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Dermal Fibroblasts Tumor Suppression of *ras*-Transformed Keratinocytes Is Associated with Induction of Squamous Cell Differentiation

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We have previously reported that tumor formation of ras-transformed keratinocytes can be suppressed by dermal fibroblasts through production of a diffusible growth inhibitory factor of the transforming growth factor-β (TGF-β) family. Keratinocytes transformed by ras and E1a oncogenes or papilloma-derived keratinocytes transformed by a ras oncogene show concomitant resistance to dermal fibroblast tumor suppression and TGF-β growth inhibition. We report here that dermal fibroblast tumor suppression is associated with a striking induction of squamous cell differentiation and that this effect is blocked in tumors resistant to dermal fibroblast inhibition. This experimental system strongly supports the notion that suppression of tumorigenicity and induction of a differentiated phenotype are closely associated events. (Am J Pathol 1994, 145:846–855)

Tumor heterogeneity represents a frequent event in human pathology. Often tumors in the same location display obvious differences in their histological, biological, and prognostic characteristics.[1–7] The differences observed within a single tumor may be secondary to genetic changes and/or environmental factors.[1] Different oncogenes can induce tumors with different histological features and distinctive malignant behavior when transfected into the same cell lines.[8,9] The influence of the surrounding tissue on the differentiation properties of tumor cells has also been postulated.[1,10–12] Furthermore, there are evidences that the malignant state is not irreversible and terminal differentiation induced by either therapeutic agents, local factors, or genetic events can convert malignant tumors to benign forms.[11–16]

In the mouse skin system, we have previously shown that primary mouse keratinocytes transformed by the v-H-*ras* oncogene are tumorigenic. Normal dermal fibroblasts (DF) can inhibit tumor growth of these cells and the tumor-suppressing effect is mediated by a diffusible inhibitory factor of the transforming growth factor-β (TGF-β) family.[15–17]

Besides primary cells, two keratinocytes lines were studied for their tumorigenic behavior after v-H-*ras* transformation. PAM 212 keratinocytes are an immortal cell line spontaneously derived from a primary keratinocyte culture.[20] These cells do not respond to the calcium-induced differentiation but they still retain their sensitivity to growth inhibition by TGF-β,[16,17] as well as other molecules such as corticosteroids and cAMP.[21,22] Unlike their parent cell line, PAM 212 cells harboring an intact or NH_2-terminally truncated *E1a* oncogene exhibit resistance to TGF-β, corticosteroid, and cAMP growth inhibition.[16,17,21,22] The p117 keratinocytes are an established cell line derived from chemically induced mouse papillomas.[9] These cells have an abnormal response to calcium and are also totally resistant to TGF-β (CM et al, unpublished observations).

In this report, we show that tumor suppression by normal DF is associated with induction of squamous cell differentiation and that different sensitivities to tumor suppression and differentiation are related to specific genetic changes of cells of the same origin.

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Materials and Methods

Cells and Tumors

Primary, PAM 212, and p117 keratinocytes were transformed by Harvey sarcoma virus (HaSV) infection and tested for tumorigenicity by subcutaneous injection into nude mice as previously described.[9,15,16] Primary DFs were obtained from newborn mice and grown as described.[16] PAM 212 keratinocytes harboring an intact or truncated *E1a* oncogene and infected with HaSV were also prepared as previously described.[16,17] The *E1a* dl787 gene carries an intact *E1a*-transforming region, whereas the mutant *E1a* NTdl598 express a truncated *E1a* protein that retains the ability of binding to the p105-Rb gene product and cyclin A as well as to other proteins but has lost its transforming ability.[18] Neo-PAM 212 keratinocytes carrying only the neomycin resistance gene were used as control.[16]

The number of cells injected of each type were 5 $\times$ 10^5 keratinocytes and 2 $\times$ 10^6 DF. Cells were injected subcutaneously in nude mice (male, 6 to 8 weeks old) and sacrificed 2 to 4 weeks later. A complete autopsy was performed in each case.

Histological and Ultrastructural Study

Tumors were fixed in 10% buffered formaldehyde and embedded in paraffin. Small pieces of tumors were frozen in liquid nitrogen and stored at −70 C. Six-micron-thick sections were stained by hematoxylin and eosin (H & E). The parameters used in the histological evaluation were the same as in human pathology. The degree of differentiation was evaluated according to the numbers of high power fields showing features of keratinization. For the electron microscopy study, 2 to 3-mm pieces were fixed in 3% glutaraldehyde for 24 hours, washed, incubated in phosphate-buffered saline (PBS) for 15 to 30 minutes, and postfixed in a 1:1 solution of 2% osmium tetroxide in PBS (pH 7.4) for 30 to 40 minutes. Dehydration was conducted in alcohol and the samples embedded in Lx resin. Sections were cut with an LKB Huxley ultramicrotome, stained with saturated uranyl acetate, and examined under a Zeiss 10C transmission electron microscope.

Immunohistochemical Study

Formalin-fixed, paraffin-embedded tumors sections were stained with an immunoperoxidase procedure following the manufacturer's instructions (Vectastatin ABC kit; Vector Laboratories, Burlingame, CA). The following antibodies were used: antikeratins AE1-AE3 (BioGenex Laboratories, Romano, CA) and CAM 5.2 (Becton Dickinson, Mountain View, CA).

Results

Primary, PAM 212, and p117 keratinocyte cultures were infected with helper associated HaSV, which causes high expression levels of the v-*ras*-p21 protein.[23] Subcutaneous injections into nude mice were used to assess tumorigenicity of the various *ras*-transformed keratinocytes alone or in combination with normal DF.[16] The number and weight of the tumors that were obtained are as previously reported,[16] and as shown in Table 1. A representative number of tumors was selected for each combination of cells for detailed histological and ultrastructural analysis.

The *ras*-transformed primary keratinocytes injected alone developed poorly differentiated tumors, with wide necrotic and hemorrhagic areas. These tumors showed noncohesive epithelioid and spindle cells arranged in a diffuse pattern (Figure 1, A). Occasionally, small epithelial cysts could be distinguished among the malignant cells. Ultrastructurally, these tumors were composed of highly undifferentiated cells with microvilli-like structures and indistinct cell junctions. Scattered keratin-positive cells were

Table 1. *Tumorigenic Behavior of Oncogene-Transformed Primary (K), PAM 212, and Papilloma-Derived p117 Keratinocytes, Plus/Minus DF*

Cells	Tumor/ Injections	Weight	Squamous Cell Differentiation
PAM/HaSV	7/7	0.45	+
" + DF	4/4	0.038	+++
PAM/neo/HaSV	20/20	0.40	+
" + DF	12/15	0.09	+++
PAM E1a787/HaSV	7/9	0.17	−
" + DF	5/5	0.13	−
PAM E1a598/HaSV	15/15	0.45	−
" + DF	7/8	0.24	−
K/HaSV	43/43	0.40	+
" + DF	0/28	−	/
P117/HaSV	9/9	0.41	+
" + DF	6/6	0.65	+
" + DF (*human*)	3/3	0.48	+

In this table, we summarize data previously reported[16] with additional experiments and the results obtained with the p117 cells. Mice were sacrificed 14 days after injection, except HaSV-transformed primary keratinocytes, which were sacrified 1 month after injection. All tumors were measured and weighed before formalin fixation. Tumors were classified as well differentiated (+++), poorly differentiated (+), or undifferentiated (−) on the basis of morphological and immunohistochemical criteria, as described in the text. K = primary keratinocytes.

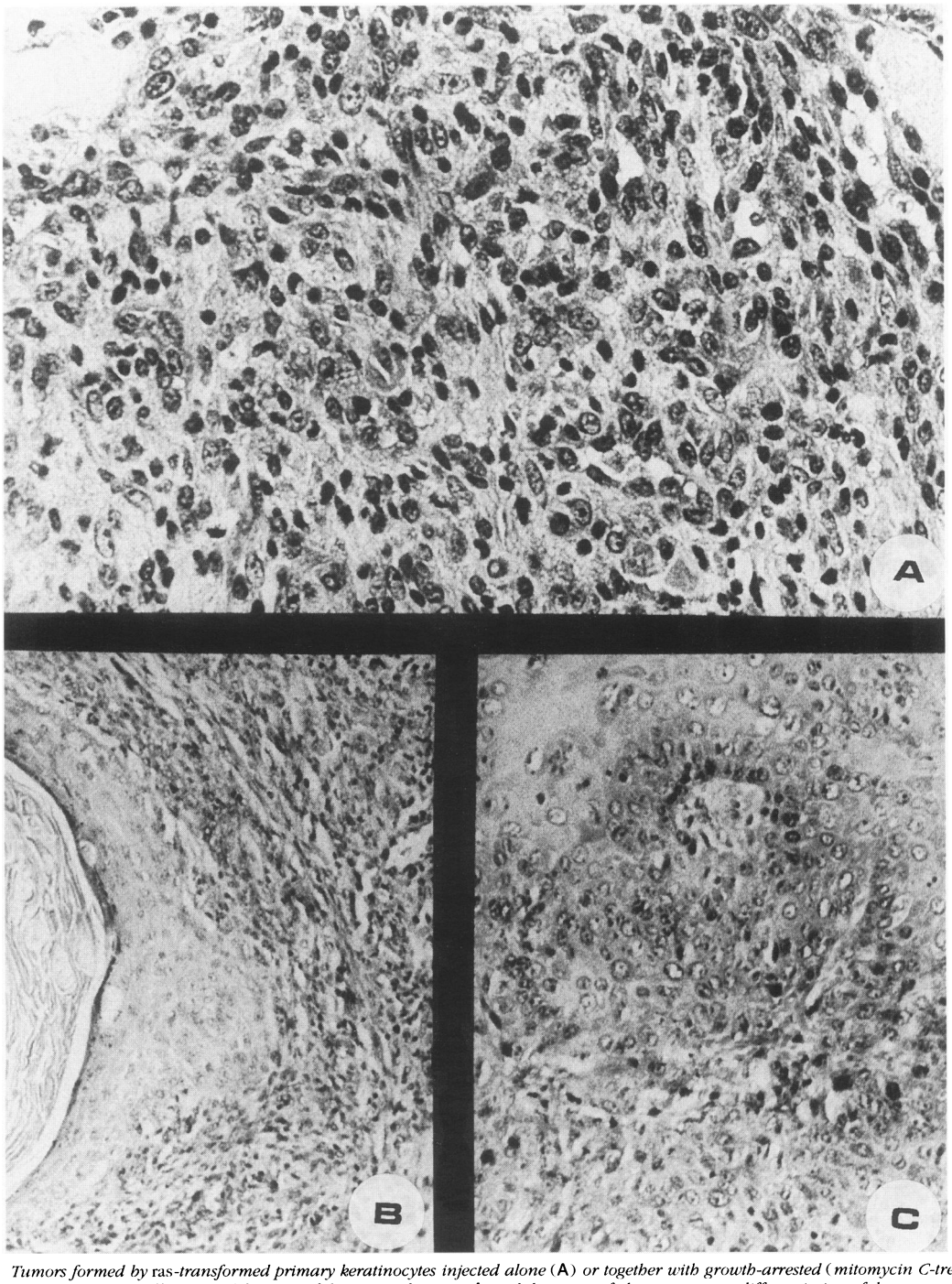

Figure 1. *Tumors formed by* ras*-transformed primary keratinocytes injected alone* (A) *or together with growth-arrested* (*mitomycin C-treated*) *DF* (B *and* C). *Note the poorly differentiated features of the tumor shown in* A *and the areas of clear squamous differentiation of the tumors in* B *and* C (*H & E,* A, ×*300;* B, ×*150; and* C, ×*250*).

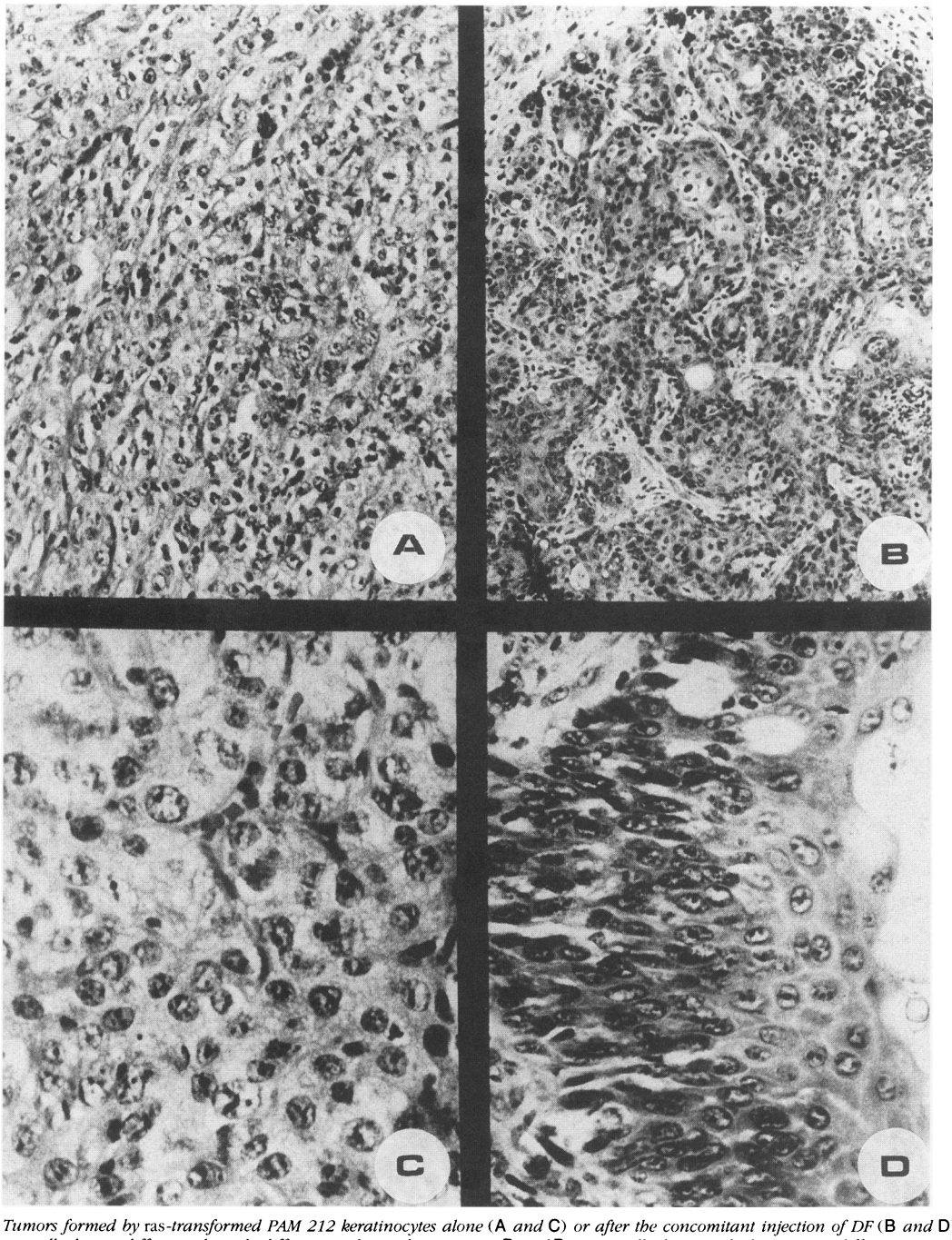

Figure 2. *Tumors formed by* ras*-transformed PAM 212 keratinocytes alone* (A *and* C) *or after the concomitant injection of DF* (B *and* D). *In* A *and* C, *tumor cells show a diffuse and poorly differentiated growth pattern. In* B *and* D *tumor cells show marked squamous differentiation* (*H & E,* A *and* B, ×*200;* C *and* D, ×*400*).

detected by immunohistochemical analysis with AE1/AE3 and CAM 5.2 antibodies (data not shown).

After addition of DF, *ras*-transformed primary keratinocytes did not develop any tumor. Only very small tumors were occasionally formed when the primary keratinocytes were mixed with DF that had been growth arrested by mitomycin C treatment.[16] The tumors formed in these cases displayed a diffuse pattern of cohesive and epithelioid cells with focal features of squamous cell differentiation (Figure 1, B-C). Polygonal cells without intercellular spaces or microvilli-like structures were observed under the electron microscope.

Like primary keratinocytes, tumors formed by *ras*-transformed PAM 212 keratinocytes injected alone were poorly differentiated, with no horn pearls or evident dyskeratotic features (Figure 2, A and C). Only scattered keratin positivity was detected by immunohistochemistry (Figure 3, A). Under the electron microscope, tumor cells displayed microvilli-like structures with some cell junctions but no well-formed desmosomes. No tonofilaments could be identified (Figure 4, A and C).

The *ras*-transformed PAM 212 keratinocytes injected together with DF formed much smaller tumors (~10-fold reduction), which were well delimited with respect to the surrounding tissue. These tumors displayed prominent features of squamous cell differentiation with well-developed intercellular bridges, horn pearls, and sheets of partially keratinized cells. Occasionally, fully keratinized centers with a granular layer epithelium could be distinguished (Figure 2, B and D). Strong and widespread positivity with both AE1/AE3 and CAM 5.2 antikeratin antibodies was observed (Figure 3, B; data not shown). Marked features of squamous cell differentiation were also observed by ultrastructural analysis (Figure 4, B and D).

Tumors formed by *ras*-transformed PAM 212 keratinocytes carrying an *E1a* oncogene displayed histological features of undifferentiated small cell carcinomas (Figure 6, A). Tumor cells were characterized by a polygonal shape, brisk mitotic activity, several small nucleoli, and little cytoplasm. They were arranged in clusters or trabeculae, which were well delimited with respect to the surrounding stroma. The histological appearance of these tumors was not affected by the

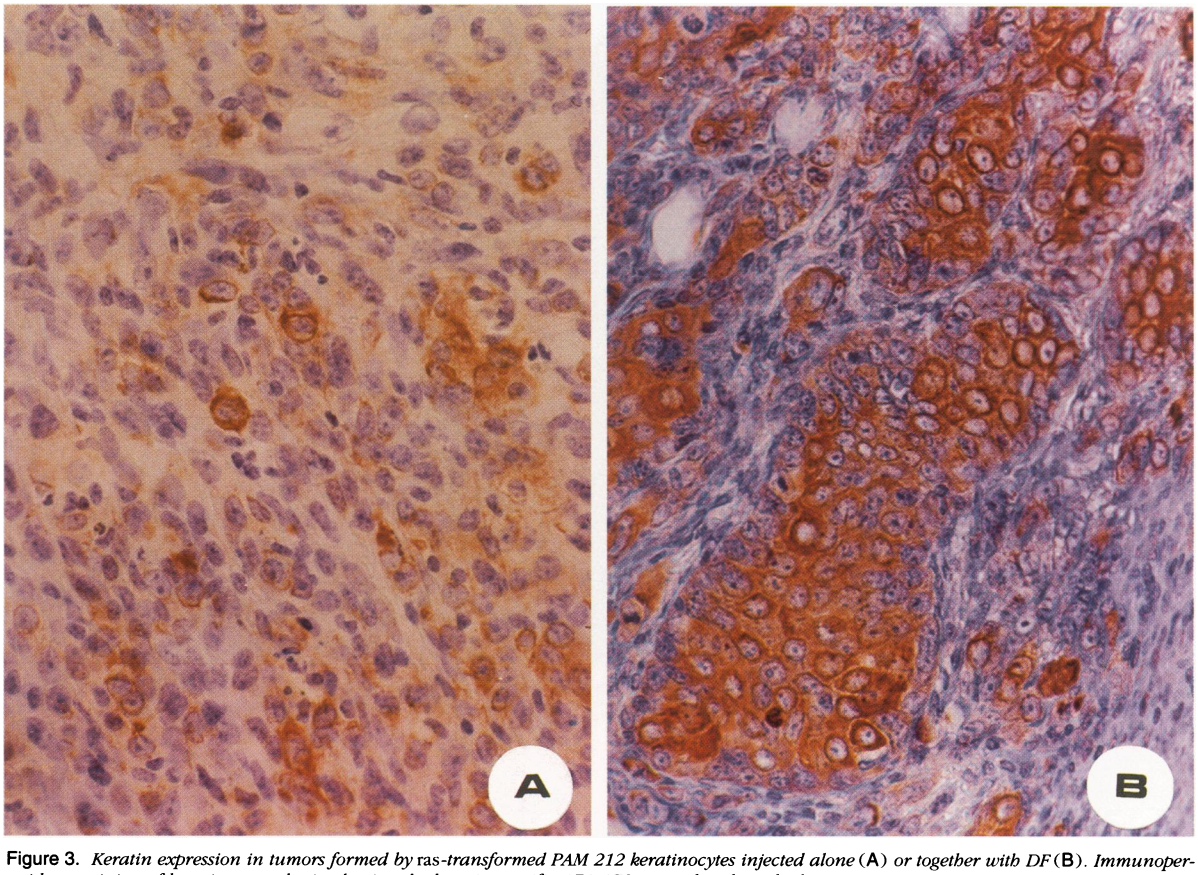

Figure 3. *Keratin expression in tumors formed by* ras*-transformed PAM 212 keratinocytes injected alone* (**A**) *or together with DF* (**B**). *Immunoperoxidase staining of keratins was obtained using the keratin-specific AE1-AE3 monoclonal antibody.*

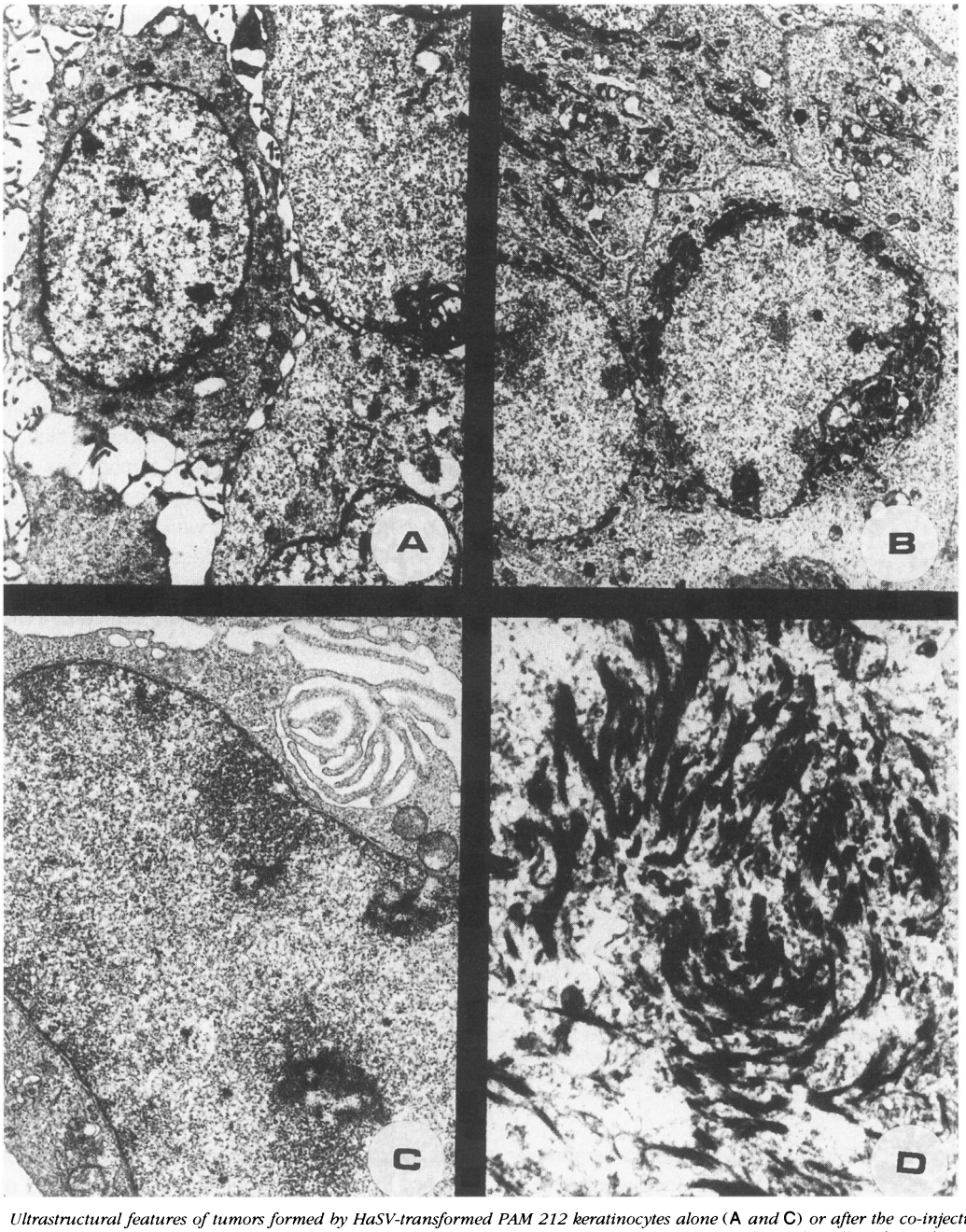

Figure 4. *Ultrastructural features of tumors formed by HaSV-transformed PAM 212 keratinocytes alone* (A *and* C) *or after the co-injection of DF* (B *and* D). *The tumors consist of cells with big nucleoli, wide cytoplasm, abundant ribosomes, and microvilli on the cell surface. The tumors obtained after the simultaneous injection of DF display abundant tonofilaments and keratin bundles* (A *and* B, ×8,000; C, ×25,000; D, ×50,000).

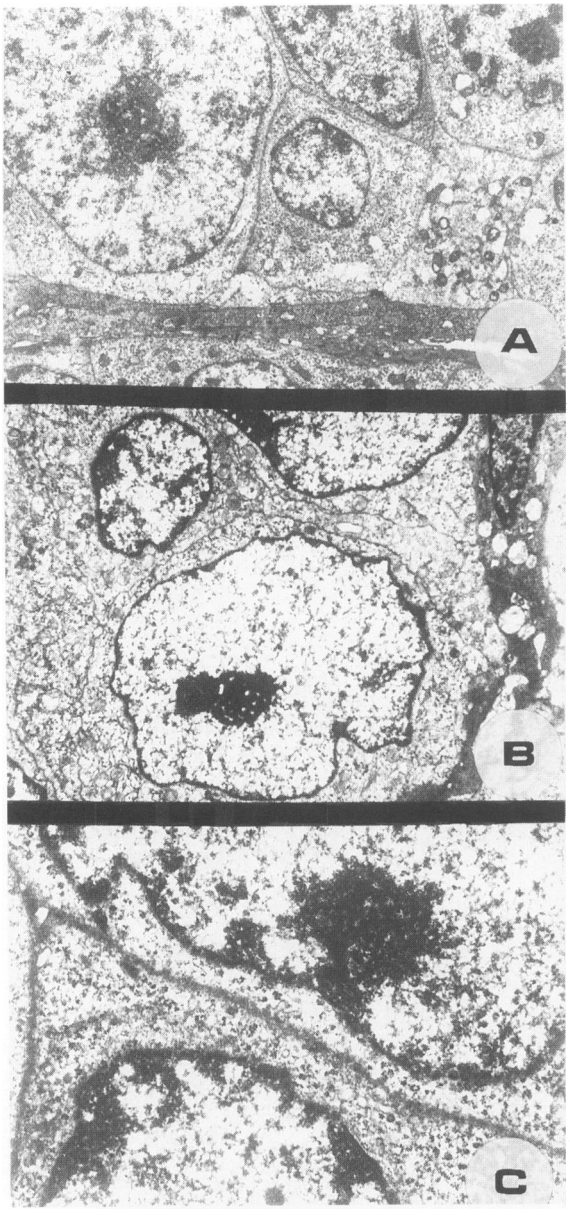

Figure 5. *Ultrastructural features of tumors formed by HaSV-transformed PAM 212 keratinocytes carrying the* E1a *dl787 gene and injected alone* (**A**) *or together with DF* (**B, C**). *The tumor cells are arranged mostly in clusters surrounded by stromal cells. The cells are attached to each other without intercellular spaces and no features of squamous differentiation are observed* (**A**, ×*12,5000*; **B**, ×*15,000*; **C**, ×*25,000*).

co-injection of DF (Figure 6, B). Scattered keratin expression was detected by immunohistochemical analysis of tumors formed with and without the addition of DF. Ultrastructurally, cells exhibited no microvilli-like structures and adhered completely to each other in the absence of any desmosome formation (Figure 5, A). As under the light microscope, similar ultrastructural features were observed in tumors formed with and without the addition of DF (Figure 5, B and C).

Tumors formed by papilloma-derived p117 keratinocytes transformed with *ras* exhibited features of poorly or moderately differentiated squamous cell carcinomas with dyskeratosis and intercellular bridges (Figure 7, A). Many desmosomes and abundant keratin bundle formation were observed under the electron microscope. The histological and ultrastructural picture of these tumors was the same, regardless of whether they were formed by keratinocytes with or without the addition of DF (Figure 7, A and B; data not shown).

Discussion

Spindle cell carcinomas appear like an extreme case of epithelial tumor development, with an highly aggressive and invasive phenotype associated with reduced or absent keratin production and lack of cadherin expression. In the mouse skin system, development of this type of tumors has been connected with high levels of expression of a transforming H-*ras* oncogene, as well as loss of some as yet unidentified tumor suppressor genes.[24,25]

We have shown here that an histological picture strongly reminiscent of spindle cell carcinomas is observed with tumors formed by either primary or PAM 212 mouse keratinocytes transformed by HaSV infection. Benign *versus* malignant behavior of these cells were found to correlate with low *versus* high levels of v-Ha-*ras* expression.[23] However, high v-*ras* expression is not sufficient for keratinocytes to escape from the restraining influences of a normal cellular environment and other genetic events are also required. In fact, tumorigenicity of HaSV-transformed keratinocytes can be suppressed by DFs through the production of a diffusible inhibitory factor of the TGF-β family, probably TGF-β3.[16,26] Introduction of *E1a* oncogenes into *ras*-transformed keratinocytes induces concomitant resistance to TGF-β growth inhibition and to DF tumor suppression.[16,17] In parallel with these findings, *in vitro* co-culturing with DF, inhibits proliferation of PAM 212 keratinocytes transformed by the single *ras* oncogene but not when these cells also carry the *E1a* oncogene (our unpublished observations).

In this study, tumor suppression of *ras*-transformed keratinocytes by normal DF was found to be closely associated with induction of squamous cell differentiation. Sensitivity to these effects was dependent on the genetic constitution of the target cells and their sensitivity to TGF-β. When co-injected with DF, PAM 212 keratinocytes transformed by the single *ras* on-

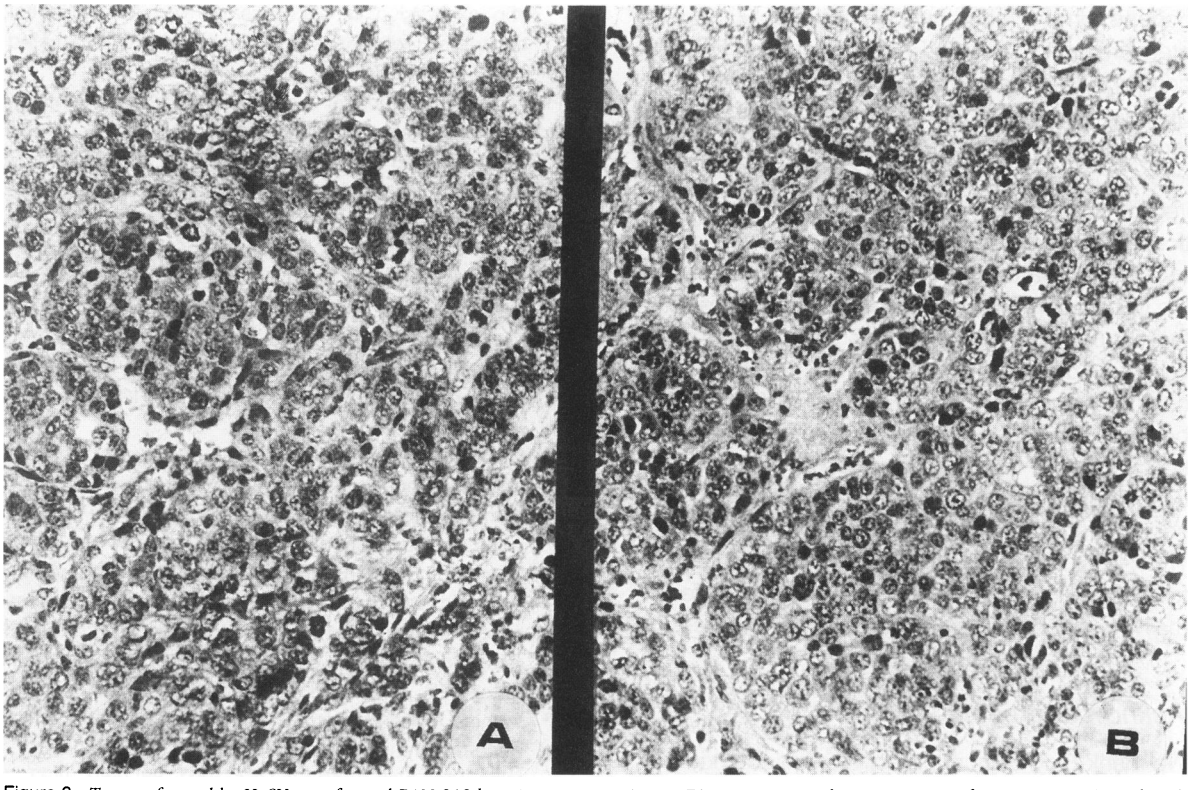

Figure 6. *Tumors formed by HaSV-transformed PAM 212 keratinocytes carrying an* E1a *oncogene with an intact transforming region (E1adl787) without* (A) *or with* (B) *the addition of DF. All tumors showed similar histological features. They were formed by small-size cells arranged in sheets or small clusters. No histological differences were observed after the co-injection of DF.* (*H & E,* A *and* B, ×250).

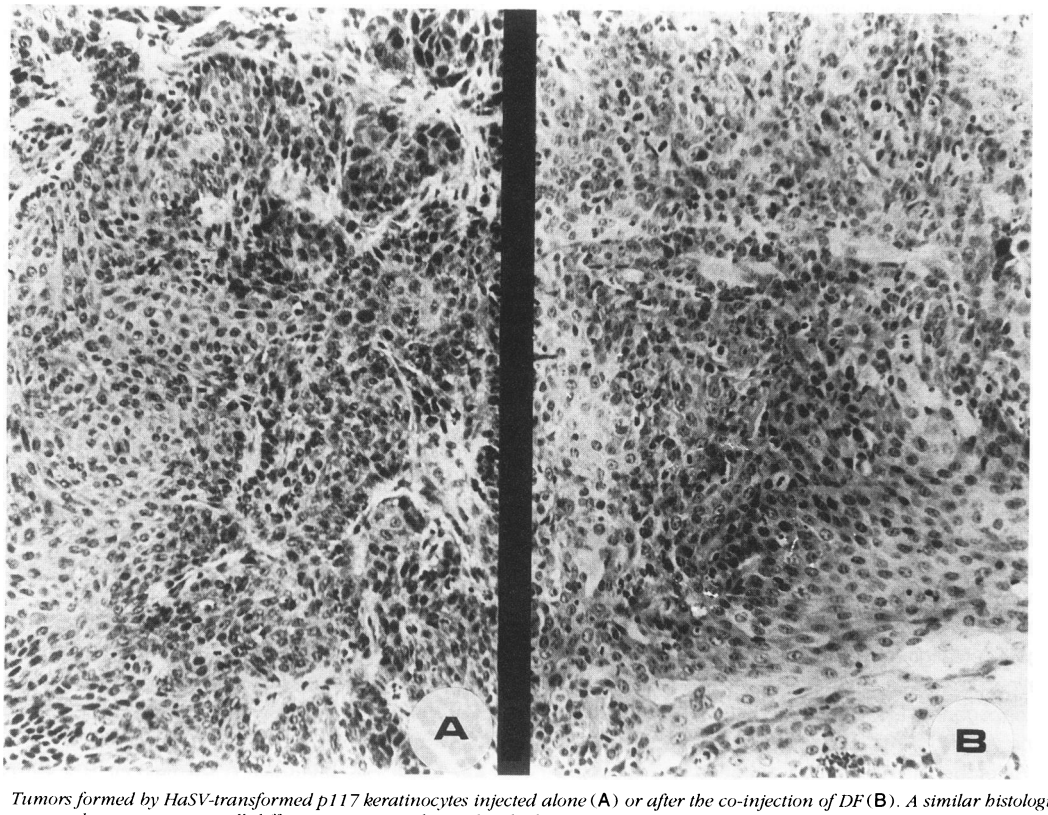

Figure 7. *Tumors formed by HaSV-transformed p117 keratinocytes injected alone* (A) *or after the co-injection of DF* (B). *A similar histological picture of poor to moderate squamous cell differentiation was observed in both cases.*

cogene formed much smaller and highly differentiated tumors. In contrast, PAM 212 keratinocytes concomitantly transformed by the *ras* and *E1a* oncogenes formed highly undifferentiated tumors, regardless of the presence or absence of DF. Tumors formed by *ras*-transformed p117 keratinocytes exhibited poorly to moderately differentiated characteristics, which were not further influenced by the addition of DF. Like E1a-transformed PAM 212 keratinocytes, the papilloma-derived p117 cells are totally resistant to TGF-β growth inhibition (our unpublished observations). Acquisition of a DF and TGF-β-resistant phenotype may be important for skin tumor development, because a number of other papilloma-derived keratinocytes have been reported to be resistant to DF tumor suppression.[27] Many high-risk papillomas and squamous cell carcinomas are devoid of TGF-β.[28] Thus, loss of expression of this factor and resistance to its growth inhibitory and differentiating effects[16] may be important events in skin tumor progression.

Acknowledgments

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