β 1 protein and transcript. Immunohistochemical analysis of TGF- β 1 protein with a peptide antibody directed against amino acids 266–278 showed that the TGF- β 1 null tumors were devoid of immunoreactive TGF- β 1 (Fig. 2B), but the protein was expressed focally in the basal compartment of tumors from both

wild-type and heterozygous keratinocytes (Fig. 2A). A second TGF- β 1 antibody (CCC 1-30-1) detected the growth factor in the tumor stroma (data not shown). In situ hybridization showed that the TGF- β 1 transcript was expressed in the basal compartment of wild-type but not in null tumors (Fig. 2C,D), and that in both control and null tumors TGF- β 1 was highly expressed in the tumor fibroblasts directly underneath the basement membrane (Fig. 2C,D).

Null tumor cells are wild-type for c-ras and p53, and are growth inhibited by TGF- β 1

It was possible that genetic changes associated previously with malignant conversion in skin carcinogenesis could have occurred during culture and contributed to the malignant phenotype of the null tumors. Previous studies have shown that cultured keratinocytes can spontaneously activate the *c-ras*^{Hs} gene at codon 61 leading to carcinoma formation in vivo after infection with the *v-ras*^{Hs} virus (Greenhalgh et al. 1989). However, the *c-ras*^{Hs} gene of 4/4 microdissected carcinomas from null tumors had a wild-type sequence at codons 12 and 61 (data not shown). Northern blot analysis also showed that *v-ras*^{Hs} expression did not correlate with the tumor TGF- β 1 genotype (Fig. 3), suggesting that increased expression of p21 *v-ras*^{Hs} was not responsible for the tumor phenotype. Tumor sections were also negative for p53

Figure 2. TGF- β 1 expression in control and TGF- β 1 null tumors. (A,B) Immunolocalization of TGF- β 1 on frozen sections of tumors derived from grafted wild-type (A) and null (B) *v-ras*^{Ha}-initiated keratinocytes using the anti-pre(266-278-1) polyclonal rabbit peptide antibody. Staining is blocked by the peptide immunogen (data not shown). Arrows in A denote focal loss of TGF- β 1 staining, and in B the basement membrane of a benign region of the tumor. In situ hybridization was performed with a TGF- β 1 riboprobe to frozen sections of grafted tumors from *v-ras*^{Ha}-transduced keratinocytes. (C,E,G,I) Dark-field illumination; (D,F,H,J) light-field illumination. The genotype of the keratinocyte/fibroblast components of the depicted tumors is wild type/wild type (C), null/wild type (E), null/wild type (G), and null/null (I). Arrows in C, E, G, I denote the tumor basement membrane. Magnification, 200 $\times$ (A–F), 400 $\times$ (G–J).

staining using antibodies CM-1 (Chemicon) and pAb122 (ATCC) that detect both wild-type and mutant p53 proteins, suggesting the absence of alterations leading to stabilization of p53 (data not shown).

Treatment of mock and *v-ras*^{Ha}-infected cultured keratinocytes from each genotype with 1 ng/ml of TGF- β 1 resulted in 80–90% inhibition of DNA synthesis, indicating that this signaling pathway was intact in the null cells before skin grafting (Fig. 4A). A similar inhibition of DNA synthesis by exogenous TGF- β 1 was also seen with

a cell line derived from a TGF- β 1 null carcinoma, indicating that loss of sensitivity was not required for the malignant phenotype (data not shown).

Tumor cell proliferation is higher in tumors derived from null keratinocytes

Previous experiments had demonstrated that the epidermis of TGF- β 1 null mice had a threefold higher labeling index compared with control animals (Glick et al. 1993).

Figure 3. Increased expression of *v-ras^{Ha}* is not responsible for the null tumor phenotype. Northern blot analysis of *v-ras^{Ha}* expression in null and control tumors. Total RNA was isolated from frozen tumors of each genotype, separated on an agarose gel, and transferred to a nylon filter. The signal obtained with the *v-ras^{Ha}* probe was quantitated by densitometry and normalized to that of GAPDH.

Figure 4B shows that cultured null basal keratinocytes have a 1.5- to twofold faster population doubling time in culture compared with wild-type or heterozygote cells. Similar results were obtained for *v-ras^{Ha}*-infected null keratinocytes using short-term measurements of DNA synthesis with a [3 H]-thymidine pulse (Fig. 4A). Thus autocrine TGF- β 1 must be an important component in the *in vitro* regulation of keratinocyte proliferation. This was confirmed *in vivo* through measurements of tumor cell proliferation using bromodeoxyuridine (BrdU) injections of the host to label S-phase tumor cell nuclei. These results show that both benign and malignant regions of tumors from *v-ras^{Ha}*-infected null keratinocytes, grafted into the TGF- β 1-positive environment of the host animal, had as much as a 2.5-fold higher

average labeling index compared with papillomas from autocrine TGF- β 1 positive wild-type and heterozygous control keratinocytes (Fig. 5A). In addition, foci of TGF- β 1-negative regions in the control papillomas (see Fig. 1A) also had a higher BrdU labeling index than TGF- β 1-positive regions (data not shown). Thus, neoplastic TGF- β 1 null keratinocytes retain their proliferative autonomy from paracrine TGF- β 1 *in vivo*, demonstrating the key role of autocrine TGF- β 1 in the suppression of tumor cell proliferation.

Tumor cell proliferation is higher when stromal fibroblasts express TGF- β 1

In the previous experiments, both null and control keratinocytes were grafted onto a TGF- β 1 wild-type nude host in combination with wild-type dermal fibroblasts, suggesting that TGF- β 1 from these sources cannot suppress the effect of either phenotypic or genotypic loss of autocrine TGF- β 1. To determine the role of fibroblast TGF- β 1 in tumor growth, we grafted *v-ras^{Ha}*-initiated keratinocytes from each genotype in combination with either TGF- β 1 wild-type or null dermal fibroblasts. *In situ* hybridization showed that the stroma of papillomas and carcinomas from null fibroblast grafts had a significantly reduced signal for TGF- β 1 (see Fig. 2E,F). In benign and malignant tumors containing TGF- β 1 null fibroblasts, there was a 15–20% decrease in the tumor cell labeling index for all genotypes (Fig. 5B). Similarly, when *v-ras^{Ha}*-infected BALB/c keratinocytes were grafted with either wild-type or TGF- β 1 null fibroblasts, the labeling

Figure 4. Growth of null keratinocytes is elevated in culture and is inhibited by exogenous TGF- β 1. (A) *v-ras^{Ha}*-Transduced or control cells of each genotype were seeded onto 24-well tissue culture trays in medium containing 50 μ M CaCl₂ and 8% FBS. After 2 days, fresh medium was added containing 1 ng/ml of TGF- β 1 and culture was continued for an additional 24 hr. DNA synthesis was measured by [3 H]thymidine incorporation as described in Materials and methods. (Hatched bar) No addition; (solid bar) 1 ng/ml of TGF- β 1. (Wt) Wild-type fibroblast tumors; (H) heterozygous tumors; (N) null tumors. (B) Primary keratinocytes from each genotype were seeded at a density of 1×10^5 cells per 60-mm tissue culture dish in medium containing 50 μ M CaCl₂ and 8% FBS. At intervals, cells in duplicate dishes were detached with 0.25% trypsin and the cell number was determined using a Coulter counter (ZBI). TGF- β 1 genotype of primary keratinocytes: (■) Wild type; (▲) heterozygote; (▼) homozygous null.

Figure 5. Autocrine and paracrine TGF- β 1 have opposing effects on tumor cell proliferation. *v-ras*^{Ha}-transduced keratinocytes of each genotype were grafted in combination with wild-type dermal fibroblasts as the stromal component (A) or with either TGF- β 1 wild type or null dermal fibroblasts (B). The labeling index of the resulting tumors was determined with injections of BrdU as described in Materials and methods. Each bar represents the average labeling index of two to three tumors $\pm$ S.E.M. In (A) the difference in labeling index between the control and null tumors was statistically significant, $P=0.018$ – 0.0017 for wild-type (open bars) vs. null tumors (solid bars), and $P=0.027$ – 0.0075 for heterozygous (hatched bars) vs. null tumors, as determined by a two-tailed *t*-test of the mean labeling index of each tumor. In B $P=0.003$ and was determined from a combined analysis of the difference in mean labeling index between tumors from all keratinocyte genotypes grafted with wild type fibroblasts (solid bar) and those grafted with null fibroblasts (hatched bar) using a two-tailed *t*-test.

index of the tumor cells in papillomas containing TGF- β 1 null fibroblasts was again reduced compared with those with TGF- β 1-expressing fibroblasts (Fig. 5B). The presence or absence of TGF- β 1 in the stromal fibroblasts did not affect malignant progression of the tumor cells (Table 2). Sixty percent of the null keratinocyte–null fibroblast grafts were carcinomas, whereas all of the wild-type/heterozygote keratinocyte–null fibroblast grafts were benign papillomas. Immunohistochemical analysis showed that regardless of the fibroblast genotype, control tumors had similar patterns of markers such as keratin 1 and 13 (data not shown), again demonstrating the lack of effect of stromal TGF- β 1 on premalignant pro-

gression. Thus, fibroblast TGF- β 1 enhances tumor cell proliferation, but autocrine TGF- β 1 status is the main determinant of premalignant progression.

Normal null keratinocytes but not tumor cells sequester and respond to paracrine TGF- β 1

Because immunodetectable TGF- β 1 protein was absent from null tumors and only focally positive in tumors derived from the control keratinocytes, neither host, tumor, nor stromal fibroblast derived TGF- β 1 could act by paracrine transfer to compensate for phenotypic (control tumors) or genotypic (null tumors) loss of TGF- β 1. In contrast, the normal epidermis produced from grafts of noninitiated null keratinocytes, together with wild-type dermal fibroblasts, contained immunodetectable TGF- β 1 in the basal compartment, a pattern identical to grafts of wild-type keratinocytes (Fig. 6A,B). Antibodies directed against the latency-associated peptide (LAP; anti-pre-266-278-1) and mature growth factor (anti-CC-1-30-1) (data not shown) detected TGF- β 1 in the null grafts, indicating that the entire TGF- β 1 molecule was transferred to the epidermis. Similar sequestration of paracrine TGF- β 1 was observed in grafts of a continuous TGF- β 1 null clonal keratinocyte cell line (not shown). Because the wild-type and the genetically null epidermis had a similar morphology and labeling indices of 9% and 8%, respectively, it is likely that the transferred TGF- β 1 was biologically active. To demonstrate that the grafted cells were derived from the null keratinocytes, *in situ*

Table 2. Comparison of malignant conversion in grafts with wild-type or null fibroblasts as the stromal component

Keratinocyte genotype	Fibroblast genotype (number of carcinomas)	
	wild type	null
Wild type	0/4	0/3
Heterozygous	0/4	0/3
Null	4/7	2/3

v-ras^{Ha}-transduced keratinocytes of each genotype were grafted in combination with either wild-type or TGF- β 1 null dermal fibroblasts. Tumors were isolated 21 days postgrafting and fixed in 70% ethanol or Carnoy's fixative; hematoxylin- and eosin-stained sections were classified according to standard pathological criteria (Aldaz et al. 1987).

Figure 6. Paracrine transfer of TGF- β 1 to grafts of normal null keratinocytes. Normal (noninitiated) null or wild-type keratinocytes and wild-type fibroblasts were cultured for 1 week and grafted to the dorsum of athymic mice. The reconstituted skin was isolated 1 month after grafting. (A,B) Immunolocalization of TGF- β 1, using anti-pre(266-278-1), to the basal cells of the graft epidermis produced from TGF- β 1 wild-type keratinocytes (A) and TGF- β 1 null keratinocytes (B). (C,D) Detection of neomycin-3'-phosphotransferase (neo^f) gene expression in graft epidermis from null keratinocytes (D) but not wild-type keratinocytes (C) using in situ hybridization with a NPTII riboprobe. Magnification, 200 $\times$ (A–D). The neo^f gene is part of the targeting vector used to make the TGF- β 1 null mutation. (C,D) Arrows denote location of the basement membrane. Hybridization to the cornified layer in control and null cell grafts is nonspecific. (E) PCR amplification of the neo^f gene from DNA isolated from null graft epidermis but not wild type graft epidermis. (Lanes 1–4) 900-bp NPTII PCR

product from microdissected null graft epidermis. Each lane represents DNA from an individual graft epidermis. (Lanes 5–6) Similar NPTII amplification with wild type graft epidermis and purified BALB/c DNA, respectively, showing absence of NPTII PCR product. (Lanes 7–10) PCR genotyping of the donor normal null and wild type keratinocytes using TGF- β 1 allele specific primers. Lanes 7 and 9 are products of the PCR with TGF- β 1 wild-type allele primers; lanes 8 and 10 are products of the PCR with targeted allele-specific primers. DNA from null cells (lanes 7,8) contains only the targeted allele, whereas wild-type DNA (lanes 9,10) contains only the wild-type allele.

hybridization was used to detect expression of the neomycin phosphotransferase (NPTII) gene that is present in the targeting vector used to make the TGF- β 1 null allele (Kulkarni et al. 1992). Figure 6, C and D, shows that the NPTII riboprobe hybridized to the entire null graft but not to wild-type grafts, indicating that the immunodetectable TGF- β 1 originated from outside the graft. In addition, we could amplify by PCR a 1.4-kb fragment of the NPTII gene from microdissected sections of null but not wild-type grafts (Fig. 6E, lanes 1–5). Figure 6E, lanes 7–10, also shows, as an additional control, that the cells used for the null grafts were homozygous null and not heterozygous. These results suggest that TGF- β 1 derived from blood, dermal cells, or adjacent host epidermis can be transferred to, and produce a biological response in, the basal cells of a nonneoplastic (in this case null) epidermis but cannot accumulate in tumor cells undergoing premalignant progression.

Discussion

Premalignant progression of tumors from the benign to the malignant stage is the major time-dependent phase in the generation of cancer but is the least understood at either the molecular or biochemical level. In vitro experiments and in vivo transgenic and gene deletion studies have established that TGF- β 1 is a potent inhibitor of

normal epithelial cell proliferation (Shipley et al. 1986; Roberts and Sporn 1990; Glick et al. 1993; Pierce et al. 1993; Sellheyer et al. 1993). Loss of negative growth regulation by TGF- β 1 has been associated with the neoplastic process either through resistance to growth inhibition (Haddow et al. 1991; Manning et al. 1991) or the inability to activate autocrine secreted TGF- β 1 (Wakefield and Sporn 1990). However, these studies have not distinguished between resistance to TGF- β 1 playing a causal role in neoplastic progression or simply providing a growth advantage to a neoplastic subpopulation. We have shown previously that chemically induced papillomas with a high frequency of malignant conversion lack TGF- β 1 peptide as soon as they erupt from the skin (Glick et al. 1993). This report now demonstrates directly that genetic deletion of autocrine TGF- β 1 results in rapid multifocal progression of benign epidermal tumor cells to squamous carcinomas, in the absence of changes in sensitivity to the growth factor. Thus, autocrine TGF- β 1 not only inhibits cell proliferation but acts to suppress the rate and frequency of cancer development.

Because human basal cell carcinomas (Stamp et al. 1993), squamous carcinomas of the hamster oral epithelium (Zenklusen et al. 1994), and some human breast and colon cancers (Mizukami et al. 1990; Gorsch et al. 1992; Mizoi et al. 1993) do not express TGF- β 1 protein, this pathway could have relevance to neoplasia in other

tissues as well as application to other cell types in which independence from negative regulation by TGF- β 1 is achieved by alternative mechanisms.

In addition, by reconstituting papillomas in which either the dermal or epithelial component was homozygous for the TGF- β 1 null allele, we have shown that tumor cell and fibroblast TGF- β 1 can have opposing effects on tumor cell proliferation. In contrast to the effect of the null allele in the epithelial component of the tumor, TGF- β 1 null dermal fibroblasts reduce tumor cell proliferation but have no effect on premalignant progression. Because dermal fibroblasts also express TGF- β 3 (Escherick et al. 1993), this factor could prevent even further reduction of the labeling index in the absence of TGF- β 1. Thus, the influence of TGF- β 1 in carcinogenesis depends on the compartment of expression. These results suggest that TGF- β 1 produced by stromal fibroblasts can act as a promoting stimulus for tumor growth and epidermal hyperplasia. This is supported by immunohistochemical observations that show elevated levels of stromal TGF- β 1 in hyperplastic conditions of the normal epidermis (Akhurst et al. 1988; Kane et al. 1990, 1991; Fowles et al. 1992; Escherick et al. 1993), as well as human and experimentally induced animal tumors (Flanders et al. 1989; Gomm et al. 1991; Fowles et al. 1992; Stamp et al. 1993; Zenklusen et al. 1994). In addition, the ability of TGF- β 1 to affect tumor cell growth indirectly through actions on the stroma has been inferred from studies in which the growth of subcutaneously injected malignant cell lines is enhanced when they express high levels of a transfected TGF- β 1 gene (Arrick et al. 1992; Steiner and Barrack 1992; Chang et al. 1993).

Because our immunohistochemical analysis showed that tumors from TGF- β 1 null keratinocytes and progressing regions of the control tumors could not acquire paracrine TGF- β 1, even when available, the effect of stromal TGF- β 1 on cell proliferation is likely to be indirect. In situ hybridization showed that TGF- β 1 expression in the tumor stroma of grafts with wild-type fibroblasts was concentrated directly underneath the dermal/epidermal junction; therefore, TGF- β 1 at this location could influence the structure and composition of the basement membrane, particularly that contributed by fibroblasts (Roberts et al. 1986; Heine et al. 1990; Roberts and Sporn. 1990.; Lankat-Buttgereit et al. 1991; Konig and Bruckner-Tuderman 1992). In addition, TGF- β 1 is a known angiogenic factor (Roberts et al. 1986), and a positive influence of stromal TGF- β 1 on tumor vascularity is possible, although there were no obvious differences in vascularity on histological sections between tumors with wild-type or null fibroblasts.

Adding another level of complexity, our results show that grafted normal null keratinocytes, but not tumor cells, were able to sequester paracrine TGF- β 1 protein and localize it to the basal cell compartment. The sequestered TGF- β 1 was apparently bioactive, as both the null and wild-type graft epidermis had the same labeling index. Because the pattern of TGF- β 1 immunolocalization to the basal compartment of the null, wild-type, and

normal epidermis was identical, the mechanism of localization of paracrine TGF- β 1 must involve specific binding, rather than passive diffusion. In addition, it is likely that paracrine transfer involves the entire TGF- β 1 molecule, as antibodies to both the precursor and mature growth factor stained the null epidermis. Paracrine transfer of various TGF- β from either an epithelium to the underlying mesenchyme or the reverse has been inferred from discordant expression between RNA and protein expression (Lehnert and Akhurst 1988; Escherick et al. 1993), but this is the first unequivocal demonstration of the phenomenon. Several cell-surface and matrix proteins have been identified that can bind either the latent or active form of TGF- β 1, including the TGF- β type III receptor (Lopez-Casillas et al. 1993), the IGFBP/mannose-6-phosphate receptor (Dennis and Rifkin 1991), and the latent TGF- β -binding protein (LTBP) (Kanzaki et al. 1990). One or all of these proteins could be involved in sequestering the growth factor from paracrine or autocrine sources. A prediction of this model is that agents promoting epidermal hyperplasia should block paracrine transfer of stromal TGF- β 1, a proposal consistent with an immunohistochemical study showing that TPA blocks the dermal to epidermal transfer of TGF- β 3 (Escherick et al. 1993).

Although TGF- β 1 protein is lost during premalignant progression of chemically induced skin tumors, in situ hybridization and Northern analysis have shown that the mRNA is abundant in basal cells of papillomas and carcinomas (Krieg et al. 1991; Fowles et al. 1992). Translational regulation of TGF- β 1 has been demonstrated in a number of systems (Kim et al. 1992); however, our data would suggest a more complex mechanism in which TGF- β 1-binding proteins involved in localization are altered during tumor progression. Thus, both autocrine and paracrine TGF- β 1 would fail to localize in the basal cell compartment, resulting in a TGF- β 1-deficient environment for the cell. Several studies have proposed that expression of the IGFBP/mannose-6-phosphate receptor is linked to the appearance of immunodetectable TGF- β 1 (Jirtle et al. 1991, 1993).

In the grafted *v-ras*^{Ha} TGF- β 1 null tumors, malignant conversion was frequently multifocal and occurred within benign papillomas, suggesting that loss of autocrine TGF- β 1 was not sufficient for malignant conversion but, rather, allowed further genetic change to occur. Elevated cell proliferation in the null tumors could accelerate malignant conversion by increasing the target population at risk for additional spontaneous mutations. However, malignant conversion of chemically induced papillomas is not enhanced by stimulating proliferation with prolonged application of tumor promoters (Henning et al. 1983). Furthermore, when null and control *v-ras*^{Ha} keratinocytes were grafted with null fibroblasts, tumor cell proliferation was reduced, but premalignant progression and malignant conversion were not affected. Because TGF- β 1 modifies the expression or the activity of a number of G₁ regulatory proteins, such as the cyclins (Geng and Weinberg 1993), cyclin-dependent kinases (Ewen et al. 1993; Koff et al. 1993), pRB (Laiho et

al. 1990), and *c-myc* (Munger et al. 1992), cell cycle regulation could be altered specifically in cells no longer expressing TGF- β 1. Interestingly, a subset of chemically induced mouse skin papillomas and all squamous carcinomas overexpress cyclin D1 (Bianchi et al. 1993), suggesting that a link may exist between loss of TGF- β 1 and deregulation of specific cell cycle proteins during epidermal tumor progression. Aberrant entry into S-phase (Hartwell 1992) could be responsible for the macrogenetic changes that are associated with progression and conversion in the mouse skin carcinogenesis system (Aldaz et al. 1987, 1989; Bremner and Balmain 1990). Further analysis of cell cycle control in the TGF- β 1 null keratinocytes should help to provide a mechanistic link between tumor progression and altered growth control.

Materials and methods

Isolation and genotyping of primary cultures

Newborn mice (1–3 days old) newborn mice from matings of heterozygous adults were sacrificed by CO₂ narcosis, and the corpses were kept overnight on ice while the genotype was determined by PCR analysis of tail DNA. Tail DNA was isolated by standard SDS–proteinase K digestion followed by extraction with phenol/chloroform/isoamyl alcohol and precipitation with ethanol. PCR primers specific for the wild-type and deleted TGF- β 1 allele were used to identify wild-type, heterozygous, and null animals (Glick et al. 1993). After genotyping, newborn skins were isolated and floated on 1% trypsin/PBS at 37°C for 1.5 hr to separate dermis and epidermis. Keratinocytes and fibroblasts from each genotype were prepared from separated skins and cultured according to standard techniques (Hennings et al. 1980). Null keratinocytes from individual mice were plated separately while genotypes were confirmed with a second PCR. Keratinocytes were cultured in standard low calcium (50 μ M CaCl₂) EMEM/8% chelexed fetal bovine serum (FBS), whereas dermal fibroblasts were cultured in the same medium with 1.4 mM CaCl₂. Primary keratinocyte cultures were infected with a replication-defective retrovirus expressing *v-ras*^{Hs} (Roop et al. 1986) on day 2. After 4–5 days of culture, 3 $\times$ 10⁶ to 5 $\times$ 10⁶ initiated keratinocytes were mixed with 8 $\times$ 10⁶ dermal fibroblasts and grafted onto a prepared graft bed on the dorsum of athymic mice (Roop et al. 1986).

To determine the doubling time of primary keratinocytes from each genotype, cells from null animals and control littermates were seeded at a density of 1 $\times$ 10⁵ cells per 60 mm tissue culture dish, and cultured in low calcium medium containing 8% FBS. At 2- to 3-day intervals, cells from duplicate dishes were removed with 0.25% trypsin and the cell number determined using a Coulter counter. For short-term assays of DNA synthesis, primary keratinocytes of each genotype were plated in 24-well tissue culture dishes. Twenty-two hours after media change, the cells were pulsed with 1 μ Ci of [³H]thymidine for 2 hr, and DNA synthesis was determined by measuring counts per minute incorporated into acid-insoluble material (Danielpour et al. 1989). In some experiments cells were incubated in the presence or absence of 1 ng/ml of TGF- β 1 for 22 hr before pulsing with [³H]thymidine. Counts incorporated were normalized to cell number that was determined from parallel wells.

Isolation and analysis of tumors

After sacrificing tumor-bearing animals by CO₂ narcosis, tumors were surgically excised from the dorsal surface of the host

animal and sections were fixed in 70% ethanol or embedded in optimal cutting temperature media (Miles) and frozen. Sections of frozen or fixed tumor were stained with hematoxylin and eosin for histological analysis. Staging of tumors was based on standard criteria of stratification, dysplasia, and invasion (Aldaz et al. 1987). Keratins 1, 13, and 8 and the α -6 integrin were localized on 5 μ m frozen sections using specific monoclonal and polyclonal antibodies and a peroxidase-based detection system with diaminobenzidine (DAB) as the chromogen (Tennenbaum et al. 1993). γ -Glutamyl transpeptidase activity was detected using a histochemical stain on frozen sections as described (Tennenbaum et al. 1993). Total RNA was isolated by grinding frozen tumor tissue in liquid nitrogen, lysis in 4 M guanidinium isothiocyanate, and purification through a cesium chloride cushion. Total RNA (15 μ g) was fractionated through a 1% formaldehyde–agarose gel and transferred to Nytran (Schleicher & Schuell). An insert of the plasmid pTRII (Matrisian et al. 1986) was used as a probe for stromelysin-1. A *Hind*III–*Bam*HI fragment of the *v-ras*^{Hs} gene was used as a probe to detect both *v-ras*^{Hs} and *c-ras*^{Hs} (Roop et al. 1986).

Quantitation of labeling index

To determine the labeling index of grafted tumors and normal skin, graft-bearing animals were injected 1 hr before sacrifice with 250 μ g/kg of BrdU in saline. Cells incorporating BrdU into DNA were localized on 5 μ m sections of ethanol-fixed, paraffin-embedded tissue, using a biotinylated monoclonal anti-BrdU antibody (Becton-Dickinson) and horseradish peroxidase-conjugated streptavidin. Sections were counterstained with hematoxylin. The labeling index was determined by counting DAB-labeled nuclei per total basal nuclei. For tumors, $\geq$ 10 randomly picked fields of >100 nuclei were counted for each section. Each labeling index is the average $\pm$ S.E.M. of two to three tumors. For grafts of *v-ras*^{Hs}-initiated BALB/c keratinocytes and null fibroblasts, DAB-positive nuclei were counted blindly, using coded slides.

Microdissection and PCR analysis of tissue sections

Foci of squamous carcinoma were localized in hematoxylin-stained sections and isolated from 50 μ m unstained, deparaffinized, serial sections under a dissecting microscope. Combined tissue from three sections was digested overnight at 56°C in 10 mM Tris (pH 7.4) and 1 mM EDTA with 400 μ g/ml proteinase K. Samples were extracted with phenol/chloroform, ethanol precipitated, and resuspended in water. Sequences of the *c-ras*^{Hs} gene flanking codon 61 and codon 12 were amplified using nested PCR primers that do not amplify the *v-ras*^{Hs} oncogene. Primer pairs for the codon 61 PCR were 5'-TGTAGGC-TGGTTCTGTGGAT, CAGGGCTCACGGGCTAGCCA and 5'-GTCATTGATGGGGAGACATG, ATGTCCTCGAAGGAC-TTGTTG. Primer pairs for codon 12 PCR were 5'-AAGG-TGTGCTTTCTCATTGG, CAGGGCTCACGGGCTAGCCA and 5'-TTGGCAGCCGCTGTAG AAGCTATGACAGAGAA, GGTCATTTACCCATGACCACT. Gel-purified PCR fragments were sequenced using a fmol sequencing kit (Promega). To isolate grafted normal epidermis for TGF- β 1 genotyping, the graft was localized on 50 μ m ethanol-fixed sections under a dissecting microscope using the absence of hair follicles as a marker, and surrounding host skin was removed with a razor blade. After a 30-min treatment with dispase (5 mg/ml) to aid separation of dermis and epidermis, the isolated epidermises from three to four serial sections for each graft were combined and digested overnight with proteinase K. The presence of the targeted TGF- β 1 allele in the graft DNA was determined by

PCR amplification of the NPTII gene, using primers 5'-ATGAT-TGAACAAGATGGATT and TCAGAAGAACTCGTCAAG-AA that flank the coding sequence.

TGF- β 1 immunolocalization

TGF- β 1 immunostaining was performed as described previously on 5 μ m frozen sections with anti-pre-266-278-1 (Wakefield et al. 1988), which does not detect dermal TGF- β 1 in frozen sections. Additional antibodies used were anti-LC-1-30-1 and anti-CC-1-30-1, which recognize the active region of the TGF- β 1 molecule (Flanders et al. 1989).

In situ hybridization

In situ hybridizations with 35 S-labeled RNA probes for TGF- β 1 and NPTII were performed on 5 μ m frozen sections of tumors and normal skin grafts with the pretreatment and hybridization protocol of Young (1990). Hybridization buffer consisted of 300 mM NaCl, 50% formamide, 10% dextran sulfate, 1 $\times$ Denhardt's solution, 1% sodium thiosulfate, 1% SDS, 100 mM DTT, and 2 $\times$ 10⁷ dpm/ml of probe. Hybridization was for 16–18 hr at 50°C in a humid chamber. Washes were done to a final stringency of 0.1 $\times$ SSC at 65°C for 1 hr, and slides were exposed to emulsion LM-1 for 4 weeks for TGF- β 1 and 7 weeks for neomycin-3'-phosphotransferase. Development was performed with Dektol developer and Kodak fixer, and the slides were counterstained with hematoxylin and eosin. The TGF- β 1 probe was derived from a 900-bp *AccI*-*KpnI* fragment of the mouse TGF- β 1 cDNA cloned into pGem3. The NPTII riboprobe construct was generated by subcloning a PCR fragment encompassing the complete coding region of the NPTII gene into the pCRII vector (Promega). Both probes were reduced to an average length of 200–500 nucleotides by partial alkaline hydrolysis (Cox et al. 1984).

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