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Escape from transforming growth factor β control and oncogene cooperation in skin tumor development

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ABSTRACT **Control of tumor development by surrounding normal cells has been suggested by a number of *in vitro* studies. *In vivo*, tumorigenicity of *ras*-transformed primary keratinocytes can be suppressed by addition of normal dermal fibroblasts. Here, we report that dermal fibroblasts produce a diffusible inhibitory factor belonging to the transforming growth factor β (TGF-β) family and possibly corresponding to TGF-β3. This factor can suppress growth of *ras*-transformed primary keratinocytes in culture and after injection into mice. As with primary cells, tumorigenicity of a *ras*-transformed, TGF-β-sensitive keratinocyte line is substantially inhibited by adding dermal fibroblasts, leading to the formation of much smaller and differentiated tumors. Introduction of an intact *E1a* oncogene into these cells induces concomitant resistance to TGF-β, to the effect of dermal-fibroblast inhibitory factor, and to dermal-fibroblast tumor suppression. Similar results are obtained with a transformation-deficient truncated *E1a* mutant, which binds to a reduced subset of cellular proteins (including the retinoblastoma gene product). Thus, genetic events such as those elicited by *E1a* transformation enable keratinocytes to escape from the inhibitory influences of a normal cellular environment and lead, together with *ras* transformation, to skin tumor development.**

Tumor development involves several genetic events, including activation of protooncogenes and inactivation of tumor-suppressor genes (1, 2). Some of these changes may be required for a cell to escape the inhibitory influences of its normal cellular environment, as suggested from a number of *in vitro* (see, for instance, refs. 3–7) and *in vivo* studies (8–12). Negative growth signals can be transmitted through several forms of intercellular communication, including direct cellular contacts, gap-junctional communication, and release of diffusible inhibitory factors. Any of these possibilities could be operative *in vivo*.

The family of transforming growth factor βs (TGF-βs) (for review, see refs. 13–15) represents one of the best-characterized examples of growth-inhibitory factors, which inhibit growth of a variety of normal cells, especially of epithelial origin, *in vitro*. This inhibition, together with the fact that epithelial-cell transformation is often associated with acquisition of TGF-β resistance, suggests an equally important role for these growth inhibitors *in vivo*.

Recent evidence has suggested that the 105-kDa retinoblastoma gene product may mediate at least some of the TGF-β growth-inhibitory effects (16, 17). The ability of certain viral oncoproteins—such as adenovirus 5 E1a, simian virus 40 large tumor antigen, and human papilloma virus type 16 E7—to induce TGF-β resistance may be explained by their association with the retinoblastoma gene product (16, 17). Retinoblastoma protein binding, however, is probably not the only factor responsible for induction of full TGF-β resistance (18, 19) and, at least in the case of E1a, binding to other cellular proteins is also required (20).

In the mouse skin system, we have previously shown that transformation of primary mouse keratinocytes by a single activated *ras* oncogene is sufficient to induce a fully tumorigenic phenotype when these cells are tested as a large and homogeneously transformed population (9). Tumorigenicity of these cells, however, can be strongly and specifically inhibited by addition of normal dermal fibroblasts (9). In the present communication, we have examined the mechanism underlying dermal-fibroblast tumor inhibition and tested whether second genetic events may be required for *ras*-transformed keratinocytes to overcome the negative influences of their cellular environment.

MATERIALS AND METHODS

Cells. Primary keratinocytes and dermal fibroblasts from newborn mice were prepared and cultivated as described (9). Infection with Harvey sarcoma virus (HaSV), Moloney leukemia virus (MuLV); and/or treatment with mitomycin C (0.4–1.2 mg/ml) were as described (9).

Injection into Nude Mice. Cells were injected alone (5×10^5 cells per injection) or in combination with other cells (in a 1:4 ratio) as described (9). Unless otherwise specified, mice were sacrificed 1 mo after injections.

Preparation of Dermal-Fibroblast Inhibitory Factor (DF-IF). Dermal-fibroblast-conditioned medium (DF-CM) was obtained from freshly confluent cultures of tertiary mouse dermal fibroblasts after washing cells with phosphate-buffered saline and incubating them with serum-free unsupplemented medium for 6 hr. This medium was discarded, and cells were washed again with phosphate-buffered saline and incubated with fresh serum-free medium for 48 hr. The conditioned medium was then collected and filtered to remove floating cells. After extensive dialysis (in phosphate-buffered saline/50 mM NH_4HCO_3) DF-CM was lyophilized and applied in 50 mM NH_4HCO_3 to Sephadex G25 columns (Pharmacia LKB) to eliminate small residual molecules. DF-IF samples were lyophilized, resuspended in serum-free medium, and tested on primary keratinocytes for mitogenic effects, as described below. DF-IF was quantified by a serial-dilution curve, one unit being defined as the amount required for 50% inhibition of DNA synthesis. One hundred to two hundred units of activity (1 unit per 20–40 μg of total protein) was consistently recovered from 350 ml of DF-CM preparations, starting from $\approx 5 \times 10^8$ cells.

Keratinocyte Mitogenicity Assay. Primary keratinocytes in 24-well trays were switched to serum-free low-calcium medium supplemented as described (20), plus or minus samples to be tested. After 20 hr of incubation, DNA synthesis was measured by a 1-hr pulse with [*methyl*-^{3}H]thymidine (20).

TGF-β-Competition Binding Assays. Competition between ^{125}I-labeled TGF-β1 and DF-IF or unlabeled TGF-β1 (porcine, crude fraction; R & D Systems, Minneapolis) was

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Abbreviations: DF-IF, dermal-fibroblast inhibitory factor; DF-CM, dermal-fibroblast-conditioned medium; HaSV, Harvey sarcoma virus; MuLV, Moloney leukemia virus.

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measured by a two-step binding assay on primary keratinocyte cultures (in 24-well trays), as described by Wakefield *et al.* (21). Briefly, cells were incubated in serum-free medium containing serial dilutions of DF-IF or unlabeled TGF-β1 for 2 hr (step 1). After being washed, cells were then incubated with 50 pM ^{125}I-labeled TGF-β1 (≈100 μCi/μg; 1 Ci = 37 GBq) for another 2 hr (step 2). Cell-associated radioactivity was solubilized (21) and counted in a LKB 1282 Compugamma CS counter. Transient acidification was done as described (21). As a control, acid base and Hepes buffer were premixed before addition.

Ribonuclease-Protection Assays. Total cellular RNA samples (30 μg) were used for ribonuclease-protection experiments with α-^{32}P-labeled RNA probes as described (22). Probes were derived from nucleotides 1–557 for TGF-β1 (23), 1511–1953 for TGF-β2 (24), and 831–1440 for TGF-β3 (25).

TGF-β Neutralization Assay. DF-IF (4 units per 150 μl) or TGF-β3 (1 ng/150 μl) were incubated in serum-free low-calcium-supplemented medium for 2 hr at 22°C either alone or together with wide-specificity-neutralizing anti-TGF-β antibodies (affinity purified; R & D Systems), or partially purified turkey anti-TGF-β1- (26) or rabbit anti-TGF-β2- (27) specific antibodies. Samples were then doubled in volume and added to triplicate wells of primary mouse keratinocytes. [^{3}H]Thymidine incorporation was measured 20 hr later (see above). A preliminary experiment with purified TGF-β1,2, and 3 was done to ensure specificity of the antibodies and that they were used in >5-fold excess relative to the amount necessary to neutralize 10 ng of pure TGF-βs. No neutralizing effects were seen with nonimmune IgGs or anti-*ras* Y13 259 antibodies, which were used in the same amount as the wide-specificity anti-TGF-β antibodies (30 μg). Anti-TGF-β1 and -β2 antibodies and TGF-β1,2,3 were from A. Roberts (National Institutes of Health).

RESULTS

Dermal-Fibroblast Tumor Suppression Is Mediated by a TGF-β Factor. These studies were aimed at determining the mechanism underlying dermal-fibroblast tumor inhibition. In a first set of experiments, dermal fibroblasts—of both murine and human origin—were found to exert similar tumor inhibition of *ras*-transformed primary keratinocytes after subcutaneous injections into nude mice (Table 1, set 1), as observed after grafting onto syngeneic animals (9). Thus, the immune system of the host—at least as impaired in the nude mouse—is probably not involved in fibroblast tumor suppression.

Among the direct inhibitory mechanisms that may be considered, cellular contacts through specific membrane determinants are an unlikely possibility because tumorigenicity of *ras*-transformed keratinocytes was suppressed by growth-arrested (mitomycin C-treated) but not by killed (freeze-thawed) fibroblasts (9; Table 1, set 1). Similarly, fibroblast-membrane preparations had no growth-inhibitory activity on keratinocytes in culture. Previous *in vitro* studies (28) revealed a total lack of gap-junctional communication between dermal fibroblasts and *ras*-transformed keratinocytes, suggesting that this function is not involved.

The third possibility, that dermal fibroblasts produce a diffusible growth inhibitor responsible for their tumor-suppressing effects, was first assessed by preparing conditioned medium from these cells (DF-CM) and testing it on primary keratinocytes in an *in vitro* mitogenicity assay. DF-CM was found to contain a specific growth-inhibitory activity that could be easily separated from salts and other small molecules through gel sizing columns. We refer to the factor(s) responsible for this activity as DF-IF (Fig. 1*A*). DF-IF activity was resistant to boiling (10 min) but was completely abolished by treatment with reducing agents [5% (vol/vol) 2-mercaptoethanol, 1 hr at 4°C]. These properties, shared by the various TGF-β factors (13, 14), suggested that DF-IF could be a member of this family. In fact, DF-IF could effectively compete with iodinated TGF-β1 in a two-step binding assay on mouse primary keratinocytes in a way similar to TGF-β1 itself (Fig. 1*B Inset*). Transient acidification—which activates TGF-β (29)—increased both DF-IF activity and binding affinity (Fig. 1 *A* and *B*).

DF-IF could be neutralized by wide-specificity anti-TGF-β antibodies but not by antibodies specific for TGF-β1 or -2 (Fig. 2). The identification of DF-IF as TGF-β3, the only other TGF-β form known so far in mammalian cells (13, 14), could not be directly proven at the protein level because specific anti-TGF-β3 antibodies are not available. However, ribonuclease-protection experiments with TGF-β1,2, and 3-specific probes revealed high TGF-β3 RNA expression in dermal fibroblasts, at levels comparable to those of TGF-β1, whereas no TGF-β2 RNA was found (Fig. 3). Interestingly, TGF-β3 RNA levels were not detectable in similar experiments with keratinocytes, whereas TGF-β1 levels were similar to those seen in fibroblasts (Fig. 3). TGF-β1 expression was significantly increased in *ras*-transformed keratinocytes, whereas TGF-β3 RNA levels were only weakly induced (Fig. 3) or remained undetectable (in a second independent experiment; data not shown).

Table 1. Tumorigenic behavior of *ras*-transformed keratinocytes with or without other cells or DF-IF factor

Cells and treatment	Tumors/injections	*n*	Weight, g
Set 1			
HaSV-K	43/43	12	0.4
HaSV-K + DF(MuLV)	0/28	8	
HaSV-K + DF(MuLV/MitC)	3/7	2	0.1
HaSV-K + DF(MitC)	11/30	5	0.1
HaSV-K + DF(freeze-thawed)	4/4	1	
HaSV-K + HuDF	2/8	3	0.1
HaSV-K + K(MuLV)	5/5	2	0.15
Set 2			
HaSV-K	12/12	3	0.12
HaSV-K + DF-IF	2/12	3	<0.03

Experiment 1: HaSV primary mouse keratinocytes (HaSV-K) were injected into nude mice either alone or in combination with other cells as described. Mouse dermal fibroblasts (DF) or primary keratinocytes (K) were made resistant to HaSV transformation by previous infection with MuLV. Similar experiments were also done with mitomycin C-treated (MitC), growth-arrested dermal fibroblasts (MuLV infected or not) (9). The weaker level of tumor inhibition that was at times seen with these cells could be from some drug-toxicity effects, as there was a narrow and somewhat variable range in the critical doses of mitomycin C necessary to arrest growth of fibroblasts without affecting their tumor-inhibitory properties. Similar inhibitory effects were also seen in experiments with early (4–6)-passage human foreskin fibroblasts (HuDF). All experiments included controls with HaSV-infected keratinocytes injected alone. Mice were sacrificed 1 mo after injections. The epithelial nature of the tumors was confirmed by immunoblotting with anti-keratin antibodies as described (9). Average tumor weight is considered a relative measure of tumor growth under the various conditions; variations in tumor weight were usually <20% of average values. *n*, Number of independent experiments done for each combination of cells. Experiment 2: primary mouse keratinocytes, 6 days after HaSV infection, were switched to serum-free supplemented medium plus or minus DF-IF (used, with similar results, at 12, 13, and 25 units/ml in three independent experiments). Cells were incubated for 24 hr, harvested, and injected into nude mice. Cell viability was not affected by the DF-IF treatment, as verified by replating some keratinocytes at the end of experiments. To minimize experimental variability, DF-IF-treated and -untreated cells were injected in parallel in the same animals in the left and right suprascapular areas, respectively. At 10 days, >80% of injected mice developed tumors in the control areas. These mice were sacrificed, and tumor formation by both control and DF-IF-treated cells was verified by accurate necropsy as well as histological analysis.

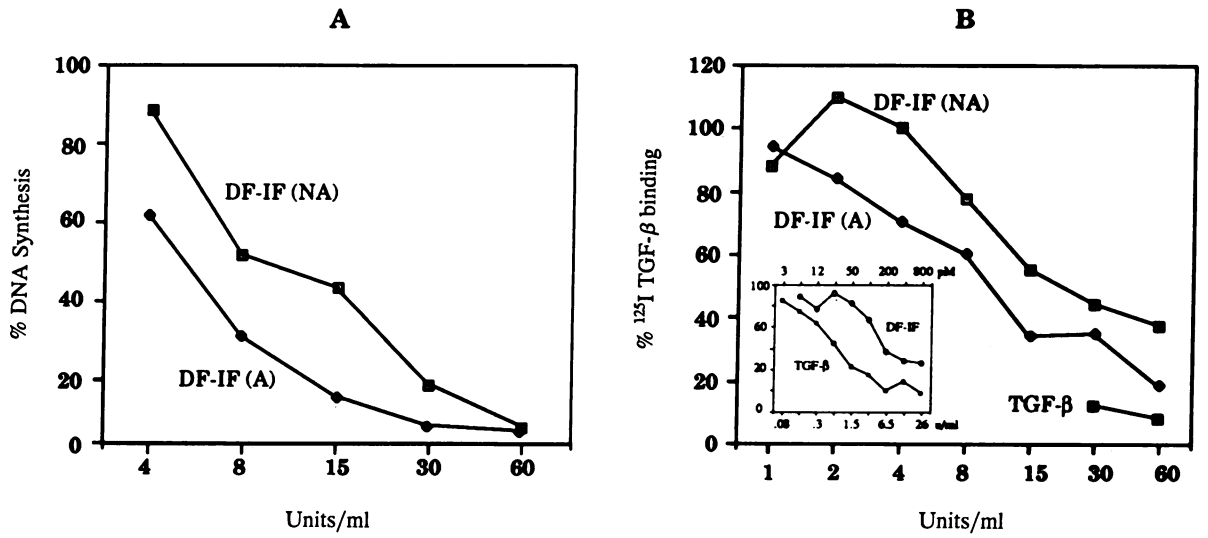

FIG. 1. DF-IF growth-inhibitory activity. (*A*) Dose–response curve of DF-IF growth inhibition on mouse primary keratinocytes. Similarly treated DF-IF that was either transiently acidified (A) or not (NA) was run through desalting columns. Samples were then lyophilized and added at 2-fold dilutions starting from a concentration of 60 units per ml to primary keratinocytes in 24-well trays. After 20 hr of incubation, DNA synthesis was measured in duplicate wells, as described. Values are expressed as percentage of DNA synthesis relative to untreated controls. Experimental variation was <10%. (*B*) Binding competition of DF-IF with iodinated TGF-β1. Competition between ^{125}I-labeled TGF-β1 (50 pM) and DF-IF was measured by a two-step binding assay on primary keratinocyte cultures. DF-IF was either transiently acidified (A) or not (NA) and tested at 2-fold serial dilutions in triplicate wells, starting from 60 units per ml. For comparison, competition with excess unlabeled TGF-β1 (800 and 400 pM) was also included. Values, expressed as percentage of maximum specific binding of ^{125}I-labeled TGF-β1, were determined in triplicate wells. (*Inset*) A second independent experiment similar to that in *B*, except that untreated, nonacidified DF-IF was used (at 26 units per ml), together with a complete dilution curve of unlabeled TGF-β1 (from 800 pM).

If DF-IF was responsible for the fibroblast tumor-inhibitory effects, it might be possible to, at least partially, reproduce these effects *in vivo*, by use of inhibitor alone. In fact, DF-IF was able to suppress growth of *ras*-transformed primary keratinocytes not only in culture but also after injection into the animal (Table 1, set 2). In this case, *ras*-transformed primary keratinocytes were exposed to DF-IF for 24 hr before injection into nude mice. Mice were examined daily and sacrificed 10 days after injection, soon after appearance of small tumors in the areas injected with control cells. Experiments were terminated this early because the reversible effects of DF-IF on primary keratinocytes in culture suggested that differential growth of treated and untreated cells would be lost with time. Relative to control cells, injection of DF-IF-treated keratinocytes significantly reduced the number and size of tumors (Table 1, set 2). Histologically, only undifferentiated malignant cells and occasional epithelial cysts were observed in the control tumors, whereas the tumors formed by DF-IF-treated keratinocytes displayed foci of moderately to well-differentiated squamous cells. These observations demonstrate that dermal fibroblasts produce a diffusible TGF-β-like inhibitor (DF-IF), which may suppress tumor formation by inducing a more differentiated phenotype.

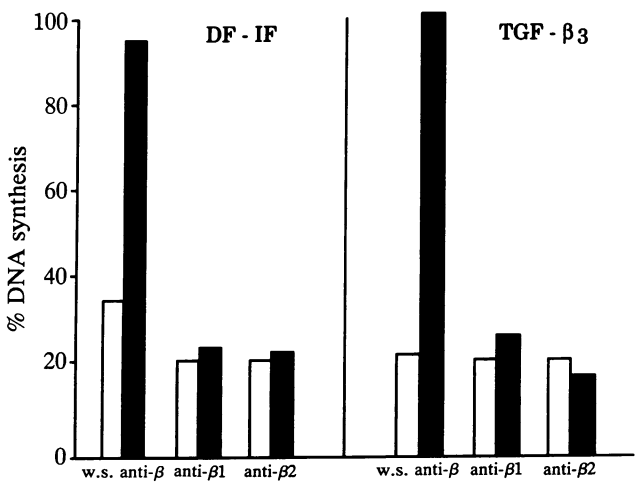

FIG. 2. Effect of anti-TGF-β antibodies on DF-IF growth-inhibitory activity. DF-IF or TGF-β3 was tested for growth-inhibitory activity on primary mouse keratinocytes, after being incubated alone (white bars) or with wide-specificity (w.s.)-neutralizing anti-TGF-β antibodies, partially purified turkey anti-TGF-β1 (anti-β1)-, or rabbit anti-TGF-β2 (anti-β2)-specific antibodies (black bars). Values are expressed as percentage of DNA synthesis relative to similarly treated control keratinocytes not exposed to the DF-IF or TGF-β3 factors. Experimental variation was <10%. Similar results were seen in two other independent experiments, including one with DF-IF, which was either transiently acidified or not, as described for Fig. 1.

Transformation of Keratinocytes with the *E1a* Oncogene Induces Resistance to Dermal-Fibroblast Tumor Suppression.

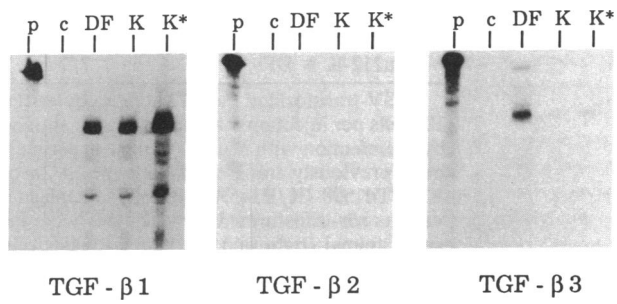

FIG. 3. RNA expression of specific TGF-β forms in mouse dermal fibroblasts and keratinocytes. Total cellular RNA was prepared from confluent cultures of tertiary mouse dermal fibroblasts (DF), primary mouse keratinocytes (K) (9 days after plating), or HaSV-infected primary mouse keratinocytes (K*) (infected 2 days after plating; RNA prepared 7 days later). Sample RNAs were analyzed by ribonuclease-protection assay with mouse TGF-β1-, TGF-β2-, and TGF-β3-specific RNA probes. Specificity of detection of these probes was confirmed by hybridization to the corresponding sense transcripts (data not shown). p, RNA probes; c, tRNA controls. Results were confirmed by two other independent experiments.

According to our initial hypothesis, second genetic changes might be required for *ras*-transformed keratinocytes to overcome the inhibitory effects of surrounding dermal fibroblasts (9). To test this model, we switched from primary keratinocytes, which cannot be transfected with foreign DNA at any reasonable frequency, to an easily transfectable keratinocyte line, Pam212 (30). These cells were spontaneously derived from a primary keratinocyte culture and, despite several other changes, were still sensitive to TGF-β1 and DF-IF growth inhibition (ref. 20; data not shown). As with primary cells, tumorigenic growth of *ras*-transformed Pam212 keratinocytes was significantly inhibited, even if not totally suppressed, by addition of normal dermal fibroblasts (Table 2). The drastic and consistent reduction in tumor size (5- to 10-fold in weight) seen after admixture of the *ras*-transformed Pam212 cells with dermal fibroblasts was accompanied by a striking induction of squamous-cell differentiation that covered >80% of the tumor areas.

The *E1a* oncogene can cooperate with *ras* in cell transformation (31, 32). We speculated that, in our system, *E1a* may contribute to neoplastic growth of *ras*-transformants by allowing escape from the inhibitory effects of a normal cellular environment and release from TGF-β growth inhibition. Recent *in vitro* experiments would be consistent with this possibility (16, 17, 20). In fact, introduction of this oncogene into Pam212 cells was sufficient to render them totally TGF-β and DF-IF resistant (ref. 20; data not shown).

In contrast to *ras*-transformed Pam212 keratinocytes, cells cotransformed with *ras* and an *E1a* gene with an intact transforming region (dl 787; ref. 33) were not inhibited by adding dermal fibroblasts, when tested in the animals (Table 2). The presence of dermal fibroblasts did not significantly change the size of the tumors formed by these cells. More importantly, these tumors displayed a very similar histological appearance with small/medium-sized cells with no keratinization features, typically disposed in clusters or trabeculae.

Expression in Pam212 cells of a truncated *E1a* oncoprotein (NTd1598; ref. 33) that binds to a reduced subset of cellular proteins (including the product of the retinoblastoma tumor-suppressor gene and cyclin A) is sufficient to confer 40–50% resistance to TGF-β growth inhibition (20). Induction of transglutaminase (a marker of keratinocyte differentiation) by TGF-β is also blocked in these cells (20). In parallel with these effects, Pam212 keratinocytes concomitantly transformed by the *E1a* NTd1598 and *ras* genes formed tumors that were only 2-fold reduced in size by the addition of dermal fibroblasts. Perhaps more significantly, a similar undifferentiated phenotype was retained in tumors formed in the presence and absence of dermal fibroblasts (Table 2).

DISCUSSION

In the present communication, we have shown that tumor suppression of *ras*-transformed keratinocytes by surrounding dermal fibroblasts is likely to be mediated by a TGF-β-like inhibitory factor (DF-IF). Genetic events such as those reproduced by *E1a* transformation appear to be required for the keratinocytes to overcome this inhibition.

The present work provides direct *in vivo* evidence that epithelial tumor development is under TGF-β control, in agreement with previous studies with normal epithelial and hematopoietic tissues (for review, see ref. 15). TGF-β can promote fibrosarcoma formation in Rous sarcoma virus-infected chickens (34), as could be expected from the growth-stimulatory effects of this growth factor on fibroblasts (the

Table 2. Tumorigenic behavior of control and oncogene-transformed Pam212 keratinocytes plus or minus other cells

Cell and treatment	Tumors/ injections	Weight, g	+DF/−DF ratio ± SD	Squamous-cell differentiation
Set 1				
Pam212-K(HaSV)	7/7	0.45	0.11 ± 0.02	−/+
Pam212-K + DF	4/4	0.038		+++
Set 2				
Pam212-K/neo(HaSV)	10/10	0.54	0.17 ± 0.07	−/+
Pam212-K + DF	9/10	0.07		+++
Set 3				
Pam212-K/E1a787(HaSV)	5/5	0.172	1.16 ± 0.4	−
Pam212-K + DF	5/5	0.144		−
Set 4				
Pam212-K/E1a598(HaSV)	7/7	0.43	0.6 ± 0.3	−
Pam212-K + DF	7/7	0.26		−

HaSV-transformed Pam212 keratinocytes (Pam212-K) were injected into nude mice either alone (5×10^5 cells per injection) or in a 1:4 combination with mouse dermal fibroblasts (DF) made HaSV-resistant by preinfection with MuLV. Similar experiments were done with HaSV-infected Pam212 cells that had been previously transfected with the SV2neo (K/neo), SV2neo/*E1a*d1787 (K/E1a787), or SV2neo/*E1a*NTd1598 (K/E1a598) recombinant plasmids (20). To minimize individual animal variations, the various *ras*-transformed keratinocytes were injected plus or minus dermal fibroblasts in parallel in the same animal (right and left suprascapular regions, respectively). Mice were sacrificed 14 days after injection, and tumor growth was measured by weight. Ratio between weight of tumors formed with and without dermal fibroblasts was calculated for each animal; the average ratio for each combination of cells is shown (+ DF/− DF ratio) ± SD. In contrast to HaSV transformants, control Pam212 cells or cells transformed by *E1a* alone produced no tumors even 1 mo after injection (0/6 injections). All tumors were analyzed histologically, and results were ≈100% consistent within each group. The well-differentiated tumors (+++), classifiable as grade I–II squamous-cell carcinomas, displayed mild-to-moderate cytologic atypia, low mitotic activity, and pronounced squamous-cell differentiation, containing basal and spinous layers and granular cells. In these tumors, undifferentiated areas covered <20% of total areas. In contrast, cells in poorly differentiated tumors (−/+), classifiable as grade III–IV squamous-cell carcinomas, showed only a few features of keratinization and high mitotic activity. Tumors formed by the *E1a*/*ras* transformants were composed, under all conditions, by small/medium-sized cells with no signs of keratinization (−), very high mitotic activity, and high nuclear/cytoplasmic ratio. The relatively smaller size of tumors formed by *ras*/*E1a*787 transformants possibly reflects the smaller size of these cells, which results in an apparently slower growth even in culture.

cells of origin of fibrosarcomas) (13–15). Thus, TGF-β can have either tumor-suppressing or tumor-promoting effects, depending on, among other things, the target-cell types.

Antibody-neutralization experiments and RNA-expression studies suggest that TGF-β3 is the main TGF-β form responsible for DF-IF activity. This factor is a 10- to 100-fold more potent inhibitor of keratinocyte growth than TGF-β1 (35). This fact, together with the differential expression of TGF-β3 in dermal fibroblasts versus keratinocytes, could explain the specificity of effects that dermal cells have in control of keratinocyte tumor development (9; Table 1). More generally, TGF-β3 production could be an important mediator of dermal–epidermal interactions and normal skin homeostasis.

Concomitant resistance to fibroblast tumor suppression and TGF-β and DF-IF growth inhibition were induced by transformation of Pam212 keratinocytes with the *E1a* oncogene. This oncogene has been shown to cooperate with *ras* in cell transformation (31, 32). The present results show how an intact *E1a* oncogene as well as a transformation-deficient mutant, which binds to a reduced subset of cellular proteins, can contribute to neoplastic growth of *ras* transformants by allowing escape from their cellular environment. The growth-inhibitory effects of TGF-β factors have been associated with induction of differentiation in a number of epithelial cells (13, 14), including some keratinocyte lines (36), even if not others (37). In our system, inhibition of tumor formation by added fibroblasts or DF-IF factor was associated with induction of squamous-cell differentiation, whereas no such effects were observed in tumors formed by *E1a* transformants. *In vitro*, we have found that epidermal transglutaminase, a marker of keratinocyte differentiation, is induced by TGF-β treatment of Pam212 keratinocytes but not of their *E1a*-transformed derivatives (20). Thus, *E1a* transformation appears to block growth-inhibitory and differentiating effects of TGF-β *in vitro* (17, 20) and *in vivo*.

Changes similar to those induced by *E1a* may occur during normal skin tumor development. In fact, a keratinocyte cell line derived from chemically induced papillomas (HaSV-p117 cells) (38) was found to be resistant to both pure TGF-β and DF-IF factors *in vitro* and dermal-fibroblast tumor inhibition *in vivo* (data not shown). Resistance to dermal-fibroblast tumor inhibition has also been reported for other papilloma-derived keratinocytes (39), and it will be interesting to test whether those cells are TGF-β and DF-IF resistant as well.

Activation of a *ras* oncogene by point mutations has been involved in the initial phase of mouse skin carcinogenesis (40), whereas increases in *ras* gene-copy number and/or gene expression have been correlated with malignant progression (41–43). Similar alterations can be induced by introducing a mutated *ras* oncogene into keratinocytes by HaSV infection (9, 38, 44–46). However, we have shown here that *ras* transformation by itself is not sufficient to allow keratinocytes to escape from the counteracting effects of a normal cellular environment and that second, independent events, such as those induced by *E1a*, are probably required.

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