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Squamous Epithelial Hyperplasia and Carcinoma in Mice Transgenic for the Human Papillomavirus Type 16 E7 Oncogene

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The human papillomavirus type 16 (HPV-16) genome is commonly present in human cervical carcinoma, in which a subset of the viral genes, E6 and E7, are expressed. The HPV-16 E6 and E7 gene products can associate with and inactivate the tumor suppressor proteins p53 and Rb (the retinoblastoma susceptibility gene product), and in tissue culture cells, these viral genes display oncogenic properties. These findings have led to the hypothesis that E6 and E7 contribute to cervical carcinogenesis. This hypothesis has recently been tested by using transgenic mice as an animal model. HPV-16 E6 and E7 together were found to induce cancers in multiple tissues in which they were expressed, including squamous cell carcinoma, the cancer type most commonly associated with HPV-16 in the human cervix. We have extended these studies to investigate the in vivo activities of HPV-16 E7 when expressed in squamous epithelia of transgenic mice. Grossly, E7 transgenic mice had multiple phenotypes, including wrinkled skin that was apparent prior to the appearance of hair on neonates, thickened ears, and loss of hair in adults. In lines of mice expressing higher levels of E7, we observed stunted growth and mortality at an early age, potentially caused by an incapacity to feed. Histological analysis demonstrated that E7 causes epidermal hyperplasia in multiple transgenic lineages with high penetrance. This epithelial hyperplasia was characterized by an expansion of the proliferating compartment and an expansion of the keratin 10-positive layer of cells and was associated with hyperkeratosis. Hyperplasia was found at multiple sites in the animals in addition to the skin, including the mouth palate, esophagus, forestomach, and exocervix. In multiple transgenic lineages, adult animals developed skin tumors late in life with low penetrance. These tumors arose from the squamous epithelia and from sebaceous glands and were characterized histologically to be highly differentiated, locally invasive, and aggressive in their growth properties. On the basis of these phenotypes, we conclude that HPV-16 E7 can alter epithelial cell growth parameters sufficiently to potentiate tumorigenesis in mice.

Human papillomaviruses (HPVs) are small DNA viruses that infect various epithelial tissues, including the epidermis and epithelial linings of the upper respiratory system and anogenital tract. A subset of anogenital HPVs, the high-risk HPVs, which include HPV type 16 (HPV-16) and HPV-18, are associated with more than 90% of cervical carcinomas, a leading cause of death by cancer among women worldwide (for a review, see reference 52). In these cancers, the papillomavirus DNA genome is commonly integrated into the host chromosome (45, 51), and a subset of viral genes, E6 and E7, are expressed. In fact, the integration of HPV-16 DNA in cervical epithelial cells leads to the increased expression of E6 and E7, a consequence at least partially due to altered stability of E6 and E7 mRNAs (28), and this correlates with a selective growth advantage of these cells (28). Thus, E6 and/or E7 are likely to be important in the development of cervical carcinoma.

An abundance of data supports the hypothesis that E6 and E7 are oncogenes. Many investigators have demonstrated E6 and E7 genes from the high-risk HPVs to be oncogenic in tissue culture; they act independently or synergistically to potentiate the immortalization of multiple cell types, including human foreskin keratinocytes and cervical epithelial and mammary epithelial cells (4, 12, 20, 25, 29, 42), and E7 cooperates with an activated *ras* oncogene to transform baby rat kidney or human cervical epithelial cells (7, 9, 33, 41). E6 and E7 have been shown to associate with the cellular tumor suppressors p53 and Rb (the retinoblastoma susceptibility gene product), respectively (13, 15, 35, 50). Interaction of E7 with Rb disrupts Rb's capacity to bind and functionally inactivate the cellular E2F transcription factor (5, 40). The E6 protein binds the tumor suppressor p53 (50) and induces its proteolysis (44) through a ubiquitin-dependent protein degradation pathway (26, 27). These activities of E6 and E7 are likely to contribute to carcinogenesis in vivo, since it has been observed that in HPV-positive cervical cancers, there is a reduced likelihood for the presence of functionally inactivating mutations in Rb and p53 (8, 43).

Our interest lies in understanding the individual contributions of E6 and E7 in carcinogenesis. Through the use of transgenic mouse technology, we and others have demonstrated the carcinogenic properties of high-risk HPV E6 and E7 in vivo (1–3, 16, 17, 19, 24, 30, 46), including the capacity for HPV-16 E6 and E7 together to induce squamous cell carcinomas, the form of cancer most commonly associated with anogenital papillomaviruses (31). In this study, we have sought to define the role of HPV-16 E7 in the development of squamous cell carcinomas. HPV-16 E7, when expressed independently of E6, could induce epithelial hyperplasia in the lenses and retinas of transgenic mice that was associated with aberrant apoptosis; the combination of these two processes led to the near-complete ablation of cells within these tissues in the adult mouse (24, 38). The presence of E6 and/or p53-null genotype partially rescued the aberrant apoptosis phenotype, and this correlated with an increased propensity for tumorigenesis in these ocular

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sites (24, 38, 39). Thus, in these epithelial tissues, E7 could contribute to carcinogenesis only under conditions in which its induction of apoptosis could be inhibited.

These latter in vivo studies predict that E7 will have a severe effect when expressed alone in stratified squamous epithelia, the natural target for papillomaviruses and the precursor cell in HPV-associated cervical carcinoma. Stratified squamous epithelia contain multiple layers of cells with distinct differentiative properties. A single-cell layer at the base of the tissue, the stratum basale, is composed of undifferentiated cells attached to a basement membrane. Only cells within the stratum basale can initiate cell division. A papillomavirus infection is thought to initiate through the infection of a cell within the stratum basale. As cell division occurs, daughter cells can lose their attachment to the basement membrane and migrate into the suprabasal layers, the stratum spinosum, the stratum granulosum, and finally the stratum corneum, during which the cells progressively undergo differentiation leading to the formation of squames. The properties of stratified squamous epithelia differ from those of the epithelia in the mouse retina and lens, making it difficult to accurately predict E7 effects in the former on the basis of its demonstrated effects in the latter.

To address the properties of E7 in stratified squamous epithelia, we generated and characterized transgenic mice containing the hK14HPV16E7 transgene, in which expression of E7 is directed from the human keratin 14 (K14) promoter, a promoter that is restricted in its activity to the stratum basale. Use of the K14 promoter permitted us to direct expression of E7 to the differentiated cell type thought to be the precursor for subsequent HPV-associated tumor development. Multiple independent lineages of mice expressing E7 only exhibited epithelial hyperplasia with high penetrance, and a fraction of these animals developed skin tumors late in life. The importance of these findings is considered in light of the potential role of E7 in the normal life cycle of papillomaviruses and its role in human cervical carcinogenesis.

MATERIALS AND METHODS

Construction of transgene and generation of transgenic mouse lineages. Plasmid pK14HPV16E7 was constructed such that the human K14 promoter was linked to HPV-16 sequences from nucleotides (nt) 79 to 883 encompassing the tandemly positioned E6 and E7 genes. pK14HPV16E7 was constructed by inserting the 814-bp *Bam*HI fragment from plasmid pαAcryHPV16E6TTL/E7 (38) into the unique *Bam*HI site in plasmid pG3Z-K14 (49). The construct was sequenced to verify authenticity of the E6 and E7 open reading frames (ORFs), the presence of the translation termination linker (TTL) in E6TTL, and the junction of the K14 promoter to the HPV sequences. For microinjection of the transgenes, this recombinant plasmid was digested with restriction enzymes *Hin*dIII and *Eco*RI, which released a 3.2-kb fragment containing the K14 promoter/enhancer region, HPV E6TTL and E7 genes, and the K14 3′ untranslated region and polyadenylation signals. The fragment was purified by gel electrophoresis as described by Griep et al. (18) and microinjected as described previously (18, 23) by the University of Wisconsin Biotechnology Center's Transgenic Mouse Facility. Genomic DNA was prepared from tail biopsies of 18-day-old founder mice, and the transgene was detected by Southern blot analysis of *Bam*HI-digested tail DNA. The hybridization probe, an approximately 800-bp E6/E7 fragment generated from PCR amplification of a full-length HPV-16 clone by using oligonucleotides 1 (5′-GGCGGATCCTTTTATGCACCAAAAGAGAA CTG-3′) and 4 (5′-CCCGGATCCTACCTGCAGGATCAGCCATG-3′), was ^{32}P radiolabeled by using random primer labeling. To assess the copy number of each of the transgenic mouse lineages, 5 μg of genomic DNA from the founder and F_1 mice was digested with restriction enzyme *Nco*I, which cuts once within the transgene, and these DNAs were subjected to Southern analysis using the probe described above. Included in the Southern analysis was a series of copy number reconstruction standards containing 1, 5, 10, and 25 pg of digested pK14HPV16E6/E7 (a plasmid similar to pK14HPV16E7 but not containing the TTL in E6) plasmid DNA, equivalent to 1, 5, 10, and 25 copies of transgene per diploid cell. The blot was quantitated with a Molecular Dynamics PhosphorImager. The multiple lineages of K14HPV16E7 mice were bred and maintained in the American Association for Accreditation of Laboratory Animal Care-approved McArdle Laboratory Cancer Center Animal Care Facility, and all offspring were screened for transgene status by Southern analysis.

Analysis of E6/E7 expression by RNA PCR. Skin and papilloma samples were collected and frozen on dry ice. The frozen samples were pulverized and immediately added to guanidinium isothiocyanate. Total RNA was prepared as described by Chirgwin et al. (6). Contaminating DNA was removed by digestion with RNase-free DNase, and the RNA samples were quantitated by UV spectroscopy. To verify the expression of E6/E7 RNA, reverse transcription coupled to PCR-mediated amplification (RT-PCR) was performed as previously described (17), using 1 μg of total cellular RNA from samples. Briefly, the HPV-16-specific cDNA was synthesized by avian myeloblastosis virus reverse transcriptase, using oligonucleotide 2 (5′-CGGGGATCCTTACAGCTGGGTTTCT CTACG-3′; with a sequence complementary to HPV-16 nt 554 to 578) as the DNA primer, and then amplified by 35 cycles of PCR using oligonucleotides 1 and 2. The samples were subjected to RNase A treatment prior to agarose gel electrophoresis and Southern blot analysis using the E6/E7-specific, ^{32}P-radiolabeled probe described above.

Quantitative RT-PCR was performed as described previously (17), using RNA isolated from skin and papillomas from 3-day-old and/or adult (>6-month-old) mice. HPV-16-specific cDNA was synthesized by avian myeloblastosis virus reverse transcriptase from 1 μg of total cellular RNA primed with oligonucleotide 2. A total of five threefold serial dilutions were prepared from each cDNA sample ranging from 81 to 0.33 ng of input RNA per reaction, and the diluted samples were subjected to PCR using oligonucleotides 1 and 2 as primers and including [α-^{32}P]dCTP as one of the substrates. The radiolabeled products were separated by polyacrylamide gel electrophoresis, and the dried gel was exposed to a Molecular Dynamics PhosphorImager screen. The amount of label in the 298-bp E6*E7 (the asterisk denotes splicing) mRNA-derived PCR product was quantitated by using a Molecular Dynamics PhosphorImager and ImageQuant software. Expression of RNA in various tissues was determined relative to the levels of expression in line 2304 adult skin. Each PCR sample was run in duplicate, and the experiment was repeated multiple times. The relative differences in RNA quantity between the multiple lines were reproducibly observed in the three experiments.

Immunohistochemical analysis of skin. Skin samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C, transferred to PBS, embedded in paraffin, and cut into 5-μm sections. Serial sections were used for staining with hemotoxylin and eosin (H&E) and immunohistochemical staining for proliferating cell nuclear antigen (PCNA), apoptosis, or cytokeratin expression.

Paraffin sections were analyzed for the extent of cell proliferation by using mouse monoclonal antibodies directed against PCNA. The sections were deparaffinized in xylene and rehydrated by brief submersion in a series of aqueous/alcohol solutions of decreasing alcohol content. Endogenous peroxidase activity was destroyed by treatment in 3% H_2O_2 in H_2O for 30 min and washed in PBS. The samples were blocked for 30 min at ambient temperature with a goat anti-mouse immunoglobulin G F(ab) fragment (catalog no. 115-007-003; Jackson ImmunoResearch Laboratories, Inc.) diluted 1:240 in 5% normal goat serum (NGS)–1% bovine serum albumin (BSA)–PBS. After washes in PBS, the samples were incubated for 3 h at ambient temperature with a PCNA monoclonal antibody (catalog no. 1170 406; Boehringer Mannheim Biochemicals) diluted 1:200 in 5% NGS–1% BSA–PBS. Following further washes in PBS, the samples were incubated for 30 min at ambient temperature with a peroxidase-conjugated, affinity-purified $F(ab')_2$ goat anti-mouse antibody (catalog no. 115-036-071; Jackson ImmunoResearch) diluted 1:250 in 5% NGS–1% BSA–PBS. After washes in PBS, the immunohistochemical staining was developed by incubating the samples in 3,3′-diaminobenzidine tetrahydrochloride for 5 min. The chromagen was quenched by submersion of the samples in H_2O; the sections were counterstained with fast green, dehydrated through a series of aqueous/alcohol solutions of increasing alcohol content and xylene, and coverslipped. The slides were examined by light microscopy and photographed with Kodak Ektachrome T160 film.

Paraffin-embedded sections were also analyzed for the presence of DNA fragmentation by using an in situ apoptosis detection kit (Apoptag; Oncor catalog no. S7110-KIT, fluorescein). The procedure was carried out as instructed by the manufacturer except that the time of incubation of samples with the fluorescein-conjugated antidigoxigenin antibody was increased to 1 h. The slides were examined by UV microscopy and photographed with Kodak Ektachrome P1600 film.

Frozen sections were analyzed for hyperproliferation by using K10 antibodies. Slides were brought to ambient temperature and fixed in 4% paraformaldehyde in PBS for 30 s. Following a PBS wash, the endogenous peroxidase activity was destroyed by incubation in 0.3% H_2O_2 in 30% methanol for 30 min at ambient temperature, and the slides were washed again in PBS. The samples were blocked with a goat anti-mouse immunoglobulin G F(ab) fragment (catalog no. 115-007-003; Jackson ImmunoResearch) diluted 1:240 in 5% NGS–1% BSA–PBS. After a 30-min incubation, the samples were washed in PBS. An anti-cytokeratin 10 monoclonal antibody (clone RSKE 60; ICN) was diluted 1:10 in NGS-BSA-PBS for 3 h at ambient temperature and washed in PBS. The samples were incubated for 30 min with a 1:250 dilution of peroxidase-conjugated affinity purified $F(ab')_2$ goat anti-mouse immunoglobulin G antibody (catalog no. 115-036-071; Jackson ImmunoResearch) in NGS-BSA-PBS. After a PBS wash, the samples were developed in 3,3′-diaminobenzidine tetrahydrochloride for 5 min, quenched in H_2O, and counterstained.

Sequence analysis of Rb and p53 genes. Tumors were collected from four K14HPV16E7 lineages, and total cellular RNA was isolated as described previously (6). RT-PCR was performed on 2 μg of RNA. Rb-specific cDNAs were synthesized by using oligonucleotides Rb-2 and Rb-4. Amplification was performed with oligonucleotide pairs Rb-2–Rb-1, Rb-4–Rb-3, and Rb-5a–Rb-6. p53-specific cDNA was synthesized by using oligonucleotide p53-2. Amplification was performed with oligonucleotide pairs p53-4–p53-1 and p53-2–p53-3. The oligonucleotides used for the generation of the Rb- and p53-specific cDNAs have been described previously (31). The products were RNase A treated, electrophoresed on a 2% agarose gel, and purified. A 4-fmol portion of each sample was sequenced by using a commercial kit (fmol DNA sequencing system; Promega Corporation) and incorporating [α-^{35}S]dATP into the products. Samples were electrophoresed on a 4% acrylamide–8 M urea–40% formamide gel. The gel was dried and exposed to film. Sequences were read for the appearance of partial or complete base substitutions in comparison to wild-type Rb and p53 samples that were run in parallel.

RESULTS

Generation of multiple lineages of K14HPV16E7 transgenic mice. To direct expression of HPV-16 E7 to squamous epithelia, a DNA fragment containing HPV-16 sequences from nt 79 to 883, which includes the ORFs for both the E6 and E7 oncogenes, was cloned into pG3Z-K14 to generate plasmid pK14HPV16E7. In this cloning, we included the HPV-16 sequences within the E6 ORF, as they contain one 5′ (nt 226) and two 3′ (nt 409 and 526) splice signals used in the generation of alternatively spliced, mature E7-specific mRNAs from the virus's own P_{97} promoter. However, only functional E7 protein can be synthesized from this DNA fragment because there is present an oligonucleotide that contains translational termination codons in all three ORFs, causing the premature termination of translation within the N terminus of the E6 ORF. A 3.2-kb DNA fragment from pK14HPV16E7 containing the HPV-16 DNA sequences positioned between the human K14 promoter and polyadenylation sequences was microinjected to produce transgenic mice. Founder candidates were screened for the presence of the pK14HPV16E7-derived DNA by Southern analysis of total genomic DNA extracted from tail biopsies (data not shown). Nine transgene-positive founder mice, hereafter referred to as K14HPV16E7 mice, were identified; six were germ line transgenic, one was non-germ line, and the other two were moribund at day 10 (Table 1). Southern analyses of tail DNAs from founder mice and their offspring (data not shown) indicated the presence of between 2 and 21 copies of the transgene (Table 1). In each germ line, all copies were integrated at a single locus, as judged from the conservation of the Southern hybridization patterns among all offspring.

Overt phenotypes of K14HPV16E7 transgenic mice. Overt phenotypes were noted in multiple K14HPV16E7 lineages (Table 1). In lines 2304, 2332, and 2350, a highly penetrant, wrinkled skin phenotype on the body and legs was apparent beginning at approximately postnatal day 3 (Fig. 1A) but less apparent once the animals developed a coat of hair. At approximately the same time, the ears of transgenic mice in multiple lines (2304, 2332, 2350, and others) took on a less translucent appearance associated with a thickening of the ear; this phenotype persisted into adult life (Fig. 1B). Older K14HPV16E7 adult mice took on a scruffy appearance associated with a less dense coat of hair (Fig. 1C). In addition, these mice often had a greasy appearance in which their hair was matted. Some animals in lines 2304, 2326, 2332, and 2341 developed tumors in the skin late in life (median age of onset, 10.6 months). The frequency of tumors was low, between 0 and 10% depending on the lineage (Table 1).

In one line of mice (2350), the transgene-hemizygous offspring were severely stunted in growth (Fig. 1D). This gross phenotype, apparent at days 3 to 28, correlated with a high mortality rate during the first 2 weeks of life. Only 2% of the line 2350 transgenic offspring survived this period, and the survivors remained stunted in growth during the first 2 months of life, though they acquired normal body weight and size as adults. No transgene-homozygous offspring of line 2350 have survived to weaning. In addition, at least two transgene-positive founder mice (confirmed to be positive for the transgene by Southern analysis) died between days 10 and 12 postbirth and had the same phenotypes of wrinkled skin, thick ears, and stunted growth as found for line 2350 (data not shown). Less severe cases of stunted growth were seen in other lines of transgenic mice (2304 and 2332), though in these lines it was evident only in transgene-homozygous offspring and at a low penetrance. At weaning, small bilateral cataracts were found in the eyes of mice in most transgenic lineages, as has been noted previously for transgenic mice expressing other exogenous genes from the human K14 promoter (2).

Expression of E7-specific mRNAs in the skin of K14HPV16E7 mice. To assess whether the K14 promoter faithfully directed expression of E7 to the epidermis, total cellular RNA isolated from skin samples from each line of K14HPV16E7 transgenic mice was analyzed by RT-PCR. Transgene-specific cDNA was synthesized by reverse transcription using oligonucleotide 2, subsequently amplified by *Taq* polymerase, using

TABLE 1. Phenotypes of K14HPV16E7 lineages

F_0 lineage	Copy no.	No. of mice exhibiting phenotype/total no. scored						
		Neonatal phenotype (≤1 mo)			Adult phenotype (>1 mo)			
		Cataracts	Thickened ears and/or snout	Wrinkled skin	Cataracts	Thickened ears and/or snout	Scruffy fur	Skin tumors
2304	2	25/30	22/30	5/30	30/30	27/30	22/30	3/30
2311[a]	ND[b]							
2319[a]	ND							
2326	2	3/48	0/48	0/48	9/48	0/48	6/48	2/48
2330	4–5							
2332	7–8	48/51	19/51	6/51	49/51	38/51	49/51	1/51
2333[c]	ND							
2341	2–3	0/18	0/18	0/18	0/18	0/18	0/18	0/18
2350	20–21	16/18	18/18	18/18	16/18	16/18	18/18	0/18

[a] Not expanded because of death of the founder.
[b] ND, not done.
[c] Lineage with non-germ line integrations.

FIG. 1. Neonatal and adult phenotypes of K14HPV16E7 mice. (A) Control (left) and transgenic (right) littermates at postnatal day 8. Note wrinkled skin on torso, legs, and jowls of transgenic animal. (B) Control (left) and transgenic (right) adult animals exhibiting thickened, less translucent ears. (C) Adult transgenic showing a paucity of fur around the snout and eyes. Also note scaliness on tail, ears, and feet as well as scruffy appearance. (D) Control (left) and transgenic (right) littermates at postnatal day 10. The transgenic mouse is stunted in growth.

oligonucleotides 1 and 2 (see Fig. 2A for relative positions of oligonucleotides on the HPV-16 genome), and then detected by E7-specific Southern analysis (Fig. 2B). An E7-specific product of 297 bp was detected in the skin of four of five lineages (one lineage was not tested). This product is of the size expected for the E6*E7 mRNAs spliced between HPV-16 nt 226 and 409, the splice event most commonly found in mature E7-specific mRNAs in HPV-16-positive cells. In addition, lower but detectable levels of the 480-bp cDNA arising from unspliced mRNAs and the 180-bp cDNA arising from the minor E6**E7 spliced mRNAs were noted (Fig. 2B). In situ hybridization analysis demonstrated transgene expression to be restricted to the epidermis, hair follicles, and sebaceous glands (data not shown).

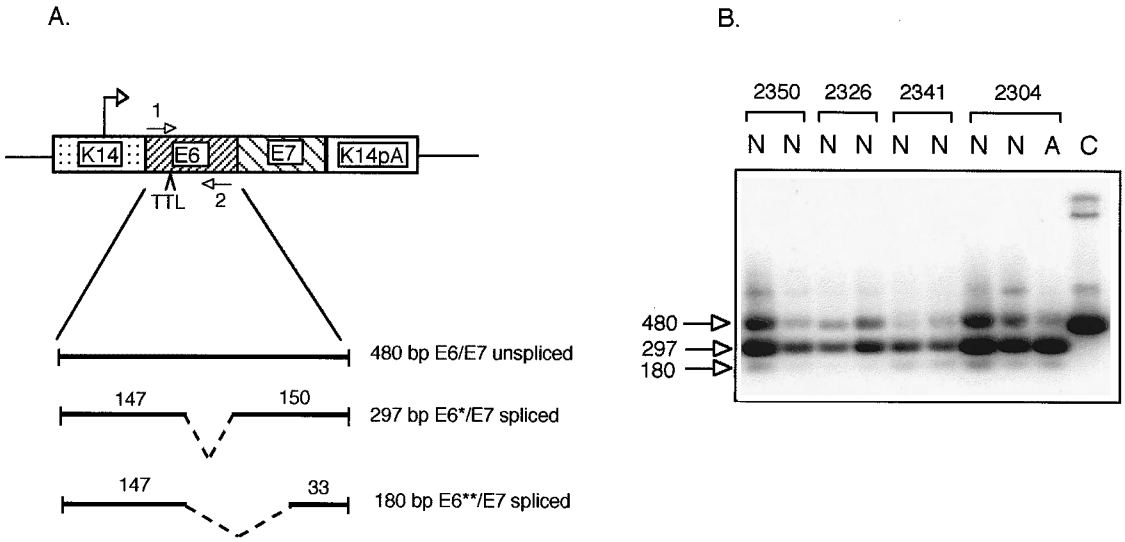

FIG. 2. Expression of HPV16E7 in transgenic mice. (A) Predicted sizes of E7 as determined by RT-PCR analysis. A schematic representation of the transgene is depicted. K14, human keratin 14 promoter/enhancer; E6 and E7, HPV-16 E6 and E7 ORFs; K14pA, human K14 polyadenylation sequences. The relative position of the TTL is indicated. Arrows 1 and 2 indicate positions at which oligonucleotides 1 and 2 anneal. The three lower lines represent the predicted sizes of PCR products from oligonucleotides 1 and 2 annealing to unspliced, E6*E7, and E6*E7 spliced messages. (B) Southern blot hybridization of RT-PCR products from K14HPV16E7 mouse skin. The predicted sizes of PCR products, shown in panel A, are indicated by arrows on the left. Four separate lineages (2350, 2326, 2341, and 2304) were analyzed. Lanes: N, neonate; A, adult; C, HPV-16 plasmid DNA control.

TABLE 2. Relative expression of E7 RNA in mouse tissue

K14HPV16E7 lineage	E7 RNA expression relative to that in line 2304 adult skin		
	Neonatal tissue (skin)	Adult tissue	
		Skin	Papilloma
2304	1.0	1.0	0.6–3.0
2326	0.07	ND[a]	ND
2341	0.09	ND	ND
2350	2.0	ND	ND

[a] ND, not determined.

To measure steady-state levels of transgene-specific mRNA, quantitative RT-PCR was performed as described previously (17). Lines 2350 and 2304 had the highest levels of E7-specific mRNA in skin samples, more than 14-fold higher that seen in the lowest-expressing line, 2326 (Table 2). Overall, the penetrance of phenotypes in the multiple lines of K14HPV16E7 mice (Table 1) correlated with levels of E7 gene expression (Table 2); lines 2304 and 2350, which displayed the multiple phenotypes described above, expressed E7 at higher levels than lines 2326 and 2341, which had less overt phenotypes. However, there is a lack of correlation between the high E7 RNA levels in the skin and the lack of skin tumors in line 2350; this is likely related to the fact that these mice are euthanized relatively early in adult life because of severe dyspnea. The underlying cause of this morbidity is currently being investigated.

E7 induces epithelial hyperplasia. To characterize further the consequences of E7 expression in squamous epithelia, multiple tissues from the K14HPV16E7 mice were analyzed histologically. At postnatal day 10, the squamous epithelia present in the skin, ears, upper digestive tracts, and forestomachs of K14HPV16E7 mice were abnormal, characterized by an increased thickness of the nucleated cell layer (compare Fig. 3A to C). The squamous epithelia in the transgenic skin (compare Fig. 3A and C) and forestomach (data not shown) were also highly invaginated, suggestive of a disproportionate lateral expansion of the epidermis with respect to the underlying connective tissue. This property correlated with the wrinkled skin phenotype (Fig. 1A). In adult mice, the epidermal hyperplasia was clearly evident on the ears (Fig. 5B). To assess the level to which thickening of squamous epithelia was due to an increased proliferative state, histological sections were subjected to immunohistochemical staining using a primary antibody specific for PCNA, a protein component of the cellular replicative apparatus specifically detected in the nucleus during late G_1 and S phases of the cell cycle (34). Whereas PCNA-positive cells are normally restricted to the stratum basale (Fig. 3B), a single layer of undifferentiated epithelial cells that make direct contact with the basement membrane separating the dermis from the epidermis, a more disorganized pattern of PCNA-positive cells was evident in the transgenic skin (Fig. 3D). A multitude of cells not in contact with the basement membrane were evidently undergoing proliferation. This expansion in PCNA-positive cells within the epidermis was observed in all neonatal skin sections analyzed from the K14HPV16E7 lines 2304, 2341, and 2350. Increased numbers and similar spatial patterns of PCNA-positive cells were also detected in the squamous epithelia at other sites in the K14HPV16E7 transgenic mice, including the forestomach and ears (data not shown). We conclude that E7 can induce hyperproliferation of epithelial cells in vivo and that this leads to both a lateral and a vertical expansion of the affected epithelium.

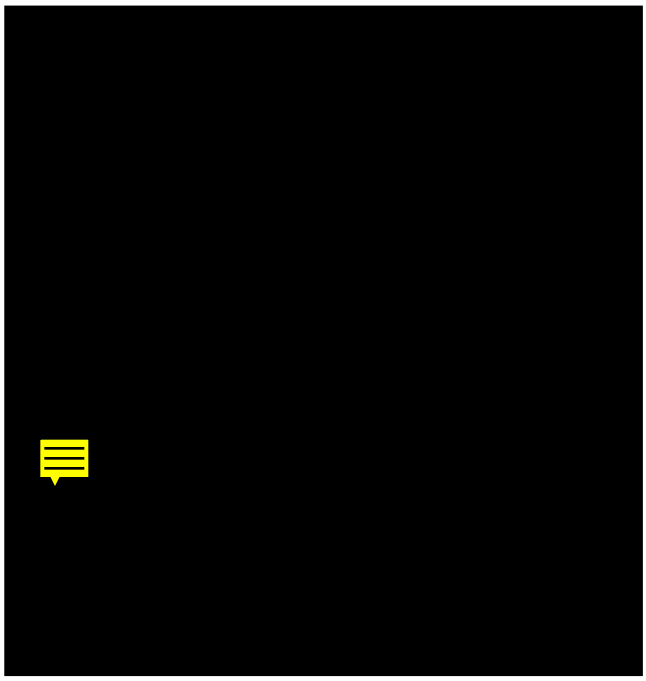

FIG. 3. PCNA and TUNEL analysis of 6-day-old nontransgenic and K14 HPV16E7 transgenic skin. All tissues were embedded in paraffin and serially sectioned. Nontransgenic skin was stained with H&E or PCNA (A and B) and with H&E or by TUNEL (E and F). Transgenic skin was stained with H&E or PCNA (C and D) and H&E or by TUNEL (G and H).

Differentiative properties of K14HPV16E7 skin. To assess the differentiative state of the cells that are undergoing aberrant proliferation in the epidermis, cryosections were subjected to K10-specific immunohistochemical staining. K10 is expressed selectively in the stratum spinosum of the epidermis but not in the stratum basale (14). The epidermis of the transgenic skin showed a vertical expansion in K10-positive staining (Fig. 4B). These findings suggest that while the E7 gene can induce aberrant epithelial hyperplasia, the cells remain sensitive to the spatial cues that induce epidermal differentiation within the suprabasal compartment of the epidermis. This finding is similar to that for the lens of αAcryHPV16E6TTLE7 mice (38), in which epithelial cells undergoing aberrant hyperplasia within the cortex of the lens expressed the β and γ crystallins, markers of terminal lens fiber cell differentiation (37).

HPV-16 E7 has recently been shown to induce aberrant apoptosis (38, 39). We therefore measured the numbers of cells in the transgenic epidermis exhibiting a hallmark of apoptosis, DNA fragmentation, that is associated with nuclear breakdown. This measurement was made by using the terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) method, in which 3′ ends of DNA within cells are labeled in situ with terminal transferase. Compared with skin from nontransgenic mice (Fig. 3F), there was an increase in the number of TUNEL-positive cells in the skin of transgenic mice (Fig. 3H). This increase was at least fivefold, as judged from direct counting of the number of TUNEL-positive cells within the epidermis in multiple frames from multiple independently stained sections of transgenic and nontransgenic skin. Interestingly, the majority of the TUNEL-positive cells in the E7 transgenic skin lay within the stratum granulosum, similar to that seen in the normal skin. The stratum granulosum is the compartment of the epidermis where nuclear breakdown nor-

FIG. 4. Expression of K10 in nontransgenic and K14HPV16E7 transgenic skin. Tissues from 6-day-old mice were coated in OCT compound and snap-frozen in liquid nitrogen, and sections were cut. Shown is the pattern of K10 staining in nontransgenic (A) skin and transgenic (B) skin.

mally occurs during terminal epidermal differentiation (14). Given this finding, we conclude that the increase in TUNEL-positive cells in the K14HPV16E7 epidermis reflects the increased rate of both growth and differentiation in the K14HPV16E7 transgenic epidermis and correlates with the presence of hyperkeratosis. It was also observed that in the majority of sections analyzed, the TUNEL-positive cells in the stratum granulosum of the transgenic skin were found in patches (Fig. 3H). The nonrandom distribution of TUNEL-positive cells in the K14HPV16E7 epidermis may reflect a lateral variability in the proliferative status within the underlying epidermis, as suggested by variability in the thickness of the epidermis in the transgenic skin, or variances in the presence of environmental cues required for nuclear degradation to occur in the epidermis. An increase in the number of TUNEL-positive cells was also detected in hair follicles (Fig. 3H) and sebaceous glands (data not shown) within the K14HPV16E7 skin. In sum, our findings demonstrate that the K14HPV16E7 mice exhibit an increase in the number of apoptotic-like cells within the skin. This finding corroborates the finding that E7 induces apoptosis in another epithelial cell type in addition to that found in the mouse lens (38).

Histopathology of skin tumors in adult K14HPV16E7 transgenic mice. Mice in multiple lineages of K14HPV16E7 mice developed skin tumors late in life (Table 1). Grossly, these tumors belong to two classes, relatively small (<1 cm) but highly keratotic lesions and large, cystic but nonkeratotic lesions. These tumors arose on the bodies and heads of the mice and appeared beginning at 9 months of age. Histological analyses indicated that the various types of tumor had different origins within the skin. The highly keratotic lesions represented low-grade squamous cell carcinomas which were relatively highly differentiated (Fig. 5D). Carcinomas of higher grade, characterized by the presence of poorly differentiated, more locally invasive cells, were also noted at low frequency. The large, cystic but nonkeratotic lesions represented aggressive sebaceous epitheliomas that were locally invasive and highly differentiated in character, giving rise to large accumulations of sebum within cysts (Fig. 5C). These cancers displayed a high proliferative index, as judged from PCNA immunohistochemistry (Fig. 6D). There was also a high number of TUNEL-positive cells, especially in the sebaceous gland epithelioma (Fig. 6H). We did not find any evidence for metastasis arising from these skin tumors in older animals, though most animals with skin tumors were euthanized within 1 month after onset of the primary tumors because of perceived discomfort. Five tumors were analyzed for the presence of mutations in the p53 and Rb genes by sequence analysis of RT-PCR

FIG. 5. Histological analysis of skin and skin lesions in K14HPV16E7 transgenic adult mice. All tissues were fixed in paraformaldehyde, embedded in paraffin, cut into sections, and stained with H&E. (A) Nontransgenic ear; (B) line 2350 transgenic ear; (C) sebaceous gland adenoma line 2304; (D) papilloma from line 2304. Magnification, ×5.

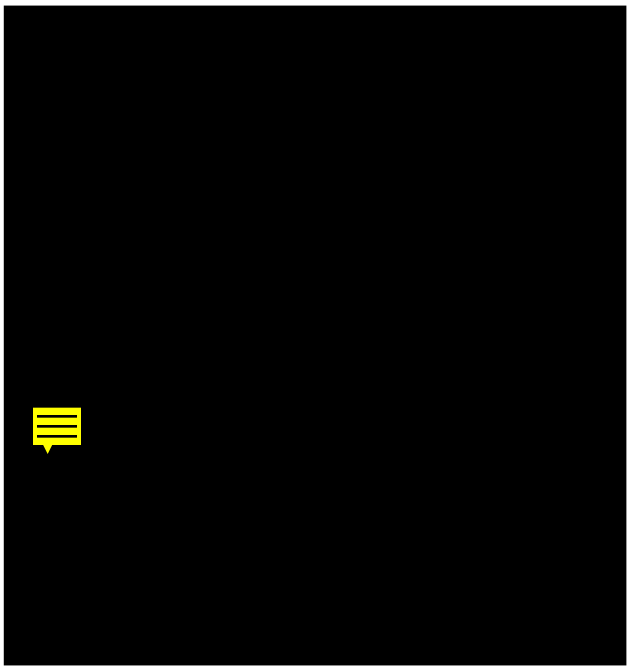

FIG. 6. PCNA and TUNEL analysis of adult nontransgenic and K14HPV 16E7 transgenic skin. All tissues were embedded in paraffin and serially sectioned. Nontransgenic skin was stained with H&E or PCNA (A and B) and with H&E or by TUNEL (E and F). Sebaceous gland tumor from an adult transgenic mouse stained with H&E or PCNA (C and D) and with H&E or by TUNEL (G and H).

products. No missense mutations could be identified in the portions of either gene known to be hot spots for such mutations in other tumors. Thus, our data demonstrate that E7, in the absence of either HPV-16 E6 or mutational inactivation of p53, can induce neoplasias in stratified squamous epithelia.

DISCUSSION

In this study, we have demonstrated the properties of the HPV-16 E7 oncogene when its expression is directed to the squamous epithelium in transgenic mice. E7 was found to efficiently induce epidermal hyperplasia. This correlated with a wrinkled skin phenotype and thickened ears. The epidermis of E7 transgenic mice also contained an increased number of cells with fragmented DNA, suggestive of apoptosis or apoptotis-like processes being induced by E7. This increased abundance of apoptotic cells may simply reflect the increased rate of turnover of epithelial cells within the K14HPV16E7 skin. Overall, the skin of the K14HPV16E7 mice bears many histopathological properties seen in HPV-induced cutaneous papillomas, including the presence of acanthosis and papillomatosis and an increased abundance of apoptotic cells localized in patches within the terminally differentiating compartment of the epidermis. Adult mice had a scruffy appearance; they had a thinning of the hair coat and often had a greasy appearance. Infrequently, adult mice in multiple lineages developed one of two types of skin tumors, low-grade squamous cell carcinoma or sebaceous gland epitheliomas. These data demonstrate that E7 is a potent inducer of squamous epithelial proliferation and potentiates tumorigenesis, further implicating HPV-16 E7 in human cervical carcinogenesis.

Role of E7 in natural infection. Expression of E7 led to an expansion of the proliferating compartment within the epidermis of transgenic mice. This result may provide insight into the potential role of E7 in the normal infective life cycle of the papillomaviruses. A papillomavirus infection is thought to take place initially though the adsorption of a virus particle to a cell within the stratum basale exposed to the surface of the skin by local trauma, followed by a clonal expansion of that cell such that daughter cells inherit papillomavirus genomes as extrachromosomal elements. While this clonally expanded population of undifferentiated cells is nonproductively infected, that is, they do not produce progeny virus, they nevertheless express a subset of the viral genes, the early genes, at low but detectable levels. E7 is one of the viral genes expressed. Eventually the infected cells undergo terminal differentiation as they rise through the epidermis, and during this process some fraction of the cells become productively infected; that is, they produce progeny virus. As a consequence of the polarized nature of the differentiating epidermis, the progeny virus is ultimately found on the surface of the papillomavirus lesion within the stratum corneum. Thus, the ultimate amplification of progeny virus within a papillomavirus infection relies not upon multiple rounds of reinfection but upon the expansion of a single, nonproductively infected cell within the stratum basale. Given that E7 is expressed in the undifferentiated cells within a papillomavirus lesion (11, 47) and that E7 induces epidermal hyperplasia within the mouse epidermis (this study), we conclude that E7 is likely to contribute to the initial expansion of nonproductively infected cells within a papillomavirus-induced lesion following the infection event. This hypothesis leads to several predictions. First, if accurate, our model posits that disruption of E7 function will reduce the severity of papillomavirus lesions. This is of clinical importance in such diseases as laryngeal papillomatosis, in which the rapid growth of the papillomavirus lesion within the upper respiratory tract sometimes necessitates frequent surgical intervention, as well as in cervical lesions, in which there exists the propensity for high-risk HPV-associated lesions to progress to carcinoma. Thus, anti-E7 drugs may be efficacious in treating such lesions. Second, one would predict that E7 must affect hyperplasia by more than the inactivation of the Rb gene product alone, given that HPVs that cause the more aggressively growing benign lesions in humans, such as the low-risk anogenital HPV-6 and HPV-11, encode E7 proteins that are demonstrably less efficient than HPV-16 E7 in binding to and inactivating this tumor suppressor protein (15, 36). Alternatively, the low-risk HPVs might rely more upon alternative viral activities than does HPV-16 to induce epidermal hyperplasia or, conversely, might be deficient for activities that in HPV-16 counteract epidermal hyperplasia, i.e., apoptosis. Experiments monitoring the activity of low-risk HPV E7 genes in the mouse epidermis will begin to distinguish between these possibilities.

Consequences of E7 gene expression in stratified squamous epithelia other than the epidermis. The mice in the K14HPV 16E7 transgenic lineage with the highest level of E7 expression were severely stunted in neonatal growth and in most cases did not survive beyond 10 days. In addition, two founder mice severely stunted in growth did not survive beyond 10 days. In all of these mice, we observed an absence of milk within the abdomen during the days preceding death and an absence of contents in the stomach upon necropsy. We believe that lack of feeding and reduced fluid intake contribute to the stunted growth and possibly to the high mortality within the first 2 weeks after birth. In this regard, we were able to increase survival of line 2350 offspring by injecting saline solution subcutaneously to reduce dehydration. Histological analysis of the digestive tract suggests that severe epithelial hyperplasia within the digestive tract, including the mouth, esophagus, and fore-

stomach (data not shown), may have contributed to an absence of feeding by these animals. We observed that the degree of hyperplasia and hyperkeratosis in the squamous epithelial compartments of the transgenic mice is most severe at an early age and reduced overall in severity in adults (data not shown). Interestingly, animals in line 2350 that survived beyond the 10-day period grew to normal size and body weight and were competent for breeding, suggesting that the underlying causes for stunted growth and neonatal mortality were transient in nature.

The cell specificity of the human K14 promoter suggested that E7 might be expressed within the squamous epithelial lining of the exocervix in the K14HPV16E7 transgenic mice. We therefore performed histological analysis of this tissue from older mice. Moderate squamous epithelial hyperplasia was noted in the exocervix in multiple adult K14HPV16E7 females. In addition, we noted the presence of hyperplasia with individual cell keratinization, a potential indicator of premalignancy. Examples of focal cystic hyperplasia and one example of squamous metaplasia were observed. In addition, a large mass compatible with a uterine stromal sarcoma was identified in a 16-month-old K14HPV16E7 mouse. While these histopathological properties are all remarkable, we are cautious about interpreting these findings since we evaluated only eight cervixes from the K14HPV16E7 mice. Nevertheless, these findings suggest that the K14HPV16E7 transgenic mice may provide a valuable animal model for evaluating directly the individual and combinatorial activities of E6 and E7 in cervical carcinogenesis.

Comparison with other animal models for HPV-16-associated carcinogenesis. Several laboratories have previously achieved expression of papillomavirus genes in the epidermis (2, 16, 31, 46). In those studies, the transgenic mice carried either the complete early region of HPV-16 (2, 46) or both E6 and E7 (16, 31). We previously demonstrated that the ectopic expression of both HPV-16 E6 and HPV-16 E7 in the skin of one HPV-16 transgenic mouse lineage, line 19, can induce squamous cell carcinomas in 25% of the adult mice (31). This heightened incidence of squamous cell carcinomas in the line 19 mice compared with the K14HPV16E7 mice characterized in the present study likely reflects the fact that E6 in addition to E7 is ectopically expressed in the skin of these transgenic mice. Supporting this view is our observation that K14HPV16E6/E7 mice, in which we have directed expression of both E6 and E7 from the K14 promoter, also have an increased incidence of squamous cell carcinomas compared with the K14HPV16E7 mice described in this study (22). Greenhalgh et al. (16) also directed expression of HPV-16 E6 and E7 together to the epidermis, though behind a suprabasal layer-specific K1 promoter. Their mice also exhibited epidermal hyperplasia and, at lower frequencies, the incidence of papillomas. The absence of frank carcinoma in the latter mice may reflect the more differentiated character of the cells to which expression of the transgenes was directed. Alternatively, it may reflect, as the authors of the study postulated (16), a selective expression of E7 from their transgene construct.

Unlike with our studies (references 22 and 31 and this study), Arbeit et al. directed expression of the entire early region of HPV-16 to the epidermis by using the same K14 promoter but failed to detect squamous cell carcinomas (2). We believe that this deficit may reflect two critical differences between their study and ours. First, we chose to perform all of our studies with the FVB/N strain of mice, a derivative of Swiss mice. As predicted by previous studies on the parental strain (10), we and others have found the FVB/N strain to be highly sensitive to skin carcinogenesis (21, 32). In contrast, Arbeit et al. (2) performed their HPV-16 transgenic analyses in C57BL/6 and BALB/c backgrounds, two strains noted for their low sensitivity to skin carcinogenesis (10). Second, we chose to clone behind the human K14 promoter only the 5′ portion of the HPV-16 early region, the segment containing the E6 and E7 oncogenes that is selectively expressed in human cervical carcinoma and therefore of relevance for study in an animal model for this disease, whereas the entire early region and a portion of the late region of HPV-16 were used in the study by Arbeit et al. (2). This larger segment of the viral genome contains an mRNA instability element in the 3′ untranslated region that would be predicted to negatively influence transgene expression (28).

An interesting difference exists between the effects of HPV-16 E7 on different epithelial cell types in transgenic mice. When expressed in the lens and retina, E7 caused a concomitant induction of hyperplasia and programmed cell death that led ultimately to the near-complete ablation of these ocular tissues (24, 38). In contrast, the expression of E7 in the epidermis (this study), while also leading to hyperplasia with concomitant induction of apoptotis-like processes, did not disrupt tissue homeostasis to the point of causing tissue ablation. This difference may reflect differences in the sensitivities of different epithelial cell types to signals that induce programmed cell death. In this regard, E7 has been demonstrated to induce apoptosis in the mouse lens through both p53-dependent and p53-independent pathways (39). Cells also possess factors such as the Bcl2 family of proteins (48) that modulate their sensitivity to signals that induce apoptosis. Differences in levels of expression among epithelial cell types may dictate differences in sensitivities to E7-induced apoptosis. Regardless of the mechanisms underlying the different sensitivities of epithelial cells to E7, it is interesting that stratified squamous epithelium, the cell type normally host to papillomavirus infection, appears most capable of tolerating the effects of E7.

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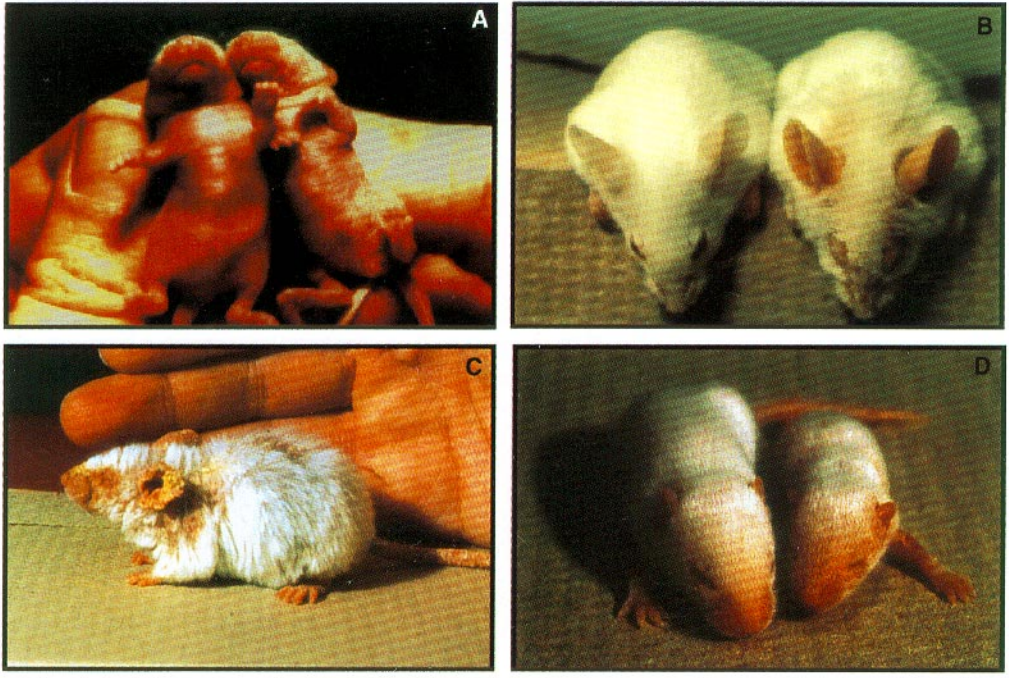
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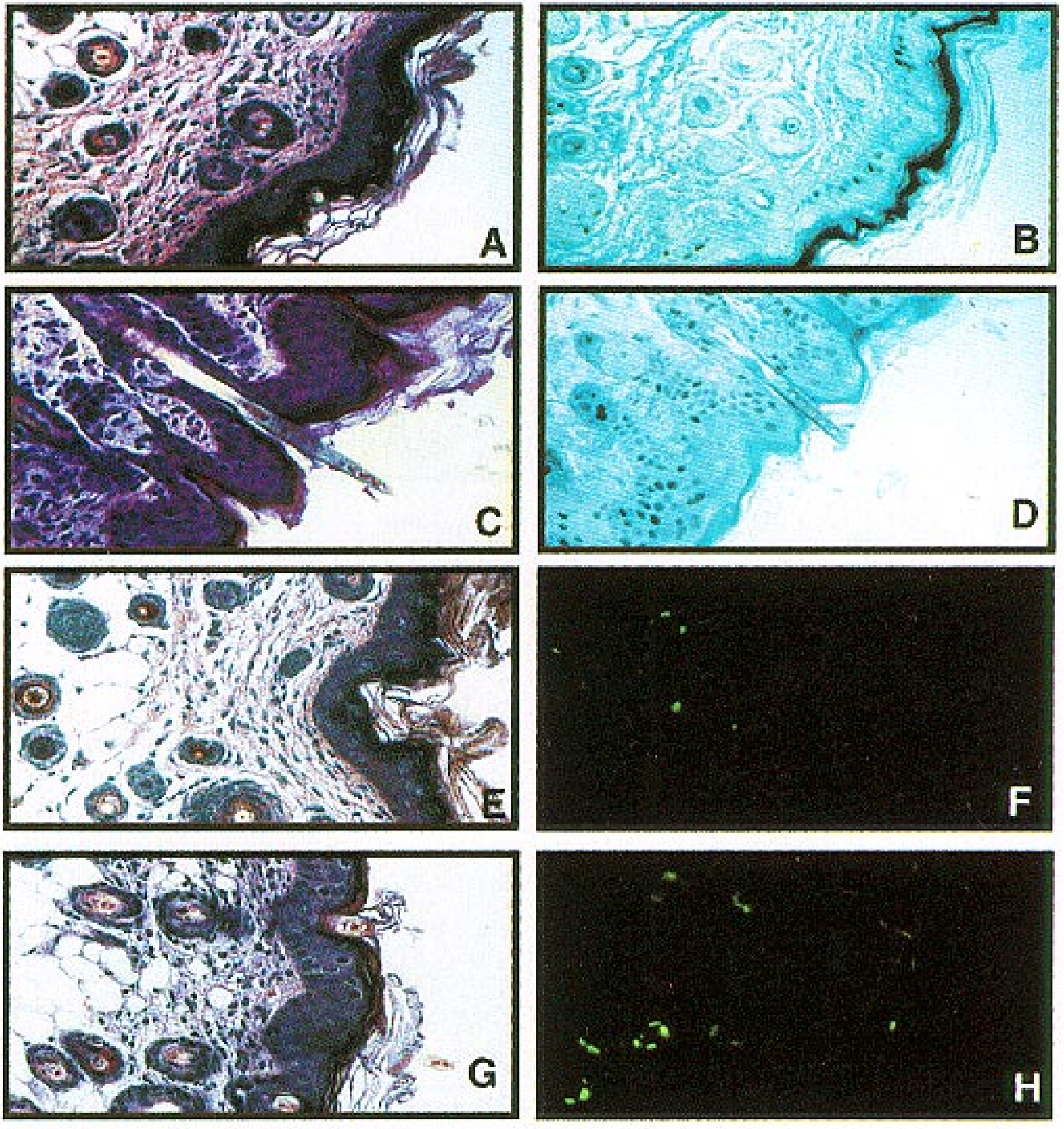
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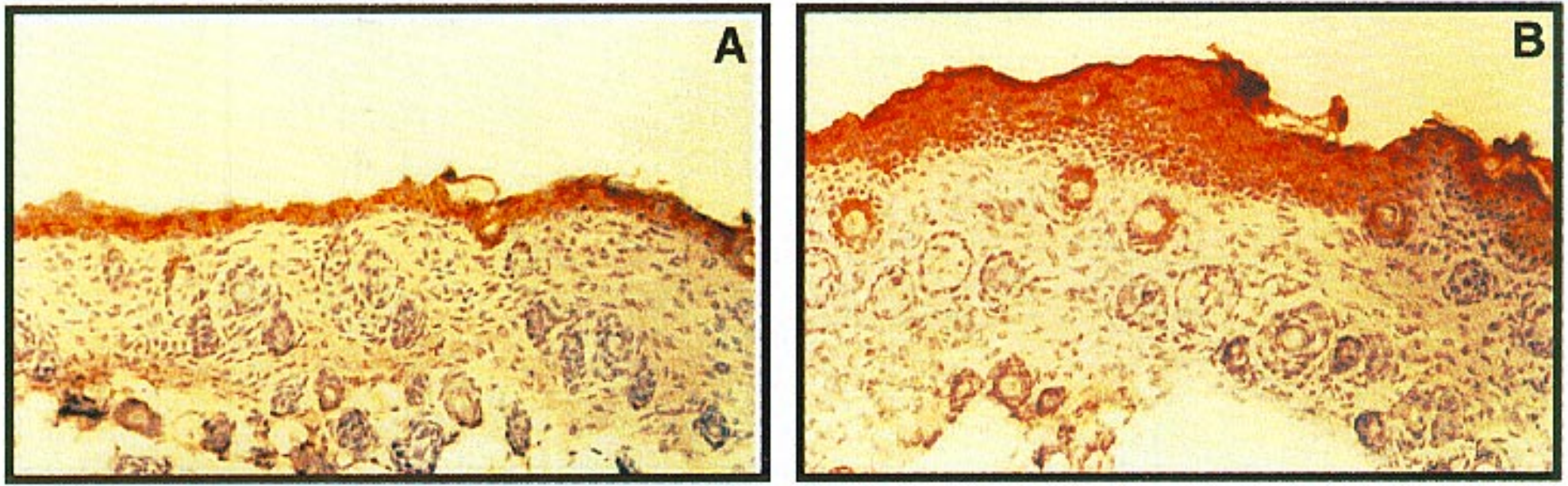
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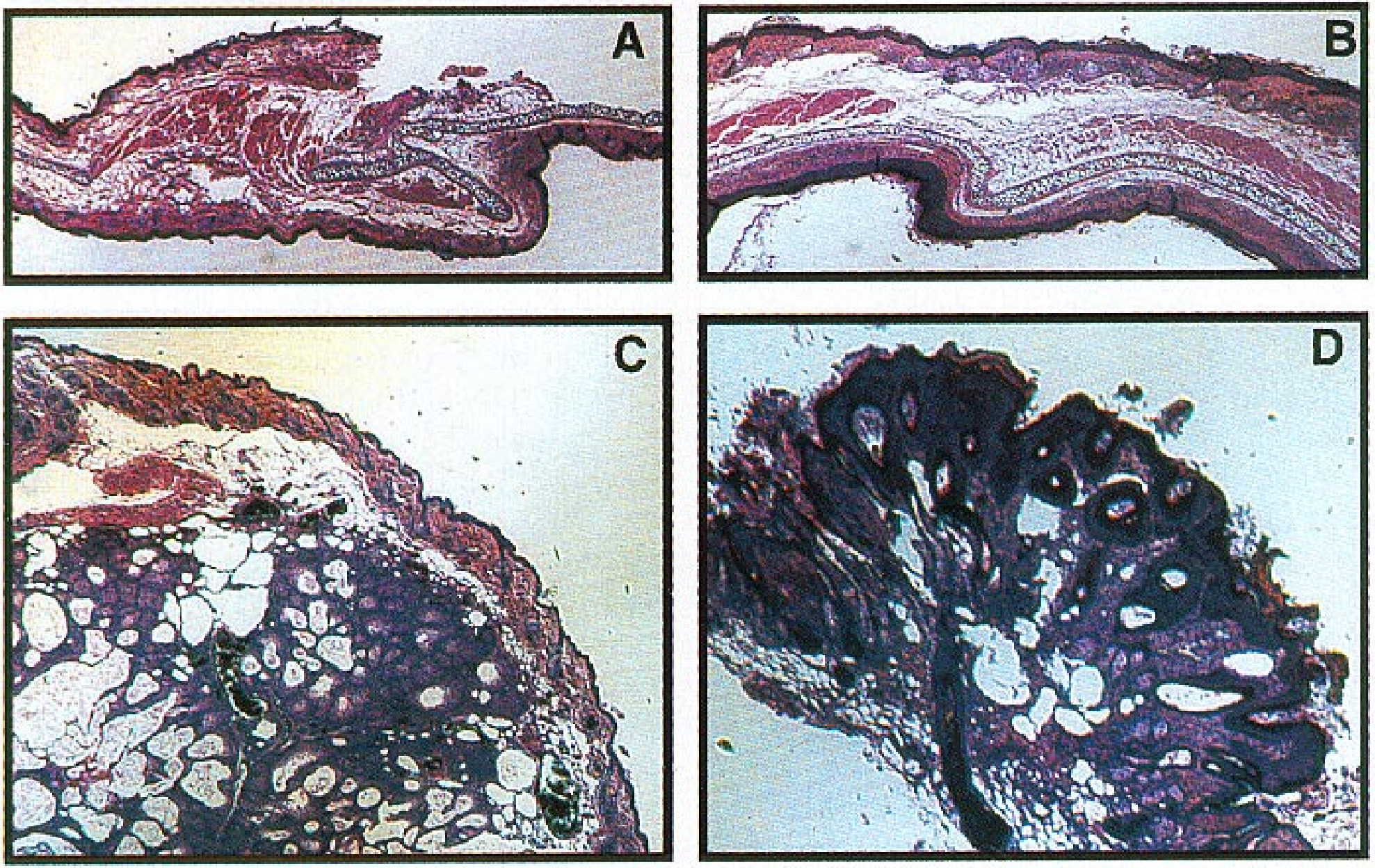
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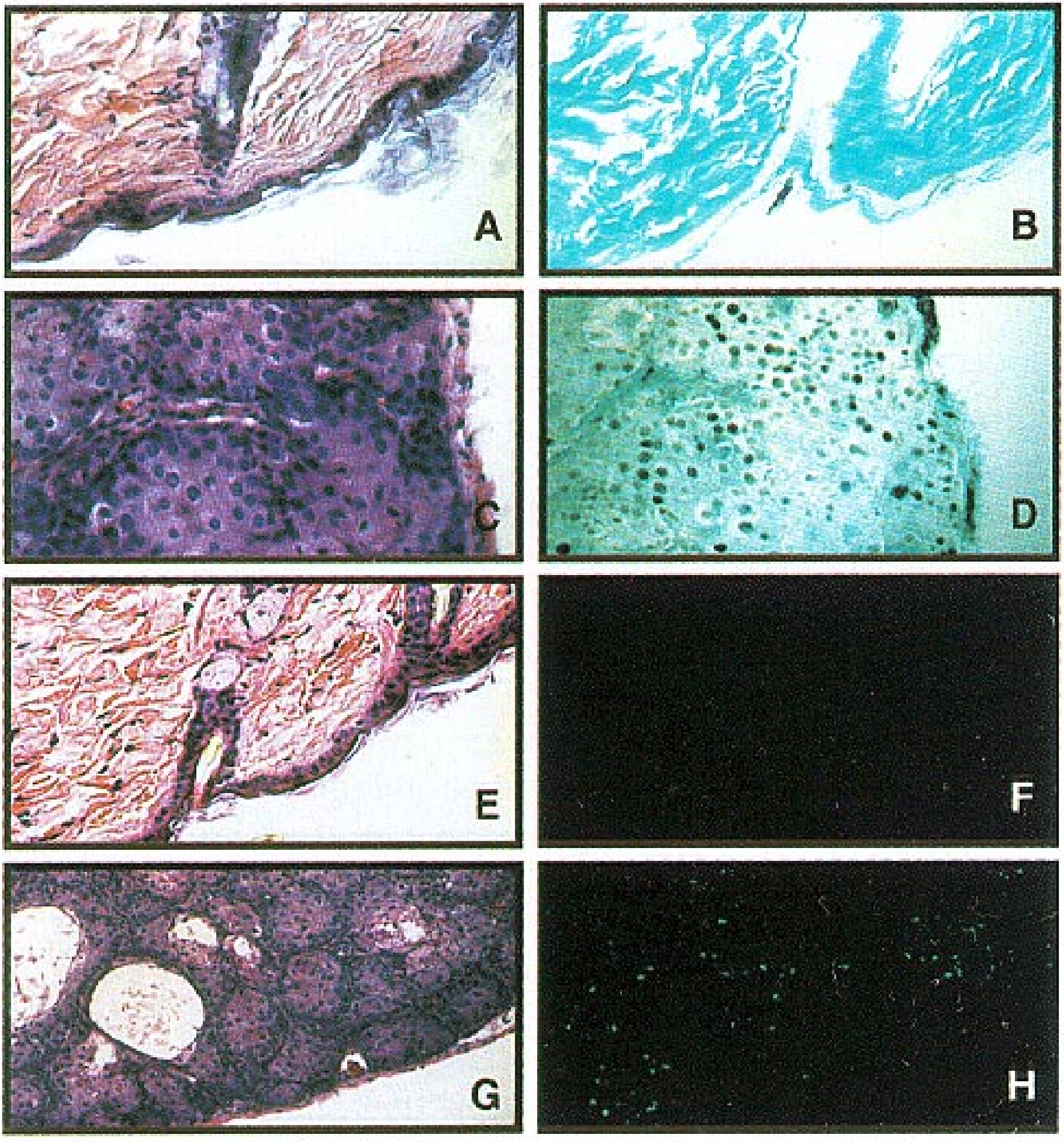
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