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Transforming Growth Factor β1 Induces Differentiation in Human Papillomavirus-Positive Keratinocytes†

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Human papillomaviruses (HPVs) are implicated in the etiology of anogenital cancers. Expression of the HPV E6 and E7 oncoproteins is believed to contribute to the carcinogenic process. Progressive loss of the ability to differentiate and resistance to the growth-inhibitory effects of endogenous signals also appear important in multistep tumorigenesis. Transforming growth factor β1 (TGF-β1) is a potent growth inhibitor for a variety of cultured cells. There have been conflicting reports on the ability of TGF-β1 to inhibit the growth of HPV-positive keratinocytes in monolayer cultures. We have employed the organotypic (raft) tissue culture system, which more accurately mimics the in vivo cellular environment and architecture. We have investigated the TGF-β1 response of HPV-positive keratinocytes derived from neoplastic cervical biopsies. Growth of these cell lines as raft tissues showed that many were altered in the ability to stratify and synthesize differentiation-specific proteins. When the organotypic tissues were treated with TGF-β1, a more complete differentiation of the keratinocytes was induced. Treatment with 12-*O*-tetradecanoylphorbol-13-acetate gave similar results. TGF-β1 treatment of HPV-positive raft epithelia led to a dose-dependent increase in E7 RNA expression in contrast to results from previous studies with monolayer cultures. Furthermore, TGF-β1 interfered with the proliferation of HPV-positive cell lines grown in monolayer cultures. Our results suggest that loss of the ability to express markers of differentiation, a characteristic of malignancy, is a two-step process. The first step is reversible; the second is irreversible.

Cervical cancer is a result of multiple alterations occurring over a long period. DNA from several human papillomavirus (HPV) types has been detected in cervical carcinomas and in their benign precursors, cervical intraepithelial neoplasias (CIN), indicating that HPV infection is a cofactor in the process of carcinogenesis (reviewed in reference 59). Of the numerous HPV types which have been identified, only a few, including types 16, 18, 31 and 33, are associated with a high risk for tumorigenic progression (28). The genome of the high-risk HPVs is generally integrated into the DNA of high-grade lesions but frequently remains episomal in premalignant lesions (9). HPV DNA integration into the host cell chromosomes has been shown to provide a cellular growth advantage in vitro (26). In most instances, viral genome integration results in the disruption of the E2 open reading frame and in an increased expression of the E6 and E7 open reading frames (26, 46). Expression of the E6 and E7 gene products from the high-risk HPV types is sufficient to immortalize human keratinocyte cultures (22, 39), an activity which correlates with the ability of the proteins to functionally inactivate the tumor suppressor proteins p53 and pRb, respectively (reviewed in reference 54). In addition, the E6 and E7 oncoproteins were found to modify the differentiation of keratinocytes in vitro (23) and synthesis of these proteins is related to the malignant state of cells derived from cervical neoplasias (45, 47, 49, 53, 59). Thus, the E6 and E7 oncoproteins are believed to have important functions in the development of HPV-associated anogenital cancers.

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† Dedicated to the memory of Christine A. Noonan.

A number of observations indicate that other cellular alterations are also necessary for the progression from high-risk HPV infection to malignancy (58, 59). Expression of the high-risk HPVs is not sufficient for cellular transformation in vitro but can be induced by the addition of an activated *ras* oncogene (15, 16). In vivo, after high-risk HPV infection, progression from CIN I to III and then to carcinoma can be assessed by the degree to which epithelial differentiation is abrogated and occurs over a period of years or decades (58). Escape from the control of endogenous growth-regulating factors long has been proposed as a hallmark of tumorigenesis (40, 55). One of the most studied proteins capable of modulating cellular growth and differentiation is transforming growth factor β1 (TGF-β1), the prototype of a superfamily of such agents (29). TGF-β1 is the strongest growth-inhibitory protein recognized for a variety of cultured cells, including those derived from epithelial, endothelial, myeloid, and lymphoid tissues (reviewed in references 10 and 29). Likewise, TGF-β1 blocks the in vitro growth of many normal human epithelial cells, including cervical epithelial cells and foreskin keratinocytes (7, 48, 57). Induction of terminal differentiation has been reported for bronchial epithelial cells treated with picomolar concentrations of TGF-β1 (31). However, induction of differentiation by TGF-β1 is not a typical observation in epithelial cells grown as monolayers (reviewed in reference 10). Although most normal epithelial cells are responsive to TGF-β1, many transformed cell lines are resistant to its effects (10). This suggests that loss of responsiveness to growth-inhibitory agents such as TGF-β1 is an important step in tumorigenesis (10, 50).

In the present study we have determined the TGF-β1 response of cervical epithelial cell lines containing integrated and/or episomal copies of high-risk types of HPV DNA. Previous studies showed that the growth inhibition brought on by TGF-β1 treatment of cultured monolayers was accompanied by a down-regulation of HPV E6 and E7 gene transcription (6,

TABLE 1. HPV-immortalized genital keratinocytes

Cell line	HPV DNA type	Derivation (reference)
AWCA (CC-1)	Integrated HPV18	Invasive carcinoma biopsy (42)
RECA (CC-2)	Integrated HPV16	Invasive carcinoma biopsy (42)
CIN-680	Integrated HPV16	CIN III biopsy (12)
CIN-612 9E	Episomal HPV31b	CIN I biopsy (24, 42)
CIN-612 6E	Integrated HPV31b	CIN I biopsy (13, 42)
pA6	Integrated HPV18	Transfected primary foreskin keratinocytes (23)

57). This regulation of HPV gene expression was proposed as a mechanism by which TGF-β1 might exert growth-inhibitory effects on HPV-infected cells (6, 57). Because monolayer cultures do not resemble the in vivo environment of stratifying epithelium, we have employed an organotypic tissue culture system which emulates the three-dimensional architecture and differentiation scheme of keratinocytes in vivo. In this system epithelial cell lines derived from cervical neoplasias resemble their in vivo counterparts (3, 42). HPV-immortalized keratinocyte cell lines grown as organotypic tissues have altered abilities to differentiate, reminiscent of HPV-positive cervical lesions in vivo (4, 23, 33). Furthermore, this system was used to demonstrate the complete, differentiation-dependent life cycle of HPV in vitro (35).

Our investigation shows that TGF-β1 inhibited the growth of HPV-positive keratinocyte monolayer cultures, consistent with the results of earlier reports (6, 57). TGF-β1 treatment of neoplastic epithelial cell organotypic cultures resulted in the renewed ability to express morphological and biochemical markers characteristic of a more normal and differentiated phenotype. These data demonstrate that TGF-β1 is able to inhibit the proliferation of monolayer cultures of HPV-containing keratinocytes; however, the growth effects elicited by TGF-β1 are not limited to the cessation of cell division. In contrast to previous results with monolayer cultures, we found organotypic epithelial tissue cultures treated with increasing concentrations of TGF-β1 to have increased levels of the oncogene E7 RNA. Our data suggest that in HPV-associated anogenital carcinogenesis, the loss of the ability to differentiate involves at least two separate steps. The first step is reversible, and is an apparent loss of the differentiation-inducing signal. The cells retain the ability to differentiate when provided with an exogenous signal. The second step involves an irreversible and progressive loss of the ability to respond to differentiation-inducing signals.

MATERIALS AND METHODS

Cell lines. The HPV-immortalized cell lines used in this study are presented in Table 1. The AWCA cell line was established from a biopsy of an invasive carcinoma of the cervix and has been shown to contain two integrated copies of HPV type 18 (HPV18) (42). The RECA cell line was established from a biopsy of an invasive cervical carcinoma and has one integrated copy of HPV16 (42). CIN-680, a generous gift of G. Wilbanks (Rush Medical College, Chicago, Ill.), was established from a CIN III biopsy and has integrated copies of HPV16 (12). The CIN-612 cell line was established from a CIN I biopsy and contains HPV31b DNA (3). The 6E clonal derivative of CIN-612 contains ≈2 to 5 copies per cell of predominantly integrated HPV31b (13). In the CIN-612 clonal derivative 9E, the HPV31b genome is episomal at ≈100 copies per cell (24). The cell line pA6 was immortalized by transfection of HPV18 DNA into primary foreskin keratinocytes (23). Two HPV-negative epithelial cell lines were also studied. The SCC-13 cell line is an established line derived from a squamous cell carcinoma of the cheek (32, 43). The HaCat cell line was established from spontaneously immortalized human epidermal cells (5).

The cell lines were maintained in E medium with mitomycin-treated murine J2 3T3 fibroblast feeders (32, 34). Epithelial organotypic (raft) tissue cultures for in vitro differentiation were maintained as previously described (23, 32, 34, 35, 42). Epithelial cells were seeded onto collagen matrices containing J2 3T3 fibroblast feeders. When the epithelial cells had grown to confluence, collagen matrices were lifted onto stainless steel grids and the cells were fed by diffusion from under the matrix. The epithelial cells were allowed to stratify and differentiate at the air-liquid interface over a 16-day period. Treatment of rafts with recombinant TGF-β1 (R&D Systems, Minneapolis, Minn.) in E medium was every other day. Treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma Chemical Co., St. Louis, Mo.) in E medium was for 16 to 20 h every 4 days as described previously (35).

Histochemical analyses. Raft cultures were grown for 16 days, harvested, fixed in 4% paraformaldehyde, and embedded in paraffin; 4-μm cross-sections were then prepared. Sections were stained with hematoxylin and eosin (35). Immunostaining was performed with the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif.) (35). Primary antibodies for immunostaining included the following: keratin 10-specific monoclonal antibody (1:100; Sera-Lab, Crawley Down, Sussex, United Kingdom); anti-human keratin 14 peptide monoclonal antibody (1:200; Sigma Immunochemicals); human-specific cytokeratin 8 monoclonal antibody (1:50; DAKO Corp., Carpinteria, Calif.); and anti-human filaggrin monoclonal antibody (1:50; Biomedical Technologies Inc., Stoughton, Mass.).

Western immunoblot analyses. Proteins were extracted from monolayer cultures or raft tissues with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris-Cl [pH 8.0], 5 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1% deoxycholate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of aprotinin per ml). Extracts were clarified by centrifugation (≈12,000 × *g*) for 15 min at 4°C. The protein concentration of supernatants was measured by Bradford assay (Bio-Rad Laboratories, Hercules, Calif.). Equal amounts of protein were separated by electrophoresis through a 10% polyacrylamide gel (3.3% crosslinker) containing SDS. The proteins were transferred to Immobilon PVDF membrane (Millipore Corp., Bedford, Mass.). The membranes were blocked for 1 h in phosphate-buffered saline (PBS) containing 0.1% Tween-20 and 5% (wt/vol) nonfat dry milk and then incubated for 1 h with either antikeratin 10 antibody (1:200; a generous gift of D. Roop, Baylor College of Medicine, Houston, Tex.) or antikeratin 14 peptide antibody (1:100; Sigma Immunochemicals). Immunodetection was performed with a 1:20,000 dilution of the appropriate antiimmunoglobulin peroxidase-linked species-specific whole antibody (Amersham Corp., Arlington Heights, Ill.) followed by the enhanced chemiluminescence (ECL) detection kit (Amersham Corp.) per the manufacturer's instructions. Results were visualized by exposure to Reflection film using intensifying screens (DuPont NEN, Boston, Mass.).

Growth properties of epithelial cells treated with TGF-β1. Cells were seeded at a density of 2.0×10^3 viable cells per 35-mm tissue culture dish. Media were changed every 2 days, and cells were either untreated or treated with 1 ng (0.4 nM) of TGF-β1 per ml. Duplicate dishes of cells were trypsinized and counted. The log of the average total cell count was plotted versus days in culture.

RNase protection analyses. The construct pHPV18-E6E7 was made by cloning the *Bam*HI-*Taq*I fragment (nucleotides [nt] 119 to 838) from pBRHPV18 (a gift of H. zur Hausen) into the *Bam*HI-*Cla*I sites of pGEM-7Zf(+) (Promega Corp., Madison, Wis.). The construct was digested with *Ava*II and produced an expected antisense RNA of 308 nt, specific for a 242-nt region of HPV18 E7. The construct pR-hGAPDH contains human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA and was created by subcloning the 548-bp *Hin*dIII-*Xba*I fragment from pHcGAP (52) into pGEM-3Zf(−) (Promega Corp.). The *Sty*I-digested template gave an expected 134-nt antisense RNA which reproducibly protected a doublet corresponding to ≈90 to 95 nt.

Total RNA was extracted from 16-day rafts and monolayer cultures using TRIzol reagent (Gibco BRL, Bethesda, Md.). Monolayer cultures were untreated or treated with 1.0, 5.0, or 10.0 ng of TGF-β1 per ml for 48 h; rafts were untreated or treated with 0.5, 1.0, 5.0, or 10.0 ng of TGF-β1 per ml every other day. Antisense RNA probes were synthesized using [α-^{32}P]CTP (800 Ci/mmol; DuPont NEN) and the MAXI script/RPA II kit according to the manufacturer's instructions (Ambion Inc., Austin, Tex.). Full-length probes were gel purified on 7 M urea–5% polyacrylamide gels. Ten micrograms of total RNA or yeast RNA was hybridized using the RPA II kit (Ambion) with 4×10^4 cpm of each probe at 43°C for 16 to 20 h. Unhybridized RNA was digested with a mixture of 2.5 U of RNase A per ml and 100 U of RNase T_1 per ml. Samples were analyzed by electrophoresis through a 7 M urea–5% polyacrylamide gel followed by autoradiography using Reflection film and intensifying screens (DuPont NEN). RNA Century standards were prepared as per the manufacturer's recommendations (Ambion Inc.). The intensity of protected fragments was measured by scanning laser densitometry.

RESULTS

TGF-β1 treatment induces morphological differentiation and stratification of invasive carcinoma cells in organotypic cultures. The invasive cervical carcinoma-derived AWCA cell line was assayed for its response to TGF-β1 treatment in the organotypic culture system. Treated and untreated AWCA rafts were harvested for paraffin embedding after exposure to

TGF-β1 as described in Materials and Methods. Thin sections of the raft cultures were histochemically analyzed and compared with raft cultures of normal primary foreskin keratinocytes. Hematoxylin and eosin staining was done to examine epithelial morphology and stratification. In vivo human epidermal cells stratify extensively and demonstrate a progressive series of morphological transitions distinctive of terminal differentiation (27). Likewise, primary human foreskin keratinocytes grown in the organotypic culture system showed morphological features similar to those of their in vivo counterparts (Fig. 1A). Untreated raft cultures of the invasive cervical carcinoma-derived AWCA cells were all basal-like and showed no evidence of stratification or differentiation (Fig. 1B and Table 2). In addition to the disorganized architecture, the basement membrane was not smooth and showed signs of invasiveness (see arrows in Fig. 1B). With respect to these characteristics, the AWCA cell line grown in the raft system faithfully mimics the morphology of its in vivo invasive carcinoma counterpart (42). In contrast, organotypically grown AWCA cells treated with TGF-β1 resulted in differentiation-specific stratification as evidenced by a distinct corneum, the appearance of a granular layer, and a basal layer which was noninvasive and more organized (Fig. 1E). Treatment with either 1 or 5 ng of TGF-β1 per ml yielded similar results in all instances (data not shown). The differentiation induced by TGF-β1 had similarities to that observed in cervical neoplastic raft tissues treated with activators of the protein kinase C pathway (35). Likewise, similar stratification and differentiation characteristics were observed in AWCA cells grown in the raft system and treated with the phorbol ester TPA, a known activator of the protein kinase C pathway (Fig. 1D).

TGF-β1 treatment induces biochemical differentiation of AWCA invasive carcinoma cells in organotypic cultures. A number of biochemical changes are associated with the process of terminal differentiation, namely, synthesis of the large keratins (56.5 and 65 to 67 kDa) (17, 19). The expression of filaggrin, a keratin filament-associated protein, is another marker specific to differentiated keratinocytes (11). To assess the expression of biochemical markers of differentiation, TGF-β1-treated and untreated AWCA rafts were harvested for paraffin embedding as described in Materials and Methods. A summary of the differentiation marker profile of AWCA raft culture tissues treated with TGF-β1 and TPA is presented in Table 2. Thin sections of the raft cultures were analyzed by immunostaining and compared with raft cultures of normal primary foreskin keratinocytes. Normal stratified keratinocytes express high levels of K10 (56.5 kDa) in the suprabasal layers (Fig. 1G) (17, 19). Rafts of the AWCA invasive carcinoma cell line lost this differentiation-specific ability (Fig. 1H). However, TGF-β1 treatment restored the ability of the AWCA raft tissues to synthesize K10 proteins in the suprabasal layers (Fig. 1I). The 50-kDa K14 protein is typically expressed in the basal and suprabasal layers of normal stratified epithelium (Fig. 1J) (17, 19). Although the AWCA carcinoma cells had a reduced ability to produce K14 (Fig. 1K), induction of differentiation by TGF-β1 was accompanied by an increased ability to synthesize K14 (Fig. 1L). Normally observed in the granulosum and corneum (11), filaggrin expression was strongly down-regulated in the untreated AWCA raft cultures (data not shown; Table 2). TGF-β1 treatment of AWCA raft cultures resulted in the detection of filaggrin, albeit at low levels, in the appropriate epithelial strata. The 54-kDa K8 protein is expressed only in simple (nonkeratinizing) epithelium and squamous cell carcinomas (36). Appropriately, normal keratinizing epithelium grown in the raft system failed to express detectable levels of K8 (Fig. 1M). In contrast, raft cultures of the AWCA carcinoma-derived cell line produced significant amounts of the K8 protein (Fig. 1N). The differentiation induced by treatment of the AWCA raft cultures with TGF-β1 resulted in the abrogation of K8 expression (Fig. 1P). Treatment of the rafts with TPA yielded results similar to those with TGF-β1 (Table 2).

Effects of TGF-β1 on the biochemical differentiation of other HPV-positive neoplastic keratinocytes in organotypic cultures. Expression of the markers of terminal differentiation was also investigated in the other HPV-positive keratinocyte lines listed in Table 1, and the results are summarized in Table 2. Similar to the histology of its invasive carcinoma counterpart, the RECA line failed to stratify in the raft system (Fig. 1C) (42). Furthermore, the RECA rafts grew fewer cell layers than did the AWCA rafts. These observations, along with the facts that both AWCA and RECA cell lines were concomitantly derived (42) and have been handled identically after establishment, suggest that the RECA cells were derived from a more highly progressed cancer tissue. Treatment with TGF-β1 did not foster any morphological stratification (Fig. 1F). In addition, treatment did not confer a change in the phenotype of the RECA rafts with respect to K10 expression (data not shown). However, compared with the untreated controls (Fig. 2G), TGF-β1 was able to slightly restore K14 synthesis in the RECA raft cultures (Fig. 2J). Filaggrin expression was also slightly restored (data not presented). Treatment with TGF-β1 resulted in a decrease in K8 expression by the RECA raft tissues (Fig. 2K and H).

CIN-612 9E cells cultivated in the raft system mimic their in vivo CIN I counterpart, both morphologically and biochemically (3, 35, 42). K10 expression and filaggrin expression were weak in the untreated CIN-612 9E raft cultures (Fig. 2A and I, respectively). Treatment with TGF-β1 induced the cells to appropriately express K10 in the suprabasal layers (Fig. 2D); TGF-β1 treatment resulted in a significant increase in filaggrin detection in the granulosum and corneum (Fig. 2L). K14 expression was slightly reduced in the untreated CIN-612 9E rafts compared with normal keratinocyte rafts; TGF-β1 restored the ability of CIN-612 9E raft tissues to synthesize K14 at more normal levels (data not shown). Similar to the case with primary keratinocyte raft cultures, K8 expression was undetectable in the untreated CIN-612 9E rafts (data not shown). In each instance tested, TPA treatment of CIN-612 9E rafts produced results similar to those observed upon TGF-β1 treatment (Table 2) (35). Raft cultures from both CIN-612 6E and pA6 were affected by exposure to TGF-β1 or TPA. Compared with the untreated controls, TGF-β1 treatment resulted in the increase of K10 expression in both CIN-612 6E (Fig. 2B and E, respectively) and pA6 (Fig. 2C and F, respectively) cells. K14 and filaggrin were more appropriately expressed in the TGF-β1-treated raft cultures of CIN-612 6E and pA6 (Table 2). The TGF-β1 responsiveness of two HPV-negative epithelial cell lines grown as raft tissues was also tested. Neither the SCC-13 nor the HaCat raft cultures were induced by TGF-β1 to stratify or to more appropriately express any of the four differentiation markers (data not shown).

Western analyses were performed to assess the relative levels of K10 and K14 protein synthesis in untreated versus treated raft cultures. Detection of K10 expression in organotypic cultures of primary human foreskin keratinocytes, treated and untreated CIN-612 9E rafts, and treated and untreated AWCA rafts was consistent with the immunostaining analyses. Raft cultures of primary human foreskin keratinocytes expressed readily detectable levels of K10 (Fig. 3A, lane 1). Untreated CIN-612 9E and AWCA raft tissues had reduced levels of K10 protein (Fig. 3A, lane 3, and data not shown, respectively). However, upon treatment with TGF-β1 or TPA,

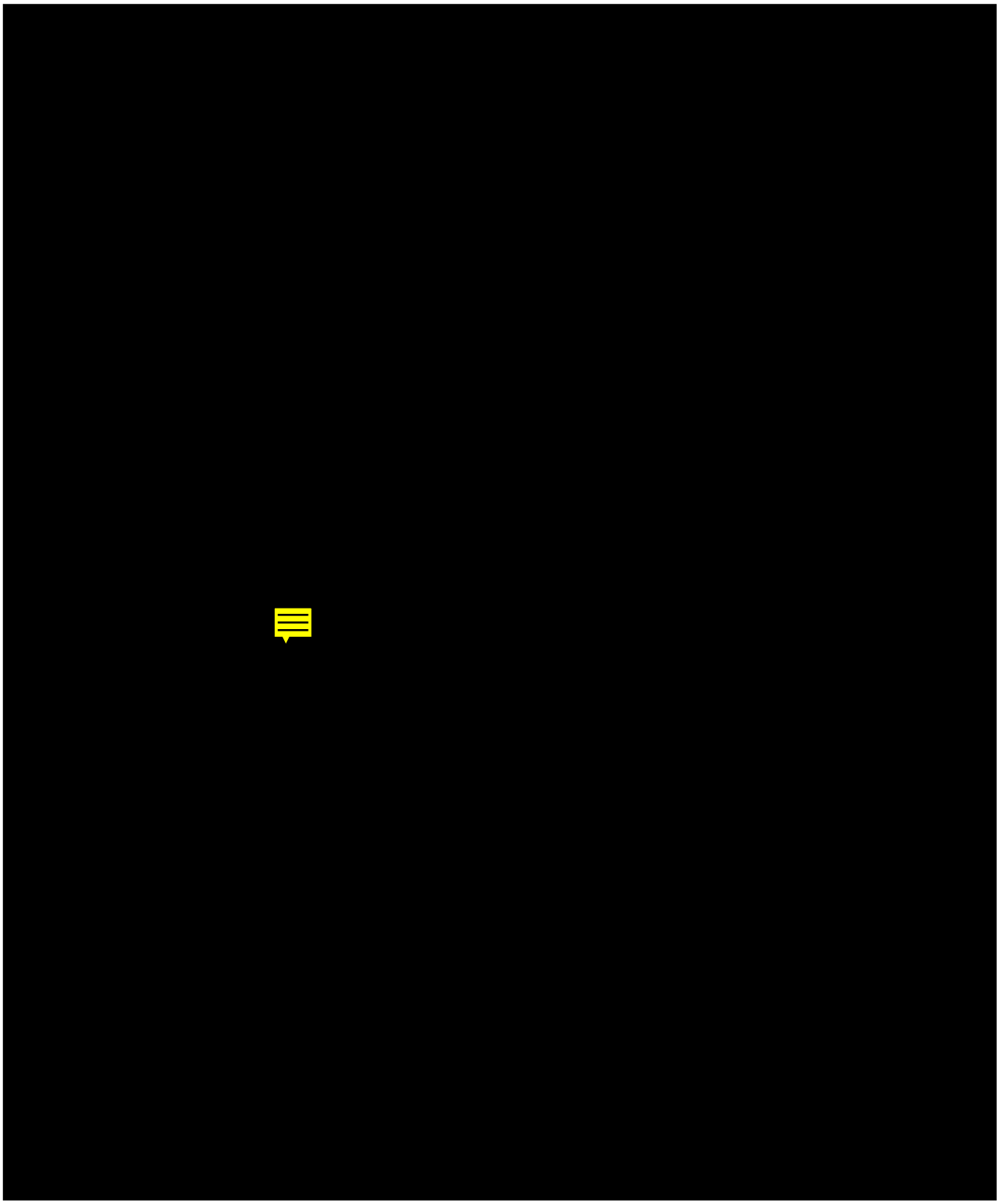

FIG. 1. (A to F) Hematoxylin and eosin staining pattern of organotypic tissue cultures. (A) Untreated primary human foreskin keratinocyte rafts; (B) untreated AWCA rafts (arrows indicate areas in which the cells are invading the collagen matrix); (C) untreated RECA rafts; (D) AWCA rafts treated with 0.16 μM TPA; (E) AWCA rafts treated with 1 ng of TGF-β1 per ml; (F) RECA rafts treated with 0.016 μM TPA. (G to I) Antikeratin 10 staining pattern of raft cultures. (G) Untreated primary human foreskin keratinocytes; (H) untreated AWCA rafts; (I) AWCA rafts treated with 1 ng of TGF-β1 per ml. (J to L) Anti-keratin 14 staining pattern of raft cultures. (J) Untreated primary human foreskin keratinocyte rafts; (K) untreated AWCA rafts; (L) AWCA rafts treated with 5 ng of TGF-β1 per ml. (M to P) Anti-keratin 8 staining pattern of raft cultures. (M) Untreated primary human foreskin keratinocyte rafts; (N) untreated AWCA rafts; (P) AWCA rafts treated with 1 ng of TGF-β1 per ml.

TABLE 2. Results of TGF-β1 and TPA treatment of keratinocyte raft tissue cultures[a]

	1°	AWCA			RECA			CIN-612 9E			CIN-612 6E			pA6		
	U	U	TGFβ	TPA	U	TGFβ	TPA	U	TGFβ	TPA	U	TGFβ	TPA	U	TGFβ	TPA
Stratification	++[b]	Ø	++	++	Ø	Ø	Ø	++	++	++	±	++	ND[c]	++	++	ND
K10 (suprabasal)	++	Ø	++	++	Ø	Ø	Ø	+	++	++	Ø	+	ND	+	++	ND
K14 (basal, suprabasal)	++	±	+	+	Ø	+	+	+	++	++	Ø	+	ND	++	++	ND
Filaggrin (corneum, granulosum)	++	±	++	+	Ø	+	+	±	++	++	Ø	+	ND	±	+	ND
K8 (simple epithelium)[d]	Ø	++	Ø	±	++	±	++	Ø	ND	ND	Ø	ND	ND	+	±	ND

[a] 1°, normal primary keratinocyte rafts; U, untreated rafts; TGFβ, rafts treated with 1 to 10 ng of TGF-β1 per ml; TPA, rafts treated with 0.016 to 0.16 μM TPA.
[b] ++, 75 to 100% of expression in normal keratinocytes; +, 50 to 75% of normal levels; ±, <50% of normal levels; Ø, not detected.
[c] ND, not determined.
[d] K8 normally is not expressed in stratifying epithelium.

both CIN-612 9E and AWCA raft tissues expressed increased amounts of K10 (Fig. 3A, lanes 4 and 5, and data not shown, respectively). Confluent monolayer cultures of CIN-612 9E and AWCA cells expressed detectable amounts of K10 and K14 protein (Fig. 3, lanes 1, and not shown, respectively), consistent with previous reports (reviewed in reference 18). The relative changes in K14 expression assessed by Western blot analysis were less than those for K10 but were consistent with the immunostaining data. CIN-612 9E raft tissues were minimally altered in their ability to appropriately express K14 compared with normal primary keratinocyte rafts (Table 2); this was also illustrated by Western analysis (data not shown). In contrast, untreated AWCA raft tissues had reduced levels of K14 protein (Fig. 3B, lanes 1 and 3, respectively). Treatment of AWCA rafts with either TGF-β1 or TPA resulted in an increase in K14 protein expression (Fig. 3B, lanes 4 and 5, respectively). Although these Western blot data are not quantitative, they corroborate the immunostaining data which demonstrated increased and more appropriate expression of the K10 and K14 proteins in the treated raft tissues.

TGF-β1 inhibits the growth of cervical cancer cells in monolayer culture. There have been conflicting reports concerning the effects of TGF-β1 on the proliferation of human keratinocytes immortalized by HPV. We chose to assess in monolayer culture whether our immortalized human keratinocytes were sensitive to the inhibitory effects of TGF-β1 on cellular proliferation. This was done to determine if TGF-β1-induced growth inhibition in monolayer cultures could be correlated with induction of differentiation in raft cultures. All cell lines contained full-length copies of high-risk types of HPV DNA and, with the exception of the pA6 cell line, all lines tested were derived from biopsies of cervical neoplasias (Table 1). This permitted the assessment of the effects of TGF-β1 on the proliferation of cervical epithelial cell lines at various stages of malignancy. Compared with untreated controls, cell lines treated with TGF-β1 at 1 ng/ml for 2 to 3 weeks were significantly growth inhibited (Fig. 4). In general, all lines tested showed some sensitivity to TGF-β1 and the effects were observed after only a few days' treatment. Although both AWCA and RECA were derived from invasive carcinomas, the cell lines differed in the extent to which they were growth inhibited by TGF-β1. After an apparent delay in proliferation after initial TGF-β1 treatment, the AWCA cells continued to divide at a rate comparable to that of their untreated controls (Fig. 4A). In contrast, proliferation of the RECA cells was effectively blocked upon TGF-β1 treatment (Fig. 4B). The CIN-680 cell line derived from a CIN III biopsy displayed a delayed, but significant, growth inhibition in response to TGF-β1 (Fig. 4C). TGF-β1-induced growth inhibition was comparable between the CIN-612 9E cell line containing episomal HPV31b DNA and the CIN-612 6E cell line harboring integrated copies of HPV31b, both derived from the same parental cell line (Fig. 4D and E, respectively). Striking growth inhibition was observed in the pA6 cell line immortalized by transfected HPV18 DNA (Fig. 4F).

TGF-β1 induces transcription of HPV18 E7 RNA. Previous reports have suggested that the growth inhibition observed upon TGF-β1 treatment of HPV-expressing keratinocytes is due to a decrease in the expression of E6 and E7 transcripts (6, 7, 57). To investigate whether this was the case in our organotypic system, AWCA cells were grown as raft tissues and treated with increasing concentrations of TGF-β1 as described in Materials and Methods. Monolayer cultures of AWCA cells were also treated with various concentrations of TGF-β1 for 48 h. RNase protection assays were performed to quantitate and compare HPV E7 RNA levels among untreated AWCA monolayers, untreated rafts, and both monolayers and rafts treated with TGF-β1 at 0.5, 1.0, 5.0, and 10.0 ng/ml (0.2 to 4.0 nM). These analyses showed that the expression of HPV18 E7 transcripts was not significantly affected by TGF-β1 treatment of monolayer AWCA cultures (Fig. 5A, lanes 1 to 4). In raft tissues, HPV18 E7 transcription was not down-regulated upon TGF-β1 treatment; moreover, E7 RNA levels increased in the rafts treated with ≥1.0 ng of TGF-β1 per ml (Fig. 5A, lanes 5 to 9). The results of the RNase protection were quantitated densitometrically, and E7 RNA levels were normalized to the RNA levels of the housekeeping gene GAPDH (Fig. 5B). This analysis showed that the amount of E7 RNA was 3.2- to 4.5-fold higher in rafts treated with ≥1.0 ng of TGF-β1 per ml than in untreated controls (Fig. 5B).

DISCUSSION

To better understand how loss of the ability to differentiate contributes to tumorigenesis, we have employed the organotypic (raft) tissue culture system. The raft system mimics important morphological and physiological aspects of epithelial differentiation (34, 35). A number of previous reports have demonstrated that the growth in this system of cell lines derived from cervical biopsies results in organotypic tissues which are morphologically similar to their in vivo counterparts (3, 12, 23, 42). Additional work has shown that supplementing TPA into the culture medium of CIN-612 9E organotypic cultures, which harbor episomal HPV31b DNA, resulted in increased expression of physiologic markers of keratinocyte differentiation (35). Coincident with this level of differentiation was the biosynthesis of HPV31b virions (35). In the present study we have analyzed the ability of TGF-β1 to induce keratinocyte differentiation in the organotypic culture system by examining

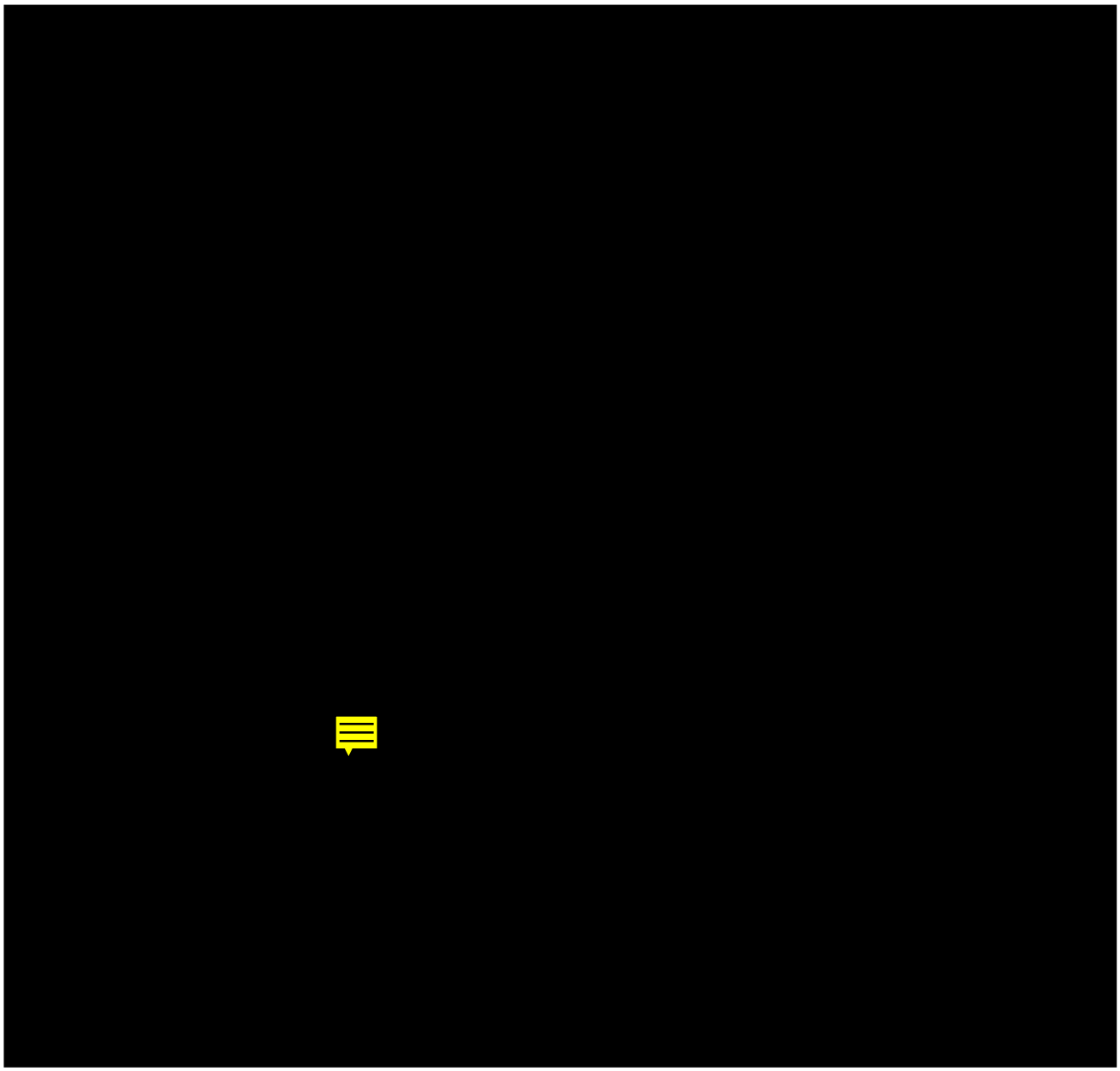

FIG. 2. (A to H) Antikeratin 10 staining pattern of organotypic cultures. (A) Untreated CIN-612 9E rafts; (B) untreated CIN-612 6E rafts; (C) untreated pA6 rafts; (D) CIN-612 9E rafts treated with 1 ng of TGF-β1 per ml; (E) CIN-612 6E rafts treated with 1 ng of TGF-β1 per ml; (F) pA6 rafts treated with 1 ng of TGF-β1 per ml. (G and J) Antikeratin 14 staining pattern of organotypic cultures. (G) Untreated RECA rafts; (J) RECA rafts treated with 10 ng of TGF-β1 per ml. (H and K) Antikeratin 8 staining pattern of raft cultures. (H) Untreated RECA rafts; (K) RECA rafts treated with 10 ng of TGF-β1 per ml. (I and L) Antifilaggrin staining pattern of raft cultures. (I) Untreated CIN-612 9E rafts; (L) CIN-612 9E rafts treated with 5 ng of TGF-β1 per ml.

the effects of TGF-β1 on a battery of high-risk HPV DNA-containing keratinocyte cell lines.

Our data show that treatment of the organotypic tissue cultures with either TGF-β1 or TPA resulted in increased differentiation, both morphologically and biochemically (Table 2). This study consisted of HPV-positive cell lines derived from biopsies of various stages of cervical carcinogenesis, including CIN I, CIN III, and invasive carcinomas. An HPV DNA-immortalized foreskin keratinocyte line was also included. In agreement with earlier reports, we found these cell lines grown organotypically to demonstrate morphological characteristics similar to those of their in vivo counterparts (3, 12, 23, 42). Untreated CIN-612 9E raft cultures were the least altered of the cell lines assayed in their ability to differentiate. After treatment with either TGF-β1 or TPA, CIN-612 9E raft tissues were able to return to a more normal phenotype of differentiation-specific marker expression. Interestingly, the CIN-612 6E raft tissues displayed a more altered differentiation marker profile; further, they were less affected by TGF-β1 or TPA treatment. This is a striking observation, considering that the cell lines were cloned from the same parental, CIN I-derived cell line and used here at the same passage; clone 9E contains

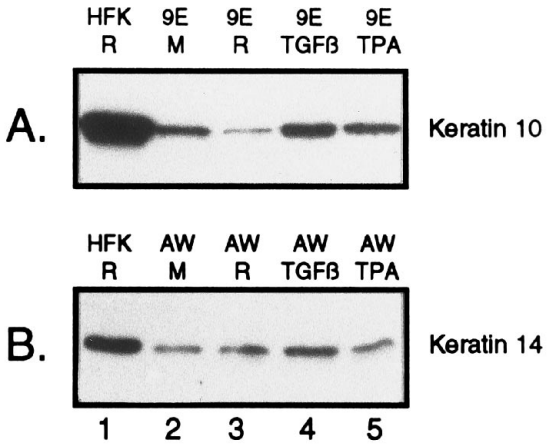

FIG. 3. Western blot analysis of K10 and K14 expression in untreated primary human foreskin keratinocyte rafts (lanes 1), untreated monolayers (lanes 2), untreated rafts (lanes 3), rafts treated with 1.0 ng of TGF-β1 per ml (lanes 4), or rafts treated with 0.16 μM TPA (lanes 5). (A) Anti-human K10 antibody detection of the 56.5-kDa K10 protein in 10 μg of total cell protein from primary human foreskin keratinocyte rafts (lane 1) and CIN-612 9E (lanes 2 to 5). (B) Antipeptide K14 antibody detection of the 50-kDa K14 protein in 20 μg of total cell protein from primary human foreskin keratinocyte rafts (lane 1) and AWCA (lanes 2 to 5).

episomal HPV31b DNA, whereas clone 6E harbors integrated HPV31b DNA. This is good evidence that HPV integration and the ensuing changes contribute to a less differentiation-competent phenotype, concordant with a role for viral integration in the progression of anogenital tumorigenesis (9, 26). Untreated organotypic tissues of the HPV-positive lines derived from higher-grade cervical lesions and HPV-immortalized keratinocytes were more altered than CIN-612 9E in their differentiation-marker expression. This is consistent with the concept that differentiation and malignancy are inversely correlated (20). We also tested two HPV-negative cell lines in raft cultures for their responsiveness to TGF-β1. Neither was induced by TGF-β1 to undergo further biochemical differentiation. However, we are unable to discern whether this is related to the HPV status, because these cell lines were established by means other than those used for the HPV-positive keratinocytes. Furthermore, the cell lines were derived from nongenital epithelium.

Our data have a number of implications. First, loss of the ability to differentiate is not immediately irreversible in cervical tumorigenesis. This was best illustrated by the finding that AWCA cells derived from an invasive carcinoma could be induced to undergo a program of differentiation when grown in the organotypic system. Treatment with either TGF-β1 or TPA induced the AWCA rafts to become more like normal keratinocytes with respect to their ability to stratify and synthesize biochemical markers of differentiation. Second, the ability to express individual markers of differentiation may become irreversibly lost during multistep carcinogenesis. As shown in Table 2, both TGF-β1 and TPA were able to affect a more normal phenotype for each marker tested, although not equally in every cell line. This suggests that loss of the ability to respond to a given differentiation-inducing signal is distinct for each marker (i.e., loss of the ability to express one marker does not imply loss of the ability to express other or all markers). Further, loss of the ability to express multiple markers appears to be progressive during tumorigenesis. This demonstrates the multifactorial nature of differentiation and supports the correlation between loss of differentiation and malignancy. Third, the lack of expression of morphologic or biochemical markers does not necessarily imply the irreversible loss of the ability to express that marker. This point illustrates the utility of the organotypic culture system for analyzing cell lines derived from cervical biopsies, rather than merely testing biopsy material directly for the expression of differentiation markers. The ability of organotypic cultures to be manipulated allowed us to determine if the loss of expression of a differentiation marker was reversible or irreversible. This permits analysis of the response to differentiation-inducing agents while the cells grow in a three-dimensional environment like epithelial tissue in vivo. Together, these findings suggest that the ability to express a given differentiation-specific marker potentially is a two-factor process. One element is the signal which stimulates the cell or tissue to express the marker. Another factor is the ability of the cell or tissue to respond appropriately to a given signal. Because we were able in the majority of cases to induce the appropriate expression of differentiation markers upon treatment with a signal (TGF-β1), we conclude that loss of the signal generally precedes loss of the ability to respond to a signal in cervical cancer. However, it is conceivable that the latter might occur in the absence of the first in some instances. Given that all of the cell lines tested showed some responsiveness to differentiation induction by TGF-β1, such an approach might be useful as a therapy for the treatment of anogenital neoplasias in vivo. In addition, the organotypic tissue culture system may provide a useful tool to test the efficacy of other potential therapeutic agents for genital, as well as other skin-related, neoplasms.

With respect to stratification and expression of differentiation markers, treatment with TGF-β1 and TPA yielded similar results in each of the HPV-positive raft tissues tested. Moreover, TGF-β1 has been shown to mimic a number of activities of phorbol esters. These include induction of angiogenesis (37, 44), induction of squamous differentiation (31, 56), inhibition of myogenic differentiation (8, 30), inhibition of adipogenic differentiation (14, 25), and in vitro tumor promotion (21). Although TPA acts through the protein kinase C pathway, there is no evidence that TGF-β1 does the same. Interestingly, Akhurst et al. found that TPA treatment in mouse skin induces high levels of TGF-β mRNA (1). These observations suggest that depending on the cell type, a subset of the activities of TPA may encompass the actions of TGF-β1, perhaps by stimulating production of TGF-β1.

We have shown that treatment with TGF-β1 results in significant growth inhibition of monolayer cultures of HPV-positive keratinocytes. Previously, there have been conflicting reports regarding the susceptibility of HPV-positive cervical epithelial cells or foreskin keratinocytes to the effects of TGF-β1. For example, monolayer cultures of genital epithelial cells containing high-risk HPV DNA were found to be similarly inhibited compared with normal cells (6, 57), less sensitive than normal cells (12, 57), or resistant to TGF-β1 (41). In our collection of cell lines were those harboring HPV16, HPV18 and HPV31b DNA, and all were sensitive in monolayer cultures to the growth inhibitory effects of TGF-β1. The disparity between our results and those of others may be due in part to different assay techniques. Pietenpol et al. (41) and Braun et al. (6) grew cells in the presence of TGF-β1 for 24 to 48 h and then assayed for DNA synthesis by [^{3}H]thymidine incorporation. Woodworth and coworkers assayed the cloning efficiency of cells grown in the presence of TGF-β1 for 10 days (57). Our cells were cultured in the presence of TGF-β1 and assayed by counting viable cells periodically for up to 18 days. We found no correlation between TGF-β1-induced growth inhibition and either the stage of lesion from which the cell lines were estab-

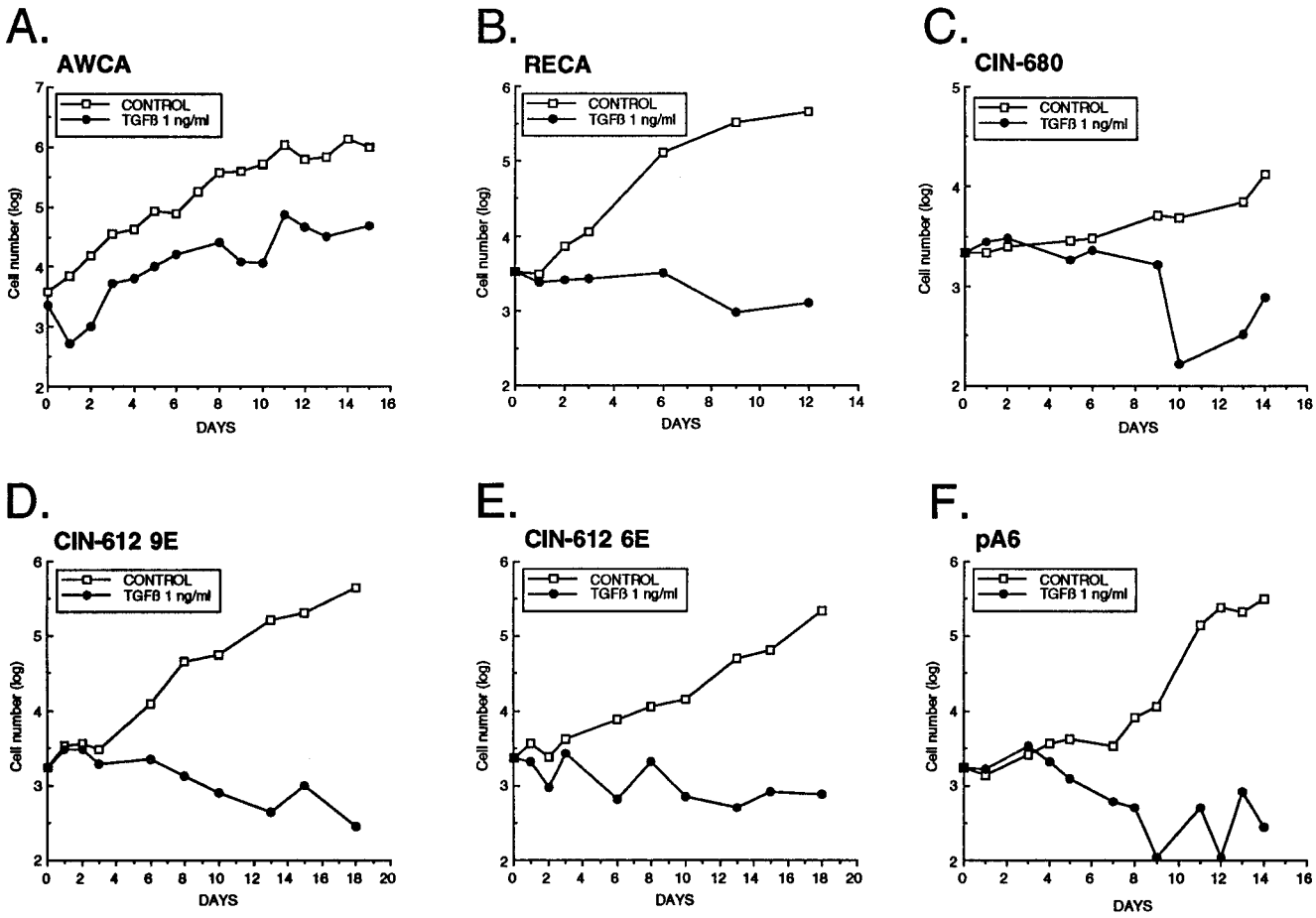

FIG. 4. Effects of TGF-β1 treatment on HPV-positive epithelial cells grown in monolayer cultures. Cells were seeded at 2×10^3 cells per plate and were either untreated or treated with 1 ng of TGF-β1 per ml. Cell number represents the log of the average of duplicate cultures counted on the indicated days. Open boxes represent control cultures grown in the absence of TGF-β1; closed circles indicate cultures grown in the presence of 1 ng of TGF-β1 per ml.

lished or the HPV DNA type in the cell lines. There was no apparent relationship between the sensitivity of a given cell line to TGF-β1-induced growth inhibition in monolayer culture and the ability of TGF-β1 to induce differentiation of the same cell line grown in the organotypic tissue system. For example, considering only the results from the monolayer growth inhibition assay, it might seem that the RECA cell line was more sensitive to TGF-β1 effects than was the AWCA line. However, in the organotypic system, the AWCA raft tissue was clearly more responsive to induction of differentiation by TGF-β1 than was the RECA raft tissue. A similar response to TGF-β1-induced growth inhibition in monolayer culture was seen in the 9E and 6E clones of the CIN-612 cell line. This suggests that the state of the viral genome (episomal versus integrated) had no significant influence on the TGF-β1 response in this assay.

A number of studies have shown that one way in which TGF-β1 may facilitate its growth-inhibitory effects is by the down-regulation of c-*myc* expression (reviewed in references 10 and 38). Furthermore, Pietenpol et al. reported that TGF-β1-induced repression of c-*myc* RNA and, subsequently, growth inhibition was blocked in immortalized keratinocytes expressing DNA tumor virus oncoproteins, including HPV16 E7 (41). Their data suggested that the tumor suppressor protein pRB or a related protein is required for the inhibition of c-*myc* transcription by TGF-β1 (41). From this they concluded that HPV16 E7 via its binding to pRb was responsible for abrogating the TGF-β1 response pathway in the transformed cell lines. However, it is noteworthy that these data were obtained with cells expressing HPV E7 from heterologous promoters. Other investigators have shown that the growth inhibition induced by TGF-β1 treatment was accompanied by a reversible, time- and dose-dependent decrease in HPV16 E6 and E7 RNA expression (6, 57). Whereas those investigations were performed with monolayer cultures, we examined the HPV E7 RNA levels in organotypic tissue cultures treated with increasing amounts of TGF-β1. In contrast to the previous findings, AWCA raft tissues treated with TGF-β1 displayed a dose-dependent increase in HPV18 E7 RNA. The levels of E7 RNA in AWCA monolayer cultures were unaffected by TGF-β1 treatment. The most obvious explanation for the disparity between TGF-β1-treated monolayer and organotypic cultures is that they represent two different phenomena. Another possibility, given the stratification of the raft tissue, is that HPV E7 expression is limited to certain strata and not present in others. It is also plausible that the increase in HPV E7 RNA expression could be a viral reaction in an attempt to subvert the terminal differentiation of the cells. Although HPV replication is tightly linked to the differentiation state of the epithelial cells (51), there likely is a delicate balance between terminal differentiation and cell proliferation maintained by

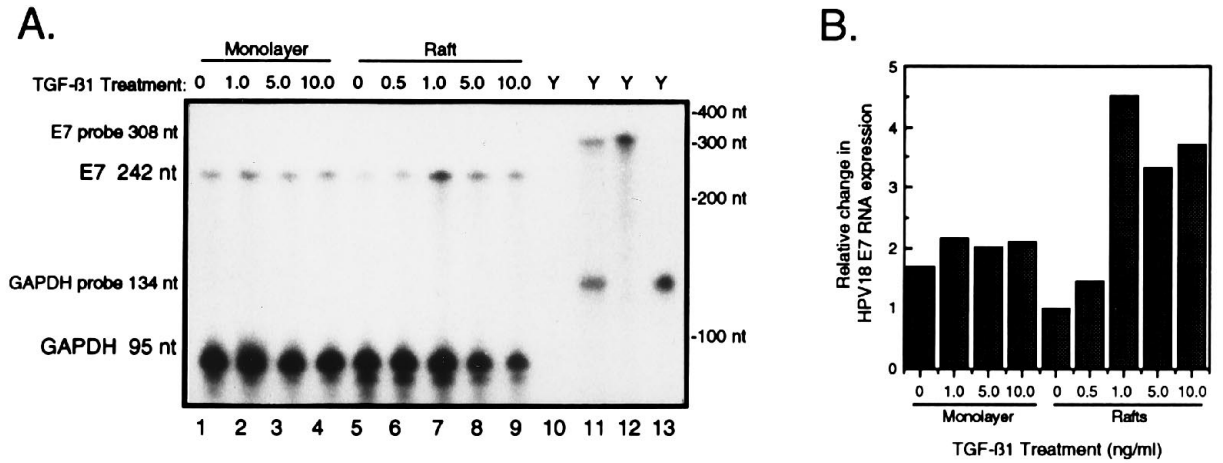

FIG. 5. RNase protection assay quantitating the RNA levels of HPV18 E7 in monolayers and rafts, both untreated and treated with increasing concentrations of TGF-β1. (A) RNase protection analysis of HPV18 E7 and GAPDH RNA expression in AWCA cultures. Total RNA was harvested from monolayer and raft cultures which were treated with concentrations of TGF-β1 from 0 to 10.0 ng/ml. Ten micrograms of total AWCA RNA (lanes 1 to 9) or yeast RNA (Y; lanes 10 to 13) was hybridized to antisense HPV18 E7 and GAPDH probes. Samples in lanes 1 to 10 were RNase treated; samples in lanes 11 to 13 were not RNase treated and represent 5×10^3 cpm of both GAPDH and E7 probes, E7 alone, or GAPDH alone (respectively). The sizes of the probes and predicted sizes of the protected fragments are indicated on the left. The positions of the RNA Century markers (Ambion) are given on the right. (B) Relative change in E7 RNA expression among the RNase-protected fragments. After densitometry scanning, each protected E7 RNA fragment was normalized to GAPDH RNA protection by dividing the sample value for E7 by the GAPDH value.

the viral oncoproteins. We are currently investigating these possibilities.

This study demonstrates that the method of culturing epithelial cells has a substantial effect on how the cells respond to TGF-β1 treatment, consistent with the cautions of Sporn and Roberts (50). TGF-β1 inhibited the growth of monolayer cultures, whereas differentiation was induced in the three-dimensional proliferating organotypic tissue cultures. This illustrates an important distinction between the two assay systems and underscores the deficiencies of monolayer cultures with respect to their inadequate analogy to the in vivo situation. There are other examples of converse effects of TGF-β1 in vitro and in vivo. For example, TGF-β1 inhibits endothelial cell growth in monolayers (2) but is strongly angiogenic in vivo (44). Although the usefulness of monolayer systems to analyze multistep tumorigenesis should not be underestimated, the limitations of such a system must be considered. We believe that the three-dimensional organotypic tissue culture technique more accurately mimics the in vivo situation and thus is better suited to study the progression of anogenital carcinogenesis.

Loss of responsiveness to TGF-β1 appears to play a role in the pathway to HPV-associated carcinogenesis; however, our data as well as those of others (57) suggest that resistance to TGF-β1 is not a prerequisite. Neither is loss of the ability to synthesize the markers of differentiation necessary (20).

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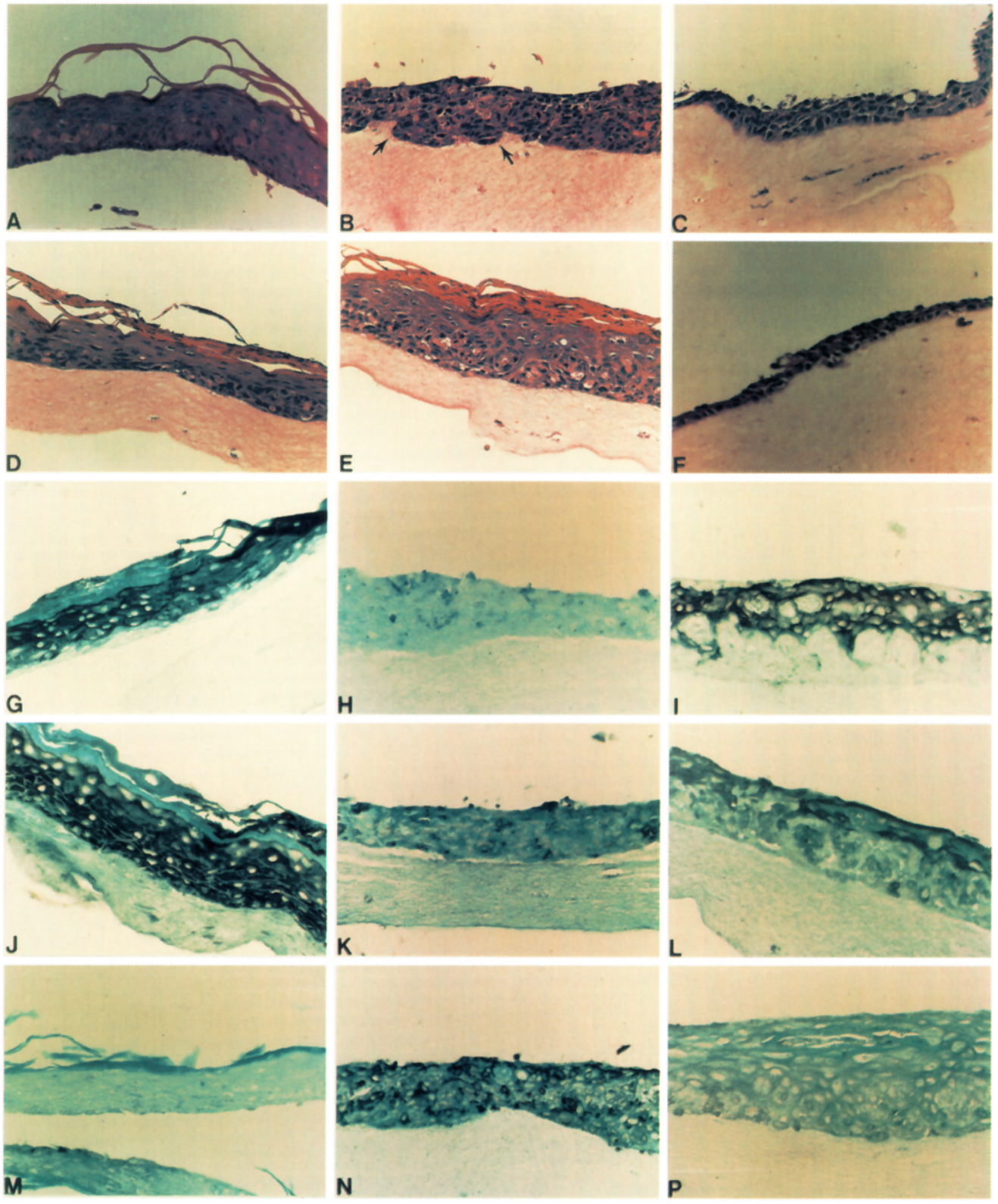
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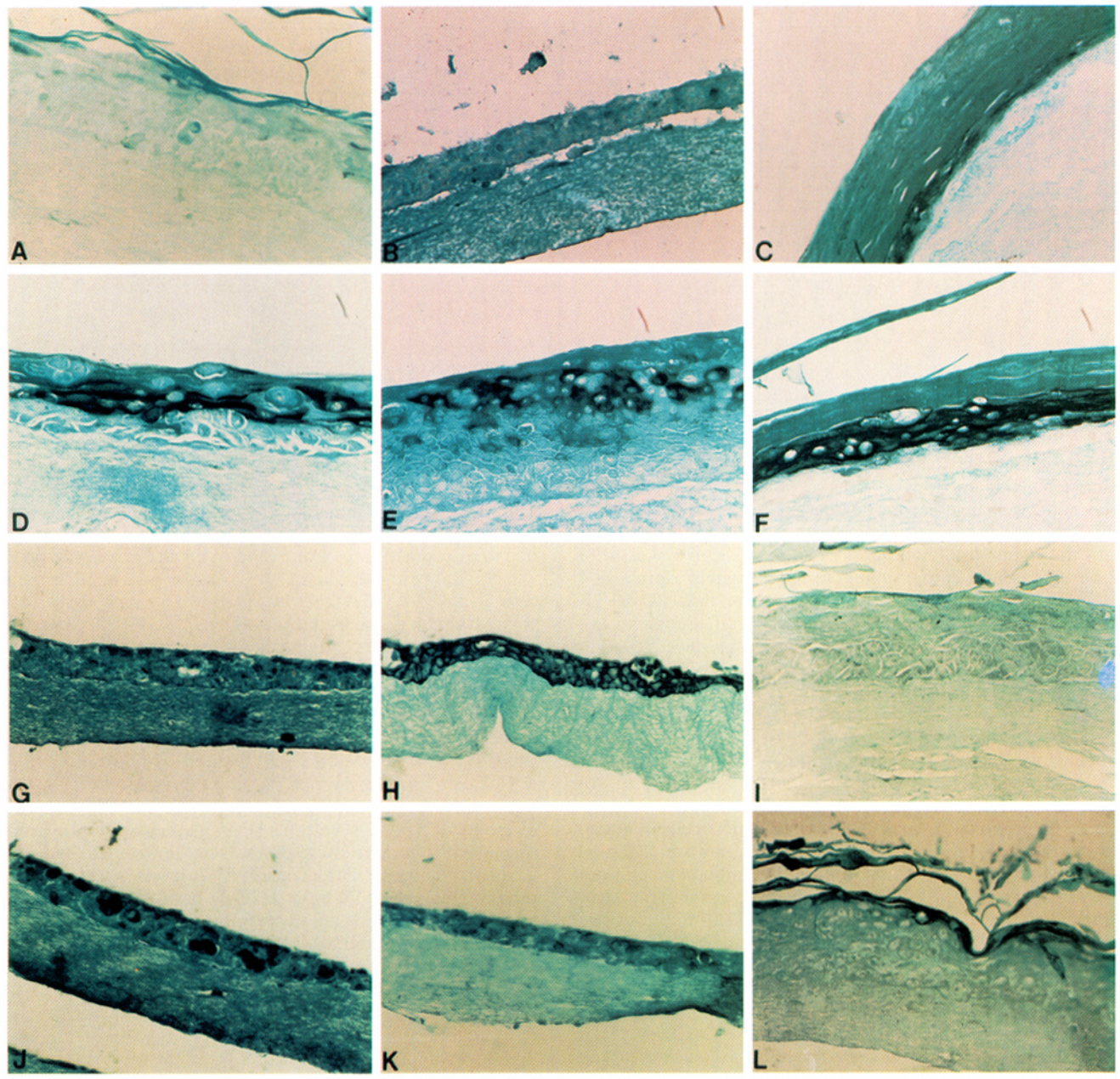
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