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REPORTS

Plasminogen Activator in Differentiating Mouse Keratinocytes*

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The activity of the serine protease plasminogen activator (PA) was measured in cell lysates from primary mouse keratinocyte cultures as well as from a number of established mouse keratinocyte lines. Enzyme activity was generally higher in the transformed lines than in the primary cultures; however, among the lines tested, those that expressed the highest degree of morphologic differentiation had the highest levels of cell-associated PA. In both the normal (primary) and transformed (established) keratinocyte cultures, PA activity increased when cultures reached confluence and morphologic evidence of differentiation was noted. The highest specific activity of the enzyme was found in cells shed from differentiating cultures, which consisted predominantly of detergent-resistant cornified envelopes. As the cultures differentiated and these cells were shed from the culture surface, the total cell-associated PA activity of the culture decreased accordingly. In both the normal and transformed keratinocyte cultures, peak PA activity occurred at a time when DNA synthesis was declining. These findings indicate that as keratinocytes differentiate, their intracellular levels of PA increase. The modulation of this endogenous keratinocyte enzyme may play an important, although as yet undefined, role in the normal maturation and terminal differentiation of these cells.

The enzyme plasminogen activator (PA), present in the blood and within a variety of cells, is a serine protease which converts the serum zymogen, plasminogen, to the active protease, plasmin. The PA in serum plays an obvious role in thrombolysis [1], while the role of cell-associated PA seems to be the production of localized extracellular proteolysis—either pathologic, as in tumor invasion [2], or physiologic, as in trophoblast implantation [3], and in postpartum involution of mammary glands [4]. In these latter cases, the expression of PA is modulated by hormones and varies with the developmental state of the cells [3,4]. Thus, PA may be considered a specific product of a differentiated cell that is involved with local destructive or remodeling processes.

A number of studies indicate that endogenous keratinocyte PA and the subsequent generation of plasmin may facilitate the terminal differentiative events of nuclear dissolution [5,6] or squame detachment [7]. These observations have led to the suggestion that intracellular levels of PA are regulated during the course of normal keratinocyte maturation, and may, in fact, serve as an indicator of the degree of keratinocyte differentiation [8].

In this study we report that PA is a product of normal and transformed mouse keratinocytes. Using both primary mouse keratinocyte cultures and established keratinocyte lines we demonstrate that the intracellular level of this protease varies with the differentiative state of the culture. In addition, in the individual keratinocyte culture, we find the highest specific activity of the enzyme in the squames shed from the culture surface. These findings indicate that as keratinocytes differentiate, their intracellular levels of PA increase.

MATERIALS AND METHODS

Cell Culture

Primary mouse keratinocyte cultures were prepared from newborn C_3H mice using a trypsinization method previously described [9,10]. Briefly, strips of mouse skin were agitated in a trypsinization vessel maintained at 37°C. The epidermal cell aggregates were separated from fibroblasts and single keratinocytes by 2 consecuous discontinuous Ficoll density gradient centrifugations. The resultant cell suspension was plated at the desired density and maintained in Eagle's minimal essential medium (MEM) containing 4-fold concentrations of amino acids (AA) and vitamins (4 × AA), and 10% fetal calf serum (FCS) (Seromed, München, Germany) in an atmosphere of 95% air and 5% CO_2 at 37°C.

The mouse keratinocyte lines used in these studies were isolated from keratinocyte cultures treated with dimethylbenzanthracene (DMBA) (line PDV in [11]), derived from an in vivo DMBA-induced skin carcinoma and cloned thereafter (line BD VII in [12]), or had developed spontaneously in vitro in long-term primary cultures maintained at 30°C (HEL lines in [13]). HEL-37 is a subline of HEL-30 which had been shifted to 37°C incubation temperature at passage 6, and exhibits reduced keratinization capacity with higher passage numbers [14]. Cells were used between passages 32–42, except line HEL-37, which was used at passage 187. Cells were maintained in Dulbecco's modification of Eagle's medium (DMEM) (Flow Laboratories, Rockville, Maryland), supplemented with 10% FCS (Rehies Chemical Company, Kankakee, Illinois) in 90% air and 10% CO_2 at 37°C.

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Abbreviations:
AA: Amino acids
BME: β-mercaptoethanol
BSA: bovine serum albumin
DMBA: dimethylbenzanthracene
DMEM: Dulbecco's modification of Eagle's minimal essential medium
FCS: fetal calf serum
MEM: minimal essential medium
PA: plasminogen activator
PBS: phosphate-buffered saline

Determination of Cell-Associated Plasminogen Activator Levels

Cells to be assayed for PA were washed twice with cold phosphate-buffered saline (PBS), scraped off the plate with a rubber policeman, and collected by centrifugation. The cell pellet was disrupted by sonification for 15 2-s bursts (Branson sonicator) in ice-cold 0.1 M Tris-HCl, pH 8.1, containing 0.5% Triton X-100. The cell extract was then cenfrifuged for 20 min at 20,000 *g* at 4°C and aliquots of the supernatant frozen for PA assay and protein determination. In some experiments, the cells were disrupted by rapid vortexing of the cell suspension in the cold Triton-containing buffer.

The [^{125}I]-fibrin-coated multiwell assay was used to determine PA levels, as previously described [15,16]. Briefly, 2 or 5 μg of cell lysate protein was added to a [^{125}I]-fibrin-coated microwell in a buffer containing 0.025% bovine serum albumin (BSA) and 12 μg/ml FCS-plasminogen, purified according to published procedures [17]. The dishes were incubated at 37°C and an aliquot of the supernatant fluid assayed for solubilized [^{125}I]-fibrin degradation products at 1, 2, and 4 h. All assays were performed under conditions of substrate (plasminogen) excess, and only values in the linear range of the assay with respect to both time and sample concentration were used. PA is expressed as milliunits (Ploug) of Urokinase Reference Standard (Leo Pharmaceutical Products, Ballerup, Denmark). All data are expressed as the mean value of duplicate assays on each of 2 samples. Control assays of cell extracts in the absence of plasminogen revealed no plasminogen-independent fibrinolysis.

Scoring of Detergent-Insoluble Cells and Cornified Envelopes

To calculate the fraction of cells containing detergent-insoluble keratin and β-mercaptoethanol (BME)-resistant cornified envelopes, dense, 7-day-old cultures of BD VII cells were vigorously washed twice with PBS to remove any adherent squames prior to the collection period. The cultures were subsequently incubated for 48 h in serum-free DMEM at 37°C. The cells shed into the medium over this period were collected by aspirating the culture medium, and vigorously washing the culture with PBS to remove loosely attached cells. The culture medium and washes were pooled, and shed cells collected by low-speed centrifugation. The cells adherent to the culture dish were removed from the plate by standard treatment with 0.25% trypsin–0.02 M EDTA, and collected by centrifugation after tryptic activity was stopped with serum.

Scoring was done following Green's published procedure [5]. The cell pellet of either the shed or the adherent cells was washed and resuspended by vigorous pipetting in 1 ml of PBS. To determine the total cell number, an aliquot of this cell suspension was counted in a Coulter counter and checked by manual hemocytometer counting. The remaining cells were collected by low-speed centrifugation and one-half of the cells resuspended in 0.05 M Tris-HCl, pH 8.1, containing 1% SDS. After incubation for 5 min at room temperature, cells were counted in a hemocytometer to determine the number of detergent-resistant cells. The number of BME-resistant cornified envelopes was determined by incubating the remaining one-half of the cell suspension for 5 min at room temperature in SDS buffer to which 1% BME had been added. Cornified envelopes appeared as clear cell ghosts and were scored in a hemocytometer.

Measurement of DNA Synthesis

An estimate of the rate of DNA synthesis was obtained by labeling with [^{3}H]-thymidine (21.8 Ci/mmol, New England Nuclear, Boston, Massachusetts) in serum-free medium (1 μCi/ml for keratinocyte lines, 5 μCi/ml for primary epidermal culture) for 1 h at 37°C. After washing the cultures twice with cold PBS, the dishes were stored frozen at −20°C until further study. When all samples of all experimental time-points had been collected, thawed cultures were incubated with ice-cold 10% TCA for 5 min and harvested by scraping the monolayer off the culture dish. Precipitated macromolecules were collected on Whatman GF/A filter paper, placed into Triton-toluene scintillant (Soluscint A, National Diagnostic, Somerville, New Jersey) and radioactivity measured in a scintillation counter.

Measurement of Protein

Protein content of the cell lysates was determined using the method of Lowry et al [18]. Total protein in lysates containing BME was quantitated by the procedure of Schaffner and Weissman [19].

RESULTS

Production of PA by Established Keratinocyte Lines

To determine whether a relationship between levels of PA and degree of differentiation could be demonstrated, the cell-associated enzyme was quantitated in a number of established mouse keratinocyte lines. These cell lines provided a good initial test system because of the variations in the extent of differentiation they expressed. Although these lines all possess the morphologic characteristics of keratinocytes, such as tonofilaments, desmosomes, keratin peptides, and cytoskeletons, and a multilayered growth pattern, the ultimate degree of differentiation achieved varies from line to line [12–14]. Line PVD, for example, differentiates poorly—as evidenced by an irregular stratification pattern, low level of cornified envelope formation, and decreased expression of keratinocyte-specific membrane antigens [20]. In contrast, line BD VII differentiates in a more normal epidermal fashion—forming organized stratified cultures complete with flat sheets of cornified squames, and possessing much keratin in disulfide-cross-linked aggregates [13,14]. The HEL lines also differ in their expression of keratinization in vitro; HEL-30 is the most differentiated line, its cloned subline HEL-C_1 is morphologically less differentiated, and the subline HEL-37 has even more reduced differentiating capacity with respect to both morphologic and biochemical criteria [13,14,21]. In all cell lines, cultures are best differentiated when they reach high cell densities.

Table I indicates that the specific activity of PA varied from line to line, with line PDV, a poorly differentiated line, expressing the lowest enzyme activity, and lines BD VII and HEL-30, well-differentiated lines, expressing approximately 2 (confluent cultures) to 10 (subconfluent cultures) times higher activity. Confluent cultures of every line tested had higher levels of PA than did their morphologically less differentiated subconfluent counterparts (Table I). This is in agreement with the observed relationship of PA and the degree of differentiation of each cell line.

Line BD VIII differentiates in culture in a fashion that closely follows the pattern of primary keratinocytes, with stratification and squame formation. PA expression was, therefore, examined in this line over a longer period of time in culture, to determine whether the specific activity of the enzyme varied as the culture differentiated. Cells were initially plated at low density and the cultures maintained until they were multilayered and contained many shedding squames. The variation of PA activity of the culture as a function of time is shown in Fig 1, and demonstrates an initial low level of this enzyme, a peak of activity at day 4, followed by a subsequent decline. The peak in activity occurred after the cultures were confluent, and after the early burst of DNA synthesis. Onset of squame formation was noted at day 3

TABLE I. *Cell-associated[a] PA activity[b] in mouse keratinocyte lines*

Line	Subconfluent (~ 1×10^4 cells/cm^2)	Confluent[c] ($1–2 \times 10^5$ cells/cm^2)
PDV	0.07 (0.06–0.08)	0.82 (0.82)
BD VII	0.77 (0.54–1.0)	2.1 (1.9–2.3)
HEL-37	0.61 (0.38–0.84)	1.6 (0.9–2.3)
HEL-30	0.76 (0.52–1.0)	1.9 (1.8–2.0)
HEL C_1	0.79 (0.50–1.1)	1.7 (1.2–2.2)

[a] Cells were plated at $1–2 \times 10^3$/cm^2 and maintained at 37°C in DMEM + 10% FCS until they were 20–30% confluent (1×10^4 cells/cm^2) or 100% confluent ($1–2 \times 10^5$ cells/cm^2), at which time they were collected and levels of PA determined as outlined in *Materials and Methods*. Cells were lysed by rapid vortexing of cell suspension in 0.1 M Tris-HCl pH 8.1 containing 0.5% Triton X-100.

[b] PA activity is expressed as milliunits urokinase per μg soluble protein after 2-h assay incubation. Values are the mean of duplicate determination done on 2 replicate plates. Values in parentheses indicate range.

[c] Early confluence: these densities are equivalent to those at which maximal values of intracellular PA are noted, as in Fig 1.

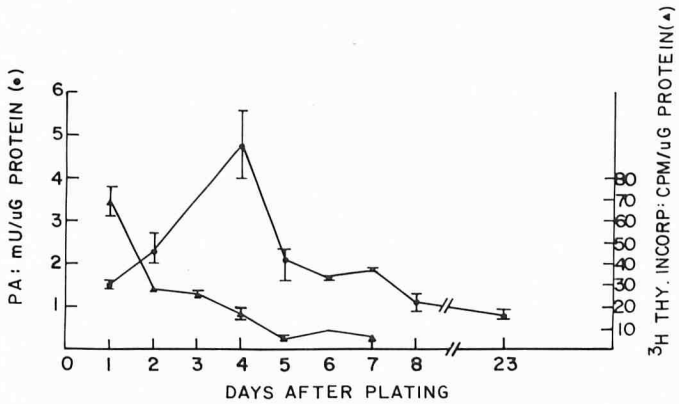

FIG 1. Intracellular PA and rate of [^{3}H]-thymidine incorporation in mouse keratinocyte line BD VII. The derivation, characterization, and maintenance of this line is outlined in text. Low-density cell cultures (7×10^4 cells/35-mm dish) were established and at the indicated times, cellular proteins were solubilized by sonification. Two micrograms of cell lysate protein were assayed for PA activity. [^{3}H]-thymidine incorporation into DNA was measured at the indicated times as outlined in the text. Each *point* represents the mean of duplicate assays on replicate plates. *Bars* indicate range; ●, PA activity; ▲, [^{3}H]-thymidine incorporation.

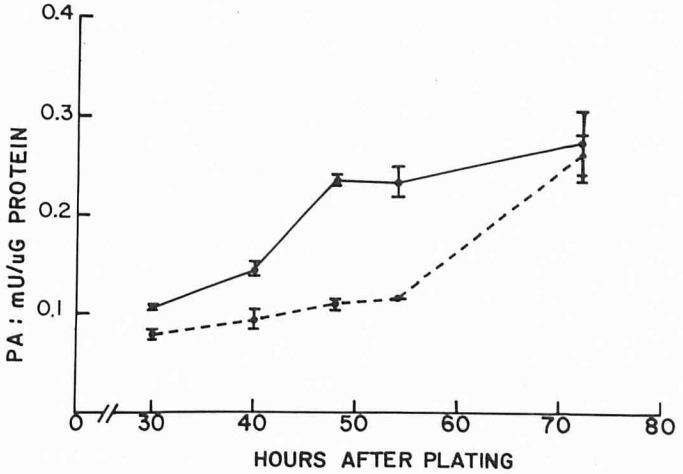

FIG 2. Intracellular PA in primary mouse keratinocytes. Primary mouse keratinocyte cultures were plated in high (5×10^5 cells/cm^2) ●——●, or low (1.25×10^5 cells/cm^2) ●- - -● density and maintained as indicated. Five micrograms of cell lysate protein, solubilized by sonification of the scraped cell layer, were assayed for PA activity at the indicated times. Each *point* represents the mean of duplicate assays carried out on 2 separate culture dishes. *Bars* represent range.

in these cultures, coinciding with the elevation of levels of PA. Thus, in this well-differentiating keratinocyte line, maximal levels of PA occurred when morphologic differentiation became evident and not at the time of maximal DNA synthesis.

Production of PA by Primary Cultures of Mouse Keratinocytes

To determine whether the observed increase in levels of PA associated with enhanced differentiation in the established keratinocyte lines could also be demonstrated in untransformed mouse keratinocytes, we examined primary cultures of mouse keratinocytes, initially plated at high ($\sim 5 \times 10^5$ cells/cm^2) and low ($\sim 1.25 \times 10^5$ cells/cm^2) densities. Primary cultures of mouse keratinocytes established by this method start as monolayers, begin to stratify after 2–3 days in culture, continue to stratify with the formation of sheets of squames, and finally, after 7–10 days in culture, consist almost entirely of sheets of large polygonal squames which spontaneously detach from the culture dish. This morphologic differentiation is density dependent; dense cultures differentiate earlier than sparse cultures. Fig 2 indicates that the cultures plated at the higher density displayed an increase in levels of PA at 46 h and maintained this elevated level until the experiment was terminated. The cultures plated at lower cell density exhibited an initially lower specific activity of PA. At 76 h after plating, however, the level of PA in both cultures was about equal (Fig 2). The increase in levels occurred after onset of visual confluence in both sets of cultures, and corresponded to the appearance of coarse fibrils within the upper layer of keratinocytes, which has been noted to be a phase-contrast morphologic indicator of early differentiation [13,14,22]. These results demonstrate that PA activity in these keratinocyte cultures is density dependent and, as in the established lines, correlates with the appearance of morphologic characteristics of differentiation.

To further substantiate the observed correlation of intracellular levels of PA with differentiation, we examined the pattern of enzyme activity during the entire lifespan of these primary mouse keratinocyte cultures. Levels of the protease were measured throughout the culture life to see whether we could demonstrate a prolonged plateau of elevated PA activity related to the sustained and continuous differentiation of the culture. The pattern of PA activity is shown in Fig 3, and demonstrates a marked peak of enzyme activity at 78 h and a subsequent

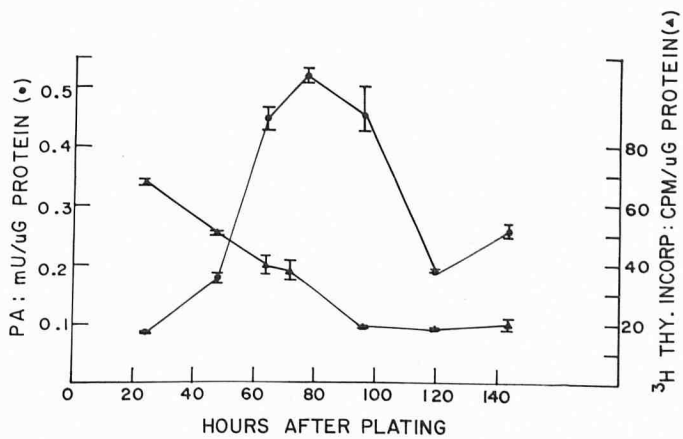

FIG 3. Intracellular PA and rate of [^{3}H]-thymidine incorporation into DNA in primary mouse keratinocyte cultures. Two hundred and five microliters of keratinocyte cell suspension ($\sim 3 \times 10^5$ cells/cm^2) were plated per 35-mm dish. Determinations of PA and [^{3}H]-thymidine incorporation into DNA were performed at the indicated times after plating, as described in Fig 1. Each *point* represents the mean of duplicate assays performed on replicate culture plates. *Bar* indicates range; ●, PA activity; ▲, [^{3}H]-thymidine incorporation.

dramatic fall in enzyme level. This pattern is qualitatively similar to the one seen with the established keratinocyte line (Fig 1). As in the keratinocyte lines, the peak in PA activity in the primary cultures was not related to a sudden burst or decline in DNA synthesis (Fig 3); it occurred when there was little [^{3}H]-thymidine incorporated into DNA. In these primary keratinocyte cultures, morphologic differentiation with stratification, formation of fibrillar cellular structures, and cornified envelopes start between days 2 and 3 of culture, while maximal DNA synthesis peaks between days 1 and 2 [22,23].

Relationship of PA to Differentiation Products

The pattern of PA production in both the keratinocyte line and the primary cultures demonstrated a peak of activity coincident with the onset of squame formation, followed by a pronounced decline in PA levels. The decreased PA activity was puzzling, for if PA were indeed related to differentiation,

no decrease in activity would be expected in cultures which still appeared to be morphologically quite differentiated. We theorized that perhaps the most differentiated cells, the squames, did indeed have high intracellular levels of PA, but were being shed into the medium by the desquamative process. These squames could have been lost during the washing of the cultures prior to PA determination, thereby lowering the total amount of assayable enzyme within the keratinocytes attached to the culture dish.

To test this hypothesis, the specific activity of the enzyme in shed squames was determined and compared to the activity of cells adherent to the surface of the culture dish. For these experiments, the keratinocyte line BD VII was used because this line differentiates in a near-normal fashion; yet, unlike the primary cultures, it consists of a uniform cloned population of cells. Primary keratinocyte cultures, on the other hand, are a mixed population of cells, consisting of aggregates of basilar and pilar unit cells, as well as some more-mature spinous or cornified cells. Another advantage of this cell line is that the squames shed from these cultures are shed as single cells rather than the sheets of squames shed in primary cultures, thus facilitating squame quantification. Table II illustrates that there is an 8-fold increase in the specific activity of the enzyme in the shed cells as compared to the adherent ones. However, the percentage of the total keratinocyte intracellular proteins that can be solubilized in detergent-containing buffers varies with the state of differentiation of the cell, i.e., more differentiated cells contain more detergent-insoluble protein [14,24]. Therefore, calculations of specific activity based on solubilized cell protein may be spuriously decreased or elevated. To obviate this problem, PA activity per 10^6 adherent or shed cells was calculated. In the experiment summarized in Table II, the shed cells demonstrated a 30-fold increase in levels of this enzyme as compared to the adherent cells. In other similar experiments, using different cell lines or different culture conditions, the calculated activity of shed cells varied from 10 to 200 times that of the adherent cells (data not shown). The activity of the shed cells, however, was always higher than that of those remaining attached to the plate.

TABLE II. *Cell-associated plasminogen activator and cornified envelope formation in shed and adherent mouse keratinocytes*[a]

	Shed cells	Adherent cells
Plasminogen activator		
milliunits/μg solubilized cell protein[b]	2.91 (± 0.62)	0.37 (± 0.8)
milliunits/10^6 cells[c]	1080	37
Percent of total cells resistant to[d]		
1% SDS	88.8 (± 11.0)	2.5 (± 0.5)
1% SDS, 1% BME	48.7 (± 7.2)	0.6 (± 0.1)

[a] Line BD VII cells, passage 35, were grown to confluency in 35-mm dishes in DMEM 10% FCS. The cultures were then washed twice with PBS, and the medium replaced with DMEM without serum. After 1 h, the medium was replaced with fresh serum-free DMEM, and the cultures were returned to the incubator for 48 h, at which time both the cells shed into the medium and those adherent to the culture dish were separately collected as outlined in *Materials and Methods*, and PA determinations and cell scorings were performed.

[b] Intracellular proteins were solubilized by sonification of collected, twice-washed cells in buffer containing 0.5% Triton, 0.1 M Tris-HCl, pH 8.1. PA determination is the mean (± SD) of duplicate assays performed on triplicate plates, using 5 μg of cell protein and a 1-h assay incubation.

[c] Calculated by determining the mean total number of milliunits of PA per culture dish (triplicate plates) and dividing by the mean number of cells adherent to the dish or shed into the medium (triplicate determinations), as outlined in *Materials and Methods*.

[d] Cells were grown as above, and adherent and shed cells collected and scored after treatment for 5 min at room temperature with 0.05 M Tris pH 8.1, 1% SDS with or without 1% BME as described in *Materials and Methods*. Determinations represent mean values (± SD) of triplicate dishes.

Previous work has shown that the terminal differentiation of the keratinocyte is marked by the accumulation of disulfide-stabilized keratin within the cell, and by the formation of an insoluble, cross-linked, "cornified" envelope [5,23–25]. These biochemical markers of differentiation can be detected easily by placing cell suspensions in a solution of 1% SDS with or without 1% BME [5]. In SDS alone, cells containing nonaggregated keratin are totally dissolved; those containing S-S stabilized keratin filaments are evident as opaque flat cells. When BME is added, the entire contents of the cells, including keratin filaments, are dissolved, leaving only the cross-linked cornified envelopes which can be scored under the microscope.

To ascertain that the cells shed into the medium were in fact squames, we scored them for the biochemical markers described above. The results, as summarized in Table II, indicate that the shed cells, which had high levels of PA, were mainly terminally differentiated squames. This observation was corroborated by the determination of protein concentration of each cell population solubilized sequentially in Tris buffer alone, 1% SDS/Tris buffer, and 1% SDS/1% BME/Tris buffer (data not shown). Thus, the increase in cell-associated PA can be definitively correlated with other biochemical aspects of keratinocyte differentiation.

DISCUSSION

Plasminogen activator is an enzyme whose production has been associated with normal cells that are involved in either local destructive processes or local remodeling. For example, in normal involuting mouse mammary glands, levels of PA are finely regulated [4], and the generation of this localized proteolytic activity represents a differentiated cell function. The findings of our study indicate that enhanced PA activity is also a feature of differentiation in the mouse keratinocyte. In cultures of both primary mouse keratinocytes and established keratinocyte lines, the level of cell-associated PA increases with the increased differentiation of the cells in culture. Increases in the specific activity of PA are demonstrated in dense keratinocyte cultures that appear more morphologically differentiated than their sparse counterparts. Monitoring the specific activity of keratinocyte PA during the life span of the culture reveals that the level of this enzyme slowly increases with the advanced differentiation of the culture, and decreases shortly after squame formation is noted. The finding of higher levels of PA in the cells shed from the culture—which consist primarily of squames—indicates that the most differentiated cells possess the highest specific activity of PA. Thus, as the keratinocytes differentiate, their measurable levels of cell-associated PA increase; the highest PA levels are found in squames that have detached from the culture surface, perhaps as a result of this increased proteolytic activity. Our findings suggest that this enzyme may be yet another marker of terminal differentiation of this epidermal cell.

Increased PA activity has also been considered a distinctive property of many neoplastically transformed cells. There are numerous examples of increased PA in virally, chemically, and spontaneously transformed cells, or in malignant tissues when compared to their normal counterparts [26–29]. However, this relationship is not absolute; there are some transformed lines that do not exhibit high PA activity [30,31], several nontransformed lines that produce relatively high levels [31,32], and some tissues (notably epithelial) where both the normal and malignant cells have high levels of this enzyme [33–35]. Our findings with mouse keratinocytes indicate that PA activity is generally higher (1.5–10×) in the transformed keratinocyte lines than in the primary cultures. Although it is difficult to determine the degree of tumorigenicity of each line, work currently in progress in one of our laboratories (N.E.F.) indicates that PA activity does not correlate with at least one indicator of tumorigenicity—growth in soft agar. The noted correlation of PA to the degree of morphologic differentiation attained by each line as well as the increased activity in shed squames

indicates that enhanced PA activity is, rather, a feature of differentiation in these cells.

It should be noted that the "squames" shed in this in vitro system, though they possess insoluble cornified envelopes and disulfide-cross-linked keratins, are, in many ways, unlike the squames found in the stratum corneum of the skin. The cells shed from the keratinocyte cultures still possess pyknotic nuclei, do not show the ultrastructural and biochemical keratin pattern of stratum corneum cells, and are generally loosely attached to the underlying layers [14,22]. What the levels of PA are within the dead squames of the normal murine stratum corneum is currently unknown.

All of the PA values in these experiments indicate the total amount of assayable enzyme in the cell lysate; relative contributions of possible intracellular PA inhibitors are not accounted for. Therefore, the increased levels of PA seen in the more mature keratinocytes may be the result of increased synthesis of the enzyme or inactivation of inhibitors of the enzyme. The possibility of a specific keratinocyte PA inhibitor is an attractive hypothesis. One could postulate, then, the presence of this inhibitor within the basal cells accounting for the low specific activity measured in that cell population, and either an inactivation, dissociation, or diffusion of the inhibitor during the process of differentiation, yielding an apparent increase in enzyme activity. Indeed, such an inhibitor of PA (urokinase-type) has been partially purified from skin [36].

The role that PA plays in the normal physiology of the keratinocyte is unclear. Evidence from other investigators indicates that PA-plasmin may play a role in the terminal differentiative event of nuclear dissolution in the keratinocyte. Though this may be true in suspension culture, as Green's work demonstrates [5], and in organ culture as others have shown [6], in most culture systems where keratinocytes are grown attached to the substratum and immersed within the culture medium, nuclear dissolution does not occur, even in the presence of serum plasminogen. Perhaps, even higher intracellular levels of PA than those observed in our submerged cultures are necessary for complete nuclear dissolution. In any event, in our experiments enhanced PA activity was not associated with nuclear dissolution.

Astrup [37] has proposed that epithelial plasminogen activator and the subsequent plasmin generated are important in squame shedding. Our findings of increased intracellular levels of PA in squames shed from the culture surface are certainly consistent with this hypothesis. However, to postulate any physiologic role for PA within the epidermis, it is necessary to demonstrate that the enzyme has access to its only known zymogen substrate: plasminogen. Since other serum proteins, such as transferrin, with about the same molecular weight as plasminogen, have been demonstrated within the keratinocytes of normal skin by immunoperoxidase staining [38], we suspect that plasminogen is likely to be present within the epidermis as well. Our preliminary evidence indicates that this is indeed the case [39]. Our findings suggest that keratinocyte-derived plasminogen activator and the subsequent generation of plasmin within the epidermis play some as yet undefined role in keratinocyte maturation.

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Epidermolytic Hyperkeratosis: Ultrastructure and Biochemistry of Skin and Amniotic Fluid Cells from Two Affected Fetuses and a Newborn Infant*

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Skin biopsy samples and amniotic fluid cells obtained in utero from two fetuses at risk for epidermolytic hyperkeratosis were examined by light and electron microscopy. Both fetuses were affected; the second was carried to term. Epidermal extracts were prepared from blisters of the newborn for analysis of keratin and filaggrin proteins. Abnormal clumps of keratin filaments were present in all layers of the prekeratinized fetal epidermis except the periderm and stratum germinativum. A significant population of amniotic fluid cells also contained the filament aggregations. Prenatal diagnosis of the disease should be possible using cells obtained at amniocentesis, thus avoiding fetal skin biopsy. Biochemical studies showed abnormalities in keratin and filaggrin proteins. The structural alterations in the tissue might be a consequence of altered interaction between these two abnormal epidermal proteins.

Epidermolytic hyperkeratosis (congenital bullous ichthyosiform erythroderma) is an autosomal dominant disorder of keratinization that is characterized histopathologically by hyperkeratosis, intraepidermal bullae, and by the presence of condensed keratin (tonofilament) bundles in spinous and granular cells [1–5]. The filamentous accumulations serve as the primary morphologic markers of the disease in the tissue. The mitotic rate of epidermal cells is increased severalfold over normal; correspondingly, the transit time is decreased [6]. A biochemical basis for the disease has been suggested by the finding of decreased amounts of fibrous proteins in the epidermis and the absence of 1 of the keratin polypeptides [7].

The clinical presentation of the disease is an initial erythroderma and blistering. The erythroderma usually fades, blistering improves, and hyperkeratosis develops. Intense buildup of scale is particularly prominent in flexural creases and other intertriginous areas [2]. Improvement is usually seen with age [1].

The disorder has been diagnosed in utero in at least 3 separate instances [8,9] on the basis of the ultrastructural identification of filament aggregations in fetal skin biopsy specimens obtained at 19–20 weeks gestation.

We have examined by light and electron microscopy the skin biopsy samples and amniotic fluid (AF) cells from 2 fetuses of the same family who were at risk for epidermolytic hyperkeratosis. Structural data from skin samples from 1 of these fetuses have been reported [8]. Biochemical studies of proteins in blister epidermis and cornified scale obtained from the affected infant (born from the second pregnancy) were also carried out. The objectives of these studies were: (1) to document more fully any structural characteristics of prekeratinized fetal epi-

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Abbreviations:
AF: amniotic fluid
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEM: transmission electron microscopy