Malignant Human Papillomavirus Type 16-transformed Human Keratinocytes Exhibit Altered Expression of Extracellular Matrix Glycoproteins

Nader Sheibani, John S. Rhim, and B. Lynn Allen-Hoffmann

Department of Pathology, University of Wisconsin, Madison, Wisconsin 53706 [N. S., B. L. A.-H.], and Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, Maryland 20892 [J. S. R.]

ABSTRACT

We found that keratinocytes immortalized with human papillomavirus (HPV) type 16 DNA and malignant converted by H-ras transfection (HPK-1A/ras) exhibit an enhanced ability to synthesize a fibronectin-containing extracellular matrix. Gene expression of fibronectin and thrombospondin was increased in tumorigenic keratinocytes compared to control and immortalized keratinocytes, 6- and 9-fold, respectively. Increased production of soluble and cell surface-associated fibronectin was not specific for HPV 16 transformed keratinocytes. Ad12-SV40-immortalized keratinocytes malignantly converted by H-ras transfection (RHEK-1/ras) also exhibited enhanced expression of fibronectin and thrombospondin, as well as pro-α1 type I collagen. Steady state mRNA levels for autocrine growth-regulatory factors, transforming growth factors α and β1, were increased in Ad12-SV40 but not HPV 16-transformed human keratinocytes. We then determined whether increased production of fibronectin was associated with aberrant differentiation of transformed keratinocytes. Less than 10% of the HPV 16-transformed cells produced cornified envelopes after suspension-induced differentiation compared to 70% of normal keratinocytes. However, immortalization by HPV 16 DNA was sufficient to confer a differentiation-defective phenotype. Both involucrin mRNA and protein levels were decreased 8-fold in HPV 16-immortalized keratinocytes compared to normal cells and malignant conversion further attenuated involucrin levels. These studies demonstrate that aberrant differentiation is an early event in the transformation of the human keratinocytes and is not the result of enhanced expression of the extracellular matrix proteins. Unlike transformed fibroblastic cell types, up-regulation of fibronectin gene expression and matrix formation is a consistent characteristic of malignantly converted human keratinocytes.

INTRODUCTION

The growth pattern of human keratinocyte cultures can be altered by infection with several HPV types (1–4). Only those HPV types implicated in the induction of cervical cancer possess the capacity to immortalize human keratinocytes. Cultured human keratinocytes can be immortalized with HPV 16, 18, 31, and 33 DNA (5–9). Generally, HPV-immortalized human keratinocyte lines are not tumorigenic in nude mice. This observation is consistent with epidemiological evidence suggesting that infection by HPV types 16, 18, 31, and 33 alone is not sufficient for progression to genital malignancy. Genital HPV infections, irrespective of HPV type, are widespread with conservative estimates of 10–30% of the normal population being affected (10, 11). A long latent period between initial infection and development of cancer is characteristic of HPV-associated cervical carcinoma. Progression to malignancy is commonly associated with various environmental factors. Therefore, it is likely that development of HPV-associated cancer is a multistep process. Recent studies have demonstrated that both HPV 16 and HPV 18 sequences encoding the E6 and E7 gene products are sufficient to immortalize primary human keratinocytes in vitro (12, 13). The events involved in malignant conversion of HPV-infected cells are currently unknown.

In normal stratified squamous epithelia, keratinocytes which are capable of proliferation are located in the basal layer in contact with a basement membrane. Cells leave the basal (proliferating) layer and undergo a series of biochemical and phenotypic changes which constitute their program of terminal differentiation (reviewed in Refs. 14–16). Ultimately, these cells are shed from the outer surface as fully differentiated squames. There appears to be a hierarchy of signals for keratinocyte terminal differentiation. These include cell cycle withdrawal, increase in cell size, decreased substrate contact, and loss of gap junctional communication. Some of these events appear to be regulated by tyrosine kinases and changes in calcium concentrations (17, 18). However, the critical events directly associated with commitment of normal keratinocytes to terminal differentiation are currently not understood.

We are particularly interested in cell-matrix interactions that may be a signaling mechanism for differentiation in stratified squamous epithelia. There is abundant evidence that disruption of cell adhesion and cell communication are critical events in neoplastic transformation. Normal keratinocytes are resistant to malignant transformation by viruses alone, chemical carcinogens, or transfection with known oncogenes (19–24). Several groups have shown that human keratinocytes can be immortalized by DNA tumor viruses (5, 6, 23, 25–34). The immortalized cells can also be malignantly converted by transfection of the H-ras oncogene (24, 26, 27). The concomitant expression of transforming virus genes from viruses (HPV and the Ad12-SV40) and other transfected oncogenes or chemical carcinogens is necessary for malignant conversion. We have used normal human keratinocytes, HPV 16-immortalized human keratinocytes, previously described by Durst et al. (5), and the corresponding H-ras transfected cell line (27) representing discrete stages of the transformation process: immortalization and malignant conversion. In this paper we have examined the ability of both immortalized and tumorigenic HPV 16-transformed keratinocytes to respond to a cell type-specific differentiation signal. We have found that fibronectin and thrombospondin genes are differentially expressed in HPV 16-transformed human keratinocytes at different stages of transformation.

MATERIALS AND METHODS

Keratinocyte Lines. Keratinocyte lines used in this study are listed in Table 1. RHEK-1 cells are human keratinocytes infected with a Ad12-SV40 hybrid virus (23). RHEK-1/ras cells are RHEK-1 cells transfected with a plasmid expressing H-ras (24). HPK-1A cells are human keratinocytes transfected with HPV 16 DNA and possess two to three complete copies of the HPV genome arranged in a head-to-tail fashion, integrated at one chromosomal site (5). HPK-1A/ras cells are HPK-1A cells transfected with H-ras (27). NS-2-EP cells are a strain of normal...
human keratinocytes that was used for comparison in these studies.

Normal keratinocytes were established from newborn human foreskin and cultured in the presence of a mitomycin C-treated 3T3 feeder cells as described by Allen-Hoffman and Rheinwald (35) with the following modifications. The culture medium was composed of Ham's F-12:Dulbecco's modified Eagle's medium (3:1; 0.66 mM Ca^{2+}) supplemented with 2.5% fetal calf serum, 0.4 μg/ml hydrocortisone, 10 ng/ml cholera toxin, 5 μg/ml insulin, 24 μg/ml adenosine, and 10 ng/ml epidermal growth factor. All transformed keratinocyte lines were cultured in the same medium as the normal keratinocytes with the exception that these cells require neither epidermal growth factor nor a 3T3 feeder layer when passed at high density. Normal keratinocytes were used for experiments prior to passage 4. Transformed keratinocytes were passaged 1:10 every 4–5 days. Cultures were maintained at 37°C in a 5% CO2 atmosphere. For low Ca^{2+}-containing medium, Ca^{2+}-free Ham's F-12 and Ca^{2+}-free Dulbecco's modified Eagle's medium were mixed (3:1) and CaCl2 was added to a final concentration of 0.05 mM.

RHEK-1, RHEK-1/ras, and HPK-1A cell lines can form colonies at clonal density only with the aid of a mitomycin C treated 3T3 feeder layer. The HPK-1A/ras cell line can form colonies at clonal densities with or without a 3T3 feeder layer. The plating efficiency ranged from 60–90% for all lines.

Induction of Terminal Differentiation. To induce terminal differentiation, cells were remixed from tissue culture plates with trypsin/EDTA, washed with serum-free medium, and resuspended in growth medium made semisolid with 1.5% methylcellulose (4000 centipoises; Fisher Scientific, Fair Lawn, NJ) at a cell density of 10⁶ cells/ml in sterile 50-ml polypropylene tubes. The suspended cells were then incubated at 37°C in a humidified 5% CO2 atmosphere. Cells were recovered from suspension at various times by diluting semisolid growth medium with 10 volumes of serum-free medium followed by centrifugation at 1000 × g.

The proliferative capacity of keratinocytes following suspension in methylcellulose-containing medium was determined by replating the cells in surface culture. The cells recovered from methylcellulose were plated at a clonal density on mitomycin C-treated 3T3 feeder layers such that approximately 100 colonies were observed on a 60-mm tissue culture dish. The cultures were allowed to grow for approximately 2 weeks. The resulting colonies were fixed with formaldehyde and stained with methylene blue, and colonies containing 30 or more cells were scored.

Measurement of Cornified Envelopes. To measure cornified envelope production, cells were washed with PBS and resuspended in PBS at a density of 1–2 × 10⁶ cells/ml. Triplicate samples of the cell suspension were boiled in the presence of 1% sodium dodecyl sulfate and 20 mM dithiothreitol for 5 min. Detergent-resistant envelopes were counted under phase contrast optics using a hemocytometer. Values from triplicate samples differed by less than 5%.

Enzyme-linked Immunosorbent Assay for Involutin. Involucrin content was determined in adherent cultures using an enzyme-linked immunosorbent assay as described by Parenteau et al. (36). Polyclonal rabbit anti-human involucrin antibody and purified human involucrin standard were obtained from Biomedical Technologies, Inc. (Stoughton, MA). Briefly, cultures were washed twice with PBS and the cells were lysed with 20 mM Tris-Cl, 2 mM EDTA, pH 7.4. Samples were sonicated for 2 min on ice (four 30-s intervals), vortexed and clarified by centrifugation in a Microfuge (Beckman Instruments, Inc., Fullerton, CA) at 14,000 × g for 5 min at 4°C. The diluted rabbit anti-human involucrin antiserum was incubated overnight at 4°C with either standards or dilutions (undiluted to 1:10,000) of a clarified cell homogenate in assay buffer (PBS containing 0.25% type A gelatin from porcine skin/175 bloom, 2 mM EDTA, 0.5% Tween 20, and 0.2% NaN3). The mixture was incubated for 30 min at room temperature in microdilution culture dishes (Nunc-Immuoplate; Maxisorp) previously coated with human involucrin (1 ng/well in 0.1 M sodium carbonate, pH 9.6) and 10 μl of 1 mg/ml 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide. After being rinsed, the wells were incubated for an additional 1 h with goat anti-rabbit IgG covalently coupled with alkaline phosphatase (Sigma, St. Louis, MO). Unbound antibody-enzyme conjugate was removed by rinsing, and the enzyme substrate, p-nitrophenyl phosphate hexahydrate, was added. ΔA₄₀₅ was read after development of sufficient color. The amount of involucrin in samples was determined by comparing values to a semilogarithmic plot of ΔA₄₀₅ versus the amount of involucrin in the involucrin standard. The readable range of the assay was 0.1–0.5 ng of involucrin per well.

Indirect Immunofluorescence Staining of Cellular Fibronectin. Keratinocytes were grown on glass coverslips. Cultures were rinsed once with 25 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid-buffered Earle's salts (pH 7.4), rinsed twice briefly with PBS (pH 7.4), and fixed for 30 min at room temperature in 0.1 M phosphate buffer containing 3% paraformaldehyde. Cells were rinsed three times with Tris-buffered saline (pH 7.4). Rabbit anti-human plasma fibronectin was diluted 1:100 in Tris-buffered saline containing 1% ovalbumin. Antiserum (100 μl) was placed on the coverslip and incubated in a moist chamber for 30 min at 37°C. The antiserum was then removed by thorough rinsing in the buffer, and 100 μl of fluorescein-conjugated goat anti-rabbit immunoglobulin G were applied at a dilution of 1:100 in Tris-buffered saline (pH 7.4) containing 1% ovalbumin. After incubation for 60 min the coverslips were rinsed and mounted with Glycergel (Sigma) containing 5 mM p-phenylenediamine. Cells were visualized with a Nikon microscope by using epifluorescence illumination and phase contrast illumination.

Enzyme-linked Immunosorbent Assay for Human Fibronectin. Cultures were washed and incubated for 24 h in 1 ml of serum-free growth medium. Soluble fibronectin in the conditioned medium was detected by a competitive enzyme-linked immunosorbent assay as described previously (37, 38). Briefly, diluted rabbit anti-human fibronectin antiserum was incubated overnight with standards or dilutions of conditioned medium. The mixture was incubated for 2 h at room temperature in microdilution culture dishes previously coated with human plasma fibronectin (4 μg/ml in Tris-buffered saline containing 0.1% bovine serum albumin) and then postcoated with 1.0% albumin. After being rinsed, the wells were incubated for an additional 2 h with goat antirabbit antibody-enzyme conjugate, then rinsed, and the enzyme substrate, p-nitrophenyl phosphate hexahydrate, was added. ΔA₄₀₅ was read. The amount of fibronectin in samples was determined by comparing values to a semilogarithmic plot of ΔA₄₀₅ versus the amount of fibronectin in the fibronectin standard. The readable range of the assay was 20–200 ng of fibronectin per ml (2–20 ng of total fibronectin).

Northern Analysis. Poly(A)⁺ RNA was isolated from logarithmically growing cells according to the method of Badley et al. (39). Poly(A)⁺ RNA (3 μg) was electrophoresed in a 1% agarose gel containing formaldehyde. The RNA was electroblotted to a Zeta-probe membrane (Bio-Rad laboratories, Richmond, CA). The membrane was hybridized and then hybridized in the presence of a random primer 32P-labeled cDNA probe as recommended by the supplier. The cDNA probes used were: human involucrin cDNA, pJ-2 (40); human fibronectin cDNA, pFH1 (41); human α1 type I collagen cDNA, pGHH3 (2800 base pair fragment from the 5'-region of complete α1 type I cDNA); human thrombospondin cDNA, M9/pGEM2 (42); monkey TGF-β1 cDNA, pTG-β2 (43); rat glyceraldehyde-3-phosphate dehydrogenase cDNA, pGPDNS (44); and human TGF-α cDNA, pT7T318 (45).

RESULTS

Immortalization Is Sufficient to Confer a Differentiation-de

TRANSFORMATION-ASSOCIATED EXTRACELLULAR MATRIX CHANGES

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<th>Cell line</th>
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<th>Transforming agent</th>
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<tr>
<td>RHEK-1</td>
<td>Ad12-SV40 virus</td>
<td>H-ras</td>
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<td>HPK-1A/ras</td>
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TRANSFORMATION-ASSOCIATED EXTRACELLULAR MATRIX CHANGES

cells to differentiate was examined following their removal from substratum attachment. Terminal differentiation, as measured by cornified envelope formation, can be induced by disaggregating adherent keratinocytes and suspending them in growth medium made semisolid with the neutral polymer, methylcellulose. Cells were resuspended in methylcellulose and incubated for different times in suspension. Following incubation, the cells were recovered and the percentage of cells producing a cornified envelope was determined (Fig. 1). Greater than 70% of the normal human keratinocytes produced cornified envelopes after 24 h suspension in methylcellulose. The HPV-immortalized (HPK-1A) and HPV-tumorigenic (HPK-1A/ras) cells were defective in the ability to differentiate in response to removal from substratum attachment. Less than 10% of these cells produced a cornified envelope after 24 h suspension in methylcellulose. Incubating the HPK-1A or HPK-1A/ras cells for an additional 3 days in suspension did not increase the percentage of the cells producing a cornified envelope (data not shown). For comparison, we also determined the response of keratinocytes immortalized with Adl2-SV40 hybrid virus and then malignant converted by transfection with H-ras. Neither the Adl2-SV40-immortalized cells (RHEK-1) nor the corresponding tumorigenic line (RHEK-1/ras) were able to produce cornified envelopes after 24 h suspension in methylcellulose. The inability of other SV40-transformed keratinocytes to produce cornified envelopes is a consistent characteristic of transformation by this virus (34, 46, 47). Compared to the normal human keratinocytes, both immortalized and malignantly converted keratinocyte lines exhibited an altered differentiation capacity in response to suspension in methylcellulose. The extent of this block in differentiation varied between SV40-transformed and HPV 16-transformed cells. Nonetheless, lack or limited response to a differentiation signal was a consistent characteristic of both immortal and malignantly converted human keratinocyte lines.

Loss of proliferative capacity is also a consistent characteristic of terminally differentiating keratinocytes. We examined the proliferation capacity of HPV 16-immortalized and tumorigenic cells, as measured by their ability to initiate colonies in surface culture following recovery from suspension in semisolid medium. A decrease in the proliferation capacity of all cells was observed with time in methylcellulose (Fig. 2). Normal keratinocytes held in semisolid growth medium lost the ability to replicate after 36 h, evidenced by the inability to form colonies in surface culture. Compared to normal keratinocytes, the transformed keratinocytes lose the ability to form colonies at a slower rate when suspended in methylcellulose-containing medium. Normal keratinocytes lost three logs of colony-forming ability after 24 h whereas HPK-1A and HPK-1A/ras cells lost 90% of their initial colony-forming ability. After 3 days in suspension these cells were still able to initiate colonies when replated in surface culture.

The formation of cornified envelopes is a multistep process. The lack of cornified envelope formation by the HPV-immortalized or tumorigenic cells may reflect deficiencies in any of approximately seven proteins which comprise the cornified envelope, as well as alterations in the three transglutaminase
were consistent with the poor ability of these lines to make cornified envelopes in response to release from substratum attachment. It is likely that the ability to form cornified envelopes is limited by the amount of the involucrin protein or other constituents of the cornified envelope present in the cells.

Human Keratinocytes Transformed by HPV 16 Have Increased Levels of Fibronectin. Since changes in cell-matrix interactions occur early during the course of terminal differentiation in stratified squamous epithelia, we determined whether HPV 16-immortalized or tumorigenic keratinocytes exhibited changes in the production of known ECM glycoproteins. Cultured normal human keratinocytes synthesize and deposit small amounts of ECM constituents, such as fibronectin, various collagens, and other glycoproteins. We examined the ECM characteristics of the HPK-1A and HPK-1A/ras keratinocyte cell lines. Using a quantitative immunosorbent assay for human fibronectin we found that medium conditioned by HPK-1A/ras cells contained 5-fold more soluble fibronectin than the HPK-1A or preconfluent normal keratinocytes (Fig. 5). Conditioned medium from HPK-1A cells and control keratinocytes contained similar amounts of soluble fibronectin. High passage HPK-1A cells did not produce increased levels of soluble fibronectin. The Ad12-SV40-tumorigenic cells (RHEK-1/ras) also produced increased levels of soluble fibronectin compared to the Ad12-SV40-immortalized cells (RHEK-1) or normal keratinocytes. Since the HPV-tumorigenic keratinocytes (HPK-1A/ras) had increased levels of soluble fibronectin present in conditioned medium, we then determined if these cells also deposited more fibronectin into their extracellular matrix. Cultures of low passage normal keratinocytes, HPK-1A, and HPK-1A/ras cells were plated on glass coverslips at low density (5 x 10^4/35-mm dish) in medium containing either high (0.66 mM) or low (0.05 mM) calcium concentrations. Immunofluorescence staining with anti-human plasma fibronectin revealed that, relative to normal keratinocytes, HPK-1A cells exhibited increased numbers of short, stitch-like fibronectin fibrils (Fig. 6, c and e). The tumorigenic HPK-1A/ras cells, however, organized and deposited a dense fibronectin-containing extracellular matrix (Fig. 6, g and i). We found that relative to HPK-1A cells the tumorigenic HPK-1A/ras keratinocytes incorporated increased levels of fibronectin into their matrices regardless of the calcium concentration of the medium. Similar results were

**Fig. 3. Analysis of steady state levels of involucrin mRNA.** Poly (A)* RNA was prepared from the indicated cell lines when cultures were approximately 90% preconfluent. Poly(A)*, 3 ng, RNA was electrophoresed through a 1% agarose-formaldehyde gel. Following electrophoresis, the RNA was electroblotted to a Zeta-probe membrane. The membrane was prehybridized and then hybridized to a random primer 3P-labeled cDNA probe for involucrin. The same blot was stripped and reprobed with a cDNA probe for GAPDH to control for loading. kb, kilobases.

**Fig. 4. Involucrin content of keratinocytes.** Preconfluent cultures of normal or transformed human keratinocytes were washed and lysed, and the involucrin content was determined by a competitive enzyme-linked immunosorbent assay. Columns, mean of three experiments; bars, SD.

**Fig. 5. Soluble fibronectin content of medium conditioned by transformed human keratinocytes.** Preconfluent cultures of normal human keratinocytes, Ad12-SV40- and HPV 16-immortalized keratinocytes, and corresponding tumorigenic cell lines in 35-mm dishes were washed and incubated for 24 h in 1 ml of serum-free medium. The conditioned medium was harvested and assayed for fibronectin content by a competitive enzyme-linked immunosorbent assay. Columns, mean of three experiments; bars, SD.
Fig. 6. Extracellular matrix fibronectin content of transformed keratinocytes. Indirect immunofluorescence staining of preconfluent keratinocytes with a rabbit polyclonal antibody to human plasma fibronectin followed by fluorescein-conjugated goat anti-rabbit IgG was performed: a and b, normal human keratinocytes (0.05 mM Ca²⁺); c and d, HPK-1A (0.05 mM Ca²⁺); e and f, HPK-1A (0.66 mM Ca²⁺); g and h, HPK-1A/ras (0.05 mM Ca²⁺); i and j, HPK-1A/ras (0.66 mM Ca²⁺).

observed with Ad12-SV40 tumorigenic keratinocytes (data not shown).

The enhanced fibronectin-containing extracellular matrix observed in the tumorigenic HPK-1A/ras keratinocyte line could be the result of increased transcription or increased stabilization of fibronectin mRNA. Alternatively, accumulation of extracellular matrix proteins may result from decreased proteolytic degradation of the matrix. To determine if fibronectin mRNA levels were enhanced in HPV 16-transformed cell lines we isolated poly(A)⁺ mRNA from populations of early passage

HPK-1A and HPK-1A/ras cells. For comparison, we also isolated poly(A)⁺ mRNA from cultures of RHEK-1 and RHEK-1/ras cells. We examined the steady state mRNA levels for several ECM glycoproteins, namely, fibronectin, thrombospondin, and type I procollagen. Densitometric scanning of the autoradiogram showed that HPK-1A/ras cells contained approximately 6-fold more fibronectin mRNA as compared to HPV 16-immortalized cells (HPK-1A) (Fig. 7). Similar results were obtained with keratinocytes immortalized by the hybrid Ad12-SV40 virus and malignantly converted with H-ras. This was in agreement with the observed elevated levels of soluble and matrix-associated fibronectin. To ensure that consistent loading of the samples was obtained, hybridization of the blot with a CDNA for glyceraldehyde-3-phosphate dehydrogenase was performed and any loading differences were accounted for when determining the increases in mRNA levels. We probed the same RNA blot with CDNA probes specific for human thrombospondin and type I procollagen (Fig. 8). The tumorigenic HPK-1A/ras cells exhibited 9-fold higher levels of thrombospondin mRNA as compared to normal keratinocytes or the HPV 16-immortalized cells (HPK-1A). There was no detectable difference in the level of type I procollagen mRNA in normal and HPK-1A cells. Unlike the tumorigenic HPK-1A/ras keratinocytes, the RHEK-1/ras cells produced high levels of type I procollagen mRNA. Type I procollagen mRNA was modestly elevated in the HPK-1A/ras cells. We did not detect changes in α1 type IV collagen mRNA levels in any of the keratinocyte lines (data not shown).

Increased Production of Autocrine Growth-regulatory Factors Is Associated with Ad12-SV40 but not HPV 16-mediated Transformation of Human Keratinocytes. To investigate the mechanisms of loss of growth control and enhanced expression of...
DISCUSSION

We have determined that human keratinocytes immortalized by HPV 16 DNA and malignantly converted by transfection of the H-ras oncogene (HPK-1A/ras) produced increased amounts of soluble and cell surface-associated fibronectin. The overproduction of fibronectin in the HPV-tumorigenic cells (HPK-1A/ras) is evidenced not only by increased mRNA for this extracellular matrix glycoprotein but also by enhanced formation of a fibronectin-containing extracellular matrix. We found that immortalization by HPV 16 DNA alone is not sufficient to increase fibronectin production in human keratinocytes. The HPV-immortalized cells (HPK-1A) produced soluble fibronectin levels similar to those of normal keratinocytes. To date, we have found that increased mRNA levels for two extracellular glycoproteins, fibronectin and thrombospondin, were consistent characteristics of malignantly converted HPV 16-immortalized human keratinocytes.

In addition to fibronectin, the malignantly converted keratinocytes exhibited an increase in the steady state mRNA levels for thrombospondin. Thrombospondin is a large, multifunctional glycoprotein secreted by growing cells. It is also released from α-granules following stimulation of platelets and accounts for the endogenous lectin activity of stimulated platelets (57). There is positive immunostaining for thrombospondin in basement membrane regions beneath glandular epithelium in skin extracellular matrix molecules we examined the steady state mRNA levels for TGF-α and TGF-β1 in normal and virally transformed keratinocytes. Normal human keratinocytes produce and respond to these growth factors (48–50). TGF-β1 has been shown to enhance production of fibronectin, thrombospondin, and type I collagen in a variety of cell lines (37, 51–53). Changes in response and production of TGF-βs during neoplastic progression of some cell types have been observed (54–56). We reasoned that the altered pattern of ECM glycoproteins expression might be a response to altered production of growth factors, particularly the members of the TGF-β family. The steady state mRNA levels for TGF-α and TGF-β1 were analyzed by Northern blot analysis (Fig. 9) and quantified by densitometric scanning. Neither the HPV-immortalized nor the HPV-tumorigenic cells produced increased levels of TGF-α or TGF-β1 mRNA. However, the tumorigenic RHEK-1/ras cells exhibited an increase in the steady state mRNA levels for both of these growth factors. The increased production of TGF-β1 is consistent with the enhanced procollagen type I mRNA production seen specifically in these cells.

Fig. 8. Analysis of steady state mRNAs for extracellular matrix glycoproteins. Poly (A)+ RNA was prepared from logarithmically growing cells when approximately 90% preconfluent and size fractionated by agarose formaldehyde-gel electrophoresis. The mRNA levels for thrombospondin and type I collagen were examined by using corresponding specific cDNA probes. This blot was also probed with the cDNA probe for GAPDH to control for loading. kb, kilobases.

Fig. 9. Expression of growth-regulatory factors. The steady state mRNA levels for TGF-α and TGF-β1 was examined by Northern blot analysis. The same blot was probed with the cDNA probe for GAPDH to control for loading. kb, kilobases.
and intense staining at the dermal-epidermal junction (58). Thrombospondin has recently been implicated in the metastatic potential of human squamous carcinoma cells (59). Steady state mRNA levels for other extracellular matrix proteins, such as procollagen type I, were not increased in the immortal HPK-1A or tumorigenic HPK-1A/ras cells. Thus, a nonspecific increase in overall synthesis and organization of extracellular matrix glycoproteins is not a consequence of malignant conversion of the immortalized keratinocytes lines used in our studies.

Unlike fibroblastic cells, viral transformation of epithelial cell types does not result in loss of cell surface-associated fibronectin or pericellular matrix (60-62). Edelman et al. (61) demonstrated that SV40-infected human keratinocytes after the period of crisis secrete increased soluble fibronectin and deposit increased amounts of fibronectin into a deoxycholate-insoluble matrix. We have confirmed and extended these early observations on changes in extracellular matrix-associated fibronectin production in SV40 T-antigen-positive postcrisis cells. In the cell system used here, immortalization by the hybrid Ad12-SV40 virus does not alter extracellular matrix production. We found that malignant conversion of RHEK-1 cells by transfection of H-ras causes a dramatic increase in not only fibronectin and thrombospondin but also procollagen type I mRNA levels. We suspect that the increases in the steady state levels of mRNAs for these extracellular matrix proteins, particularly procollagen type I, in Ad12-SV40-tumorigenic keratinocytes are mediated, in part, by the overexpression of TGF-β1 and possibly other TGF-β family members. Our observation of increased steady state mRNA levels for TGF-β1 in the RHEK-1/ras cells is consistent with a TGF-β-mediated enhancement of procollagen mRNA levels.

The mechanisms by which events associated with malignant conversion alter gene expression of fibronectin and thrombospondin in human keratinocytes are not known. TGF-β1 is known to increase gene expression of several extracellular matrix glycoproteins as well as cell surface receptors of the integrin superfamily (51, 52, 63). It is interesting to note that we observed no changes in the steady state mRNA levels for two known autocrine regulators of keratinocyte growth, TGF-α and TGF-β1 in HPV 16-immortalized or tumorigenic keratinocytes. We did, however, observe increases in steady state mRNA levels for both TGF-α and TGF-β1 in the Ad12-SV40 tumorigenic cells (RHEK-1/ras). Therefore, overproduction of autocrine growth factors, particularly TGF-β1, does not appear to be a consistent characteristic that could account for altered expression of extracellular matrix components in the malignantly converted human keratinocytes used in our studies. Another interpretation of our data is that malignant conversion of immortalized keratinocytes by H-ras or transfection of H-ras alone elicits an increase in extracellular matrix production. We have found that RHEK-1 cells malignantly converted with v-fos also produced increased amounts of fibronectin and thrombospondin but not procollagen type I. Therefore, H-ras-specific induction of extracellular matrix proteins does not appear to be a likely explanation.

Since increased expression of fibronectin and thrombospondin was a consistent characteristic of HPV 16 tumorigenic keratinocytes, we asked whether these extracellular matrix glycoproteins possibly contributed to altered differentiation characteristics of the HPV 16-keratinocyte lines at different stages of neoplastic transformation. Interaction of these molecules with their cognate cell surface receptors appears to be important events during terminal differentiation of stratified squamous epithelia; for example, suspension-induced differentiation, as measured by the number of involucrin expressing cells, can be reduced by high concentrations of plasma fibronectin (64). The effect of thrombospondin on terminal differentiation is currently unknown. The effects of exogenously added plasma fibronectin on differentiation appear to be mediated by the cell surface integrin, α5β1. Watt et al. (65) have shown that suspension-induced terminal differentiation in vitro involves loss of adhesiveness to several extracellular matrix glycoproteins, specifically fibronectin, laminin, and collagen types I and IV. Changes in the adhesive function of several integrins were observed in these studies. Interestingly, we found that both the HPV 16-immortalized and malignantly converted keratinocytes exhibited aberrant suspension-induced differentiation. Therefore, poor differentiation potential is associated with the immortalization stage in vitro and occurs prior to changes in production of keratinocyte fibronectin. The HPV 16-immortalized keratinocytes (HPK-1A), although differentiation-defective, did not overproduce soluble or cell surface-associated fibronectin. HPK-1A cells produced 8-fold less involucrin protein when compared to preconfluent normal keratinocytes. This decreased production of involucrin protein is reflected in decreased steady state levels of involucrin mRNA. Malignant conversion further attenuated involucrin protein as well as mRNA levels. Similar results were obtained with the Ad12-SV40 immortal and tumorigenic keratinocyte lines. Neither RHEK-1 nor RHEK-1/ras cells produced cornified envelopes in response to suspension culture and expressed very low levels of involucrin mRNA and protein. However, RHEK-1 cells overexpressed fibronectin and thrombospondin when malignantly converted by H-ras transfection. Therefore, overexpression of fibronectin does not directly contribute to maintenance of the differentiation-defective phenotype in vitro.

There have been no previous studies on extracellular matrix characteristics of HPV-transformed human keratinocytes. Extracellular matrix properties of neoplastic cells have been well characterized in fibroblastic cell types. From these studies we know that transformed fibroblastic cells present several adhesive abnormalities including: (a) deficient deposition of fibronectin and other extracellular matrix proteins into matrix; and (b) reduced and/or altered integrins, specifically α5β1 (66, 67). Unlike fibroblastic cell types, the HPV 16-transformed keratinocytes we have studied exhibit increased deposition of fibronectin and most likely other extracellular matrix glycoproteins such as thrombospondin. From our studies it appears that virally induced alterations in the expression of fibronectin and thrombospondin and perhaps their cognate cell surface receptors may contribute to the acquisition or maintenance of malignant phenotypes in stratified squamous epithelia. We are currently screening human cervical biopsies to determine if our in vitro observations of increased fibronectin and thrombospondin expression have in vivo correlates. Our purpose is to determine whether changes in extracellular matrix molecules or their cognate cell surface receptors are also observed in cervical samples from patients with confirmed condylomata or cervical intraepithelial neoplasia (CIN grades I-III).

**ACKNOWLEDGMENTS**

We wish to sincerely thank Dr. M. Durst for the generous gift of the HPK-1A and HPK-1A/ras cells. We also thank Dr. D. F. Mosher for the kind gift of anti-human plasma fibronectin antibody. We are very...
grateful to Dr. H. Green, Harvard Medical School, Boston, MA, for the human involucrin cDNA, pl-2; Dr. F. E. Baralle, University of Oxford, Oxford, England, for the human fibronectin cDNA, pFH1; Dr. D. Greenspan, University of Wisconsin, for the human α1 type I collagen cDNA, pGGH03; Dr. J. Lawler, St. Elizabeth's Hospital and Tufts University, Boston, MA, for the human thrombospondin cDNA, M9/pGEM2; Dr. A. F. Purchio, Oncogene, Seattle, WA, for the monkey TGF-β cDNA, pTGFS-2; Dr. P. Jeantet, Laboratoire de Biochimie, Center Paul Lamarque, France, for the rat glyceraldehyde-3-phosphate dehydrogenase cDNA, pGPDNS; and Dr. Jeffrey Kudlow, University of Alabama, for the human TGF-α cDNA, pT7T318. We are grateful to Dr. H. Green, Harvard Medical School, Boston, MA, for the human thrombospondin cDNA, pFH1; 24 the human involucrin cDNA, pI-2; Dr. F. E. Baralle, University of

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Nader Sheibani, John S. Rhim and B. Lynn Allen-Hoffmann


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