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Optimization of Filaggrin Expression and Processing in Cultured Rat Keratinocytes

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Introduction

In normal mammalian epidermis, cell division and expression of keratins K5/K14 in the basal layer is followed by K1/K10, the keratin bundling protein filaggrin, and cornified envelope proteins such as involucrin and loricrin [1,2]. However, hyperproliferative epidermal cells can be motile and phagocytic, and can sustain cell division in the suprabasal layers. The hyperproliferative differentiation phenotype, seen during wound healing or in certain disease states results in production of keratins K6/K16, high levels of involucrin, and parakeratotic cornified cells [3,4].

For elucidation of structures and functions of the different types of epidermal cells, keratinocyte culture models were developed [5].

These models of epidermal differentiation are complicated by the phenotypic plasticity of keratinocytes. In the extreme, this is represented by the “activated or hyperproliferative” vs “normal” differentiation programs. These programs are superficially similar in that a stratified epidermis with cornified cells forms, but each involves the expression of different structural proteins [6]. It appears that these two pathways are not mutually exclusive, so that cells can express a mixed phenotype in some situations.

Terminal differentiation in both phenotypes involves degradation or remodeling of phospholipids, cytoplasm, cell organelles, and the nonkeratin cytoskeletal components [7]. Little is understood about the regulatory mechanisms for initiation of these global degradative processes. Lysosomal breakdown and release of lysosomal proteinases to the cytosol is unlikely, as there is no evidence that the terminally differentiating cell has a decreased pH and lysosomal enzymes are not very active at neutral pH. In fact, a major

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differentiation specific protein, cystatin, is a known inhibitor of lysosomal proteinases [8]. The enzymes responsible for specific processes in terminal differentiation have been shown to have neutral pH optima. For example, the cross-linking of proteins at the internal cytosolic face during formation of the cornified cell envelope involves a transglutaminase having a neutral pH optimum [9]. Proteolytic processing of profilaggrin in normal terminal differentiation apparently also involves a chymotrypsin-like proteinase having a neutral pH optimum [10]. More recently, it has been suggested that epidermal differentiation resembles apoptosis [11,12] with terminal differentiation being only a variation of these pathways involving activation of cytoplasmic and nuclear proteinases having neutral pH optima. Indeed, the morphology of epidermal cells indicates occurrence of orderly disassembly, although the precise similarity of these events to apoptosis is disputed [7].

Differentiation in cultured keratinocytes is less clearly defined [13]. Cultured keratinocytes predominantly follow the hyperproliferative differentiation pathway and are inefficient at the final steps in cornification. Pre-confluent cells produce basal cell keratins, consistent with their status as dividing cells, and are usually considered analogous to basal epidermal cells. These cells stratify at confluence and begin producing keratins K6/K16 and high levels of involucrin. Under some culture conditions, low amounts of K1/K10, loricrin and profilaggrin are produced, but processing of profilaggrin to filaggrin, thin cornified cell envelopes, and poor barrier function due to absence of specialized lipids are seen [14]. When cultured on reconstituted dermis at the air/medium interface, keratinocyte differentiation is more normal suggesting that growth and differentiation factor gradients are important in ordering the tissue and determining the balance between the two phenotypes [6,15]. Thus cultured cells have a mixed phenotype, and the balance between the two extremes can be modulated by external signals.

A number of models are currently available for studying in-vitro keratinocyte differentiation. When cells become confluent in media with high calcium concentration, both cell-cell contact and increased calcium levels initiate differentiation [16]. A second approach is to induce keratinocytes to differentiate by suspending the proliferating cells in a matrix, typically methyl cellulose [17]. In a third approach, the organotypic method, [18] where the epithelium is cultured on a substrate at the air-liquid interface, the keratinocytes form layers representative of each stage of differentiation, much like that observed in vivo. Each approach has its own advantages and disadvantages. For example, in the first two approaches, differentiation can be observed in a relatively short period of time. The three dimensional characteristics of the organotypic culture provide the unique advantage of temporal and spatial study at the expense of technically difficult methods that limit the extent of studies.

Several authors have used a confluence-induced differentiation model for the study of in vitro epidermal differentiation [19,20]. Profilaggrin is expressed but not processed to filaggrin in submerged cultures of normal human keratinocytes [21] or in long-term mouse keratinocyte cultures [22].

The HaCaT cell line, a spontaneously immortalized human epidermal cell line from adult skin that maintains epidermal differentiation capacity [27], has a transformed phenotype in vitro but remains nontumorigenic. Despite altered and unlimited growth potential, these cells show differentiation similar to normal cells expressing keratins 1 and 10, as well as involucrin, providing a promising tool for studying regulation of keratinization in human cells. Though this cell line is widely used to study keratinocyte differentiation [28], profilaggrin expression is variable depending on the line studied and processing to filaggrin is not seen (unpublished results).

While profilaggrin is expressed in lifted cultures, the model is not ideal for biochemical studies because of the difficulty of the technique and cell heterogeneity. The ideal culture system would be the one where cells carry out differentiation in a synchronous manner or in response to changes in the medium. A rat keratinocyte cell line developed by Kubilus and Baden closely meets these criteria; this immortalized cell line stratifies at confluence and produces differentiation specific proteins when grown in submerged culture directly on tissue culture plastic dishes [23]. These are the only keratinocytes capable of processing profilaggrin to filaggrin in submerged culture, and these processing events appear to be partially synchronized [24,25,26].

In this work using the rat immortalized cell line, we show that when profilaggrin expression decreases as a function of passaging, these keratinocytes undergo a type of senescence which can be blocked by an inhibitor of fibroblast senescence. Furthermore, high levels of nonspecific proteolysis of profilaggrin, occurring most likely via autophagocytosis, reduces filaggrin yield. By adjusting culture conditions, down-regulation of nonspecific proteolysis of profilaggrin can be decreased with concomitant increase in filaggrin yield, presumably shifting the balance towards normal differentiation.

Materials and Methods

Cell Culture methods

A rat keratinocyte cell line derived from newborn rat epidermis [23] was generously provided by Drs. Kubilus and Baden (Dept. of Dermatology, Massachusetts General Hospital, Boston, MA). The initial cell line was received at passage 23 and expanded through passage 25, at which time the original line was frozen and stored over liquid nitrogen. For subsequent studies, cells were thawed and seeded in six-well plates (Corning Glass Works, Corning, NY) at 1.4×10^3 cells/cm^2 in Dulbecco's modified Eagle medium (DMEM; low glucose, Life Technologies Inc.) (calcium concentration in DMEM is 1.2 mM) with 10% fetal calf serum (HyClone, Logan UT) (approximate calcium concentration of FBS is 3.3 mM [29]). 0.4 μg/ml hydrocortisone (Sigma), 100 units of penicillin/ml and 100 μg of streptomycin/ml (Penstrep, Life Technologies, Inc.). Cells were cultured at 36.7°C in a humidified atmosphere containing 5% CO_2. Cultures were fed for 3 or 4 days after plating and cells became confluent after 6 days, as judged by phase microscopy. Cells were transferred to keratinocyte growth medium containing growth factors (KGM; Clonetics, San Diego CA) at confluence and fed every second day, except as indicated. Pharmacological agents were mixed into fresh medium at the time of feeding. Aminoguanidine nitrate (Aldrich, Milwaukee WI) was prepared as a 0.75 M stock solution in distilled water and stored as a precipitated salt at 4°C. Before diluting into media, the precipitated salt was resolubilized by heating to 37°C.

Cornified cell envelopes

To determine the number of cornified cell envelopes, two ml of 1% SDS, 50 mM β-mercaptoethanol, and 50 mM Tris at pH 6.8 was added to the culture dish to solubilize the cells. Samples were boiled for 5 min, and an aliquot taken for counting cornified envelope ghosts in a hemacytometer. After pelleting the cell remnants, samples were analyzed by Western blotting to determine the extent of profilaggrin processing.

Analysis of Cell Extracts by SDS-PAGE and Western blotting

Following experimental manipulation, extracts were analyzed by SDS-PAGE as described previously [26]. Briefly, the medium was removed and the cells harvested by scraping the dish. Cells were homogenized (30 μl of buffer/cm^2 plate area) in 9M urea, 50 mM Tris at pH 8.0, 10 μg/ml phenylmethylsulfonyl fluoride, 1 mM EDTA, and 0.1 mg/ml aprotinin

(Sigma), and 5 μL of the supernatants were analyzed by SDS-PAGE on 4-15% gradient gels. Since differentiation of keratinocytes involves degradation of cellular contents, as well as synthesis of large and variable amounts of keratins [25] a constant proportion of each dish was loaded into each gel lane [30]. Gels were stained with Coomassie Blue R-250 or Western blotted using protease enhanced transfer, as previously described [10]. Blots were probed with a rabbit antiserum raised against purified rat filaggrin which also reacts with profilaggrin and the large processing intermediates [31]. The antigen antibody complexes were visualized with peroxidase conjugated goat antibody using 4-chloro-1-napthol as substrate.

To quantify the transfer efficiency and reactivity of profilaggrin and filaggrin, ^{3}H-histidine proteins were labeled in vivo and purified [32]. Protein concentrations of the purified profilaggrin and filaggrin were determined by amino acid analysis. Samples were either run on gels for electrotransfer or dot blotted. Efficiency of transfer or binding was assessed by quantifying bound radioactivity. Immunoreactivity of the transferred protein was determined by reacting with filaggrin antibody in the same manner as for Western blots, followed by scanning the peroxidase reacted blots to determine the relative reactivity normalized to the protein bound.

RNA isolation, northern blot analysis, and nuclear run off assays

Procedures were as described earlier [33].

Lipid aggregation

Phosphatidylserine/Triton X-100 mixed micelles were prepared by the method of Lee and Bell [34]. A phosphatidyl serine stock solution in chloroform (Avanti Polar Lipids Inc. Alabaster AL) was dried under a nitrogen stream and the lipid was resuspended in 0.3% Triton X-100, 20 mM HEPES, pH 7.4 with vortexing. The micelle solution was incubated at 37°C for 10 min., and an equal volume of filaggrin in water was mixed. The mixture was incubated at room temperature for 1 hr and centrifuged in a microfuge at 15 K rpm for 15 min. The supernatants were removed and both pellets and supernatants were analyzed by SDS-PAGE.

Electron microscopy

To visualize the cell structure, the culture medium was removed, and cells were fixed with one-half strength Karnovsky's fixative [34] in 0.1 M cacodylate buffer, postfixed in 2% OsO_4 in distilled water, dehydrated with ethanol, and embedded in situ in Epon as previously described [35,36]. Thin sections were stained with uranyl acetate and lead citrate, and viewed with a Philips 420 scanning/transmission electron microscope.

The two types of granules present in the keratinocytes (similar to the L-granules containing loricrin, and P-granules containing profilaggrin) were detected as described in [37].

Formation of lipid/filaggrin aggregates

Phosphatidylserine (PS)/Triton X-100 mixed micelles were prepared by mixing PS and the nonionic detergent at different mole percents. The mixed micelles were added to filaggrin and incubated at room temperature for 1 hr. Samples were spun at 15K rpm for 15 minutes. Supernatants and pellets were analyzed separately by SDS-PAGE.

Results

Processing of profilaggrin to filaggrin vs nonspecific proteolysis and decrease in profilaggrin with passaging in the rat cell line

As previously reported [24,25], Western blots of extracts of immortalized rat keratinocytes showed that cells synthesized profilaggrin at confluence. Similar expression of K1 and K10 as a function of confluence in medium with relatively high calcium concentration has been shown previously [19].

Fig.1a shows the time course of profilaggrin expression and processing. Below profilaggrin, a “smear” of immunoreactive material was seen, migrating on nonequilibrium isoelectric focusing in a manner similar to that of profilaggrin (data not shown). Since normal processing involves extensive dephosphorylation before proteolysis, altering the protein's pI, this suggests that the smear was due to nonspecific proteolysis of profilaggrin. Typically, processing intermediates became apparent one to two days after confluence and Filaggrin was observed between 2 to 6 days post confluence (compare Fig. 1, 5, and 6). Cornified cell envelopes could also be identified in the SDS extracts of the cultures (data not shown). Quantitative conversion of profilaggrin to filaggrin cannot be measured using Western blotting because the large difference in charge and size differentially affects both transfer, and detection (pI of profilaggrin is ~6, and pI of filaggrin is >10.5). Parallel experiments using radiolabeled mouse profilaggrin and filaggrin showed that immunological detection of filaggrin is 5-10 fold less efficient than profilaggrin (not shown). Furthermore, in the Western blotting protocol used for these studies, profilaggrin transfer was facilitated by electroeluting a protease into the gel, thus breaking profilaggrin and its large processing products down to more easily transferred products. While complete transfer of profilaggrin could be achieved under optimal conditions, more commonly often up to 50% of the profilaggrin might remain in the gel. Using a 7.5 fold correction factor for immunological detection and assuming 50% of the profilaggrin was transferred, the efficiency of “normal” processing to filaggrin in the rat cultures ranged from undetectable to 10%, averaging 5%. Maximum processing to filaggrin was reached at 5 days, and a longer time of culture did not increase this.

Fig. 1b & c show decreased profilaggrin expression as a function of passage. With passaging, less profilaggrin was produced (Fig. 1b). The cells still proliferated and stratified to the same extent in early vs late passage cultures. This down-regulation was not due to variation in culture conditions, as a second frozen aliquot of cells, thawed and grown in parallel with the late passage cultures, produced a similar amount of profilaggrin as the early passages of the first aliquot (Fig. 1c), and the same decrease was seen during passaging of several frozen aliquots.

Role of aminoguanidine in profilaggrin/filaggrin expression in the rat cell line

Fig. 2a & b show the role of aminoguanidine on profilaggrin expression in passaging. Although cell viability was not affected, it seemed reasonable to regard the decrease in profilaggrin (a late stage differentiation marker) as a type of senescence. We therefore tested whether the down-regulation of profilaggrin could be prevented by aminoguanidine, which blocks senescence of cultured fetal fibroblasts [38].

Cells (passage 26) were thawed into medium containing aminoguanidine and subcultured into several lines, each of which was passaged independently. Every four passages, one dish derived from each line was grown to six days post-confluence (one day after feeding) [33], and cell extracts analyzed for profilaggrin expression by Western blotting. Blots show that in the absence of aminoguanidine, profilaggrin expression was markedly decreased as a function of increasing passage number (Fig. 2a). In the presence of aminoguanidine,

profilaggrin expression remained high in some, but not in all of the independently maintained cell lines (data not shown). We have repeated this last observation with 12 thaws of the original cell line. Thus, culturing in aminoguanidine was necessary, but not sufficient, for sustained ability to express profilaggrin at confluence.

Dose-response curves showed the optimal aminoguanidine concentration for preserving profilaggrin expression was 0.75 mM (not shown). After the initial selection for an expressing subcultured line, we observed stable expression of profilaggrin for an additional 50 or more passages. Removal of aminoguanidine from stable subcultured lines resulted in decreased profilaggrin expression over the ensuing ten passages. Addition of aminoguanidine to the medium failed to recover profilaggrin expression in cell lines where expression had decreased, suggesting that the change is irreversible (not shown). mRNA levels mirrored protein levels seen by the corresponding immunoblot (Fig. 2b), suggesting either transcriptional regulation or message stability to be implicated in this process. Nuclear run off assays showed no differences in profilaggrin transcription, indicating regulation must be at the level of message stability (data not shown).

Decrease in profilaggrin processing and autophagocytosis

The large amount of nonspecific proteolysis of profilaggrin as evidenced from figure 1, suggested autophagocytosis was occurring. To test this, cells were cultured in presence of 3-methyladenine (Fig. 2c), an inhibitor of PI-3 kinase that inhibits maturation of autophagosomes [39]. Expression of profilaggrin was enhanced, with an optimum concentration of 6 mM. Above 75 mM, decreased cell viability was detected. Nonspecific proteolysis was still observed (blot at 72 hrs. in Fig. 2a shows very little filaggrin signal due to almost complete nonspecific profilaggrin processing) even over a wide dosage range (not shown), probably because this inhibitor does not completely block autophagocytosis (see Fig. 3).

Evidence for autophagocytosis was also observed in electron micrographs of cultured cells. After confluence, when profilaggrin processing was well underway, a few cells were observed that still contained keratohyalin granules with normal cell morphology (Fig. 3a, & b). These cells presumably had not begun processing profilaggrin. In most areas of the dish, most of the intermediate filaments were bundled near the nucleus. In addition, large, round aggregates of what appeared to be lipid bilayers were seen (Fig. 3c & d). These were much larger than lamellar bodies and did not have the internal flat disks associated with those structures [40]. In the presence of 3-MA, there were large numbers of lipid bound areas that appeared to be phagosomes, with cytoplasm in them (Fig. 3e & f). This is consistent with the expected accumulation of phagosomes in 3-MA treated cells, due to the inhibition of autophagosome maturation, but not their formation. Within these vesicular structures, there were small bilayer aggregates similar to those seen in the nontreated cells, supporting the idea that these deposits are the remnants of the autophagocytic process. In a few cases, these appeared to contain intact keratohyalin granules. In some areas, we observed lipid vesicles apparently associating with the phagosomes.

Association of filaggrin with lipid

We hypothesized that the formation of lipid aggregates might be due to association of profilaggrin processing products with phospholipid vesicles. In vitro studies showed that phospholipid liposomes precipitated filaggrin efficiently (Fig. 4). When filaggrin was held constant at 0.1 mg/ml, there was little precipitation below 10 mole percent phosphatidylserine in the liposomes; maximum precipitation was observed around 15 mole percent, which is the typical concentration of phosphoserine in vivo. Above 30 mole percent, precipitation was again absent. These results are consistent with the presence of

several phospholipid binding sites on filaggrin with aggregation occurring at a fairly narrow filaggrin/liposome ratio, similar to the phenomenon of antibody precipitation with an antigen. Electron microscopy of these mixed filaggrin/phospholipid vesicles showed formation of stacked structures with spacing similar to that of the membranes in the aggregates observed in the cultured cells Fig. 3d & e (data not shown). Generated in vitro, by mixing sonicated liposomes with filaggrin, mixed vesicles with elongated structures are produced, whereas mixed vesicles with rounded structures are produced in culture. Spatial constraints during aggregate formation are a possible explanation for the differences in the shapes of the vesicles.

Maximizing processing by mimicking tissue [Ca^{2+}] gradients

These results suggest that down-regulation of vesicular transport and/or autophagocytosis occurs in epidermal differentiation before release of filaggrin, and that this is not occurring in the cultured cells. We hypothesized that known factor gradients might coordinately regulate this postulated event with filaggrin release. Two known gradients in epidermis are decreasing serum components and increasing calcium concentrations in the upper cell layers. To test the effects of serum and external calcium concentration, cells were cultured in serum/DMEM or in serum free KGM (Fig. 5). In KGM vs. serum/DMEM, there is a decrease in nonspecific proteolysis of profilaggrin, and significantly higher yield of filaggrin. Raising external calcium concentrations above the level (1 mM) found in serum, enhances processing with maximum processing occurring at $[Ca^{2+}]_{ext}$ = 5 mM [41].

In mouse keratinocyte cultures, optimum production of early differentiation markers is observed at $[Ca^{2+}]_{ext}$ lower than 1 mM, which is the level of calcium present in serum [13,42]. In an attempt to mimic this more closely in the rat keratinocyte cultures, cells were cultured in intermediate calcium concentrations for several days in order to induce profilaggrin processing before shifting to 5 mM $[Ca^{2+}]_{ext}$. Better processing was achieved when confluent cells were first grown at an intermediate $[Ca^{2+}]_{ext}$ =300-450 μM for several days before shifting to $[Ca^{2+}]_{ext}$ = 5 mM, rather than growing first at an intermediate $[Ca^{2+}]_{ext}$ = 150 μM and then shifting to $[Ca^{2+}]_{ext}$ =5mM (Fig. 6).

Discussion

These studies show that in comparison to <5% of the profilaggrin being processed to filaggrin using previously described culture conditions, medium composition for submerged rat keratinocyte cultures can be modulated to achieve near complete processing of profilaggrin to filaggrin. Optimal expression of profilaggrin was found when the cell line was maintained in serum/DMEM supplemented with aminoguanidine, although maintenance in aminoguanidine had no effect on the extent of processing that could be achieved after confluence. The observation that aminoguanidine was necessary, but not sufficient, for developing cell lines that continue to express profilaggrin suggests that this compound may facilitate selection of profilaggrin expressing cells. Because the proliferative potential was not affected, this loss of profilaggrin expression in untreated cultures might be similar to the reduced profilaggrin expression observed in aging skin (unpublished observations), and the effect of the drug may be similar to reversal of senescence in fibroblasts. These observations were further strengthened when a recent study suggested that the anti-oxidant property of aminoguanidine might have an anti-aging effect on cultured fibroblasts [43]. Other roles of aminoguanidine include inhibiting nonenzymatic glycosylation [44], polyamine formation and NO production [45]. Though the medium used in our studies has 5.6 mM glucose (comparable to serum glucose levels), sequestration of epidermis from serum might mean that this level is still too high. Inhibition of polyamine formation protects mouse embryo cell lines from early senescence. NO signaling has been implicated in transcriptional silencing by DNA methylation.

The large amount of nonspecific proteolysis suggested autophagocytosis; this hypothesis is supported by the observation of enhanced profilaggrin expression in the presence of 3-MA, an inhibitor of PI-3 kinase that slows maturation of autophagosomes [39].

The evidence argues that autophagocytosis is being induced, probably by membrane aggregation. In support of this model, electron microscopy of cells treated with 3-MA demonstrated an accumulation of late stage autophagosomes, consistent with the expected effect of 3-MA on autophagosome maturation, but not on formation. These contained small lipid aggregates similar to the larger ones seen in the nontreated cells, suggesting that the larger aggregates are remnants of the autophagocytosis process. Autophagocytosis involves sequestering of cytoplasm and cell organelles into membrane bound structures formed by vesicle fusion [46]. After formation, the nascent autophagosomes acquire H^+-ATPases and acidic hydrolases by fusion with lysosomes [47,48,49] or late endosomes/pre-lysosomes [50,51] to form late acidic autophagosomes where the included contents are degraded. It is this latter fusion that is regulated by PI-3 kinase, and inhibited by 3-MA.

Little is known about the role of autophagy in keratinocyte physiology; however, autophagy at a low level is thought to play a reparative function for disrupted areas of all cells and makes a significant contribution to normal protein turnover [52,53]. The trigger for autophagocytosis in the rat keratinocytes may be disruption of intermediate filaments by filaggrin, because disruption of the cytoskeleton is known to induce autophagocytosis [54]. Another possible trigger became apparent when it was noted that filaggrin, which has a pI of ~10, aggregated acidic phospholipid vesicles in vitro, and these filaggrin/phospholipid aggregates resembled the aggregates seen in cultured keratinocytes.

These data suggest that profilaggrin processing products might disrupt vesicular trafficking, which is also known to induce autophagocytosis [55]. Additional evidence that release of filaggrin induces membrane dysfunction in cells can be seen in studies where an expression construct that encodes filaggrin is transfected into cells [56].

Significantly improved processing to filaggrin was observed when cells were shifted to serum-free artificial medium at confluence. Nonspecific proteolysis decreased and filaggrin yield increased when cells were shifted first to an intermediate $[Ca^{2+}]_{ext}$ for 1-2 days, then to 5mM $[Ca^{2+}]_{ext}$. The improved expression and processing of profilaggrin achieved by removal of serum and by the two phase calcium treatment may mimic gradients of growth and differentiation factors and calcium in epidermis that regulate epidermal differentiation [57,58,59], or by down-regulation of the DAPk family of kinases, (calcium/calmodulin regulated serine/threonine kinase implicated as a positive mediator of autophagocytosis) [60]. Raising external calcium concentrations above physiologic (serum) levels enhances profilaggrin processing [41]. Although 5 mM Ca^{2+} is supraphysiologic- and the effects on processing may be nonspecific, the requirement of high $[Ca^{2+}]_{ext}$ for maximum processing may also reflect the differences between skin and this in vitro model system. The serum effect may be related to the phenotype of cells in inflammatory diseases. In cultured human keratinocytes, serum induces tissue plasminogen activator [61] a marker for inflammatory response. Thus, serum appears to shift keratinocytes towards a hyperproliferative phenotype, similar to the effect of serum factors in a wound healing situation. The turn-over of profilaggrin by autophagocytosis and the unexpected avidity with which filaggrin aggregates phospholipid may shed new light on why keratinocyte lipids are so extensively remodeled, and suggests that down-regulation of cell processes, such as vesicular transport, are probably tightly regulated by the differentiation program. In conclusion, we have shown that a keratinocyte cell line maintained in a medium supplemented with aminoguanidine and 3-methyl adenine, following a two phase calcium treatment synthesizes and processes profilaggrin at confluence providing a culture model for studying epidermal differentiation.

This is particularly true in the transition stage, where organelle and cytoplasmic degradation, and lipid remodeling must be coordinated with specific and regulated protein processing events.

Profilaggrin, a major structural protein of the epidermis, aggregates the keratin cytoskeleton resulting in the formation of the cornified cell envelope. Loss or reduction of this major structural protein leads to varying degrees of impaired keratinization [62] implicating a number of diseases like ichthyosis vulgaris, atopic eczema, psoriasis, and a number of autoimmune disorders [63,64]. Hence, studies on filaggrin expression/profilaggrin processing provide new insights into the etiology of atopic diseases and might pave the way for development of new therapeutic approaches.

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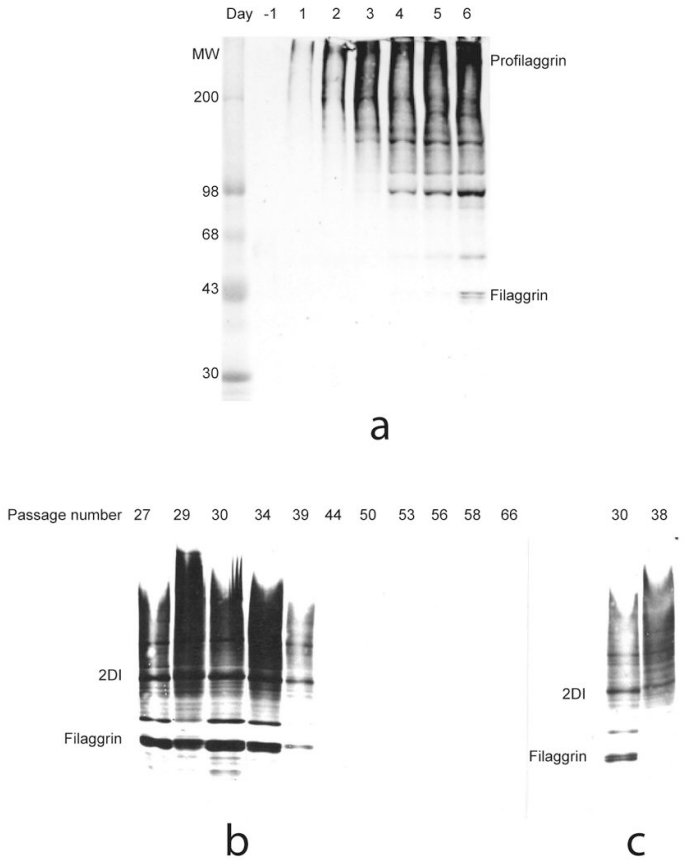


Figure 1.

Nonspecific proteolysis and decrease in profilafilaggrin expression with passaging. a. Western blot of extracts of cultured rat keratinocytes reacted with antibody to filaggrin from one day pre-confluent (-1) through several days of confluence (1 through 6). The band at 43 kDa comigrates with rat epidermal filaggrin, while the bands near 98 and 150 kDa are the sizes of the processing intermediates consisting of two or three copies of filaggrin joined by linker peptides (2DI and 3DI). Higher processing intermediates are probably also present, but obscured by the nonspecific proteolysis. A clear band for the intact profilaggrin was not observed (ProFG is marked where an intact profilaggrin band is expected). Processing to filaggrin occurred rather late, in this particular experiment (compare with Fig. 5, and 6). b. Western blot of extracts of keratinocytes on day 5 post-confluence at various passage numbers. c. Western blot of extracts of a second thaw of keratinocytes at passage 30 & 38 cultured in parallel with cells from the first thaw at passage 50 and 58 as shown in panel b.

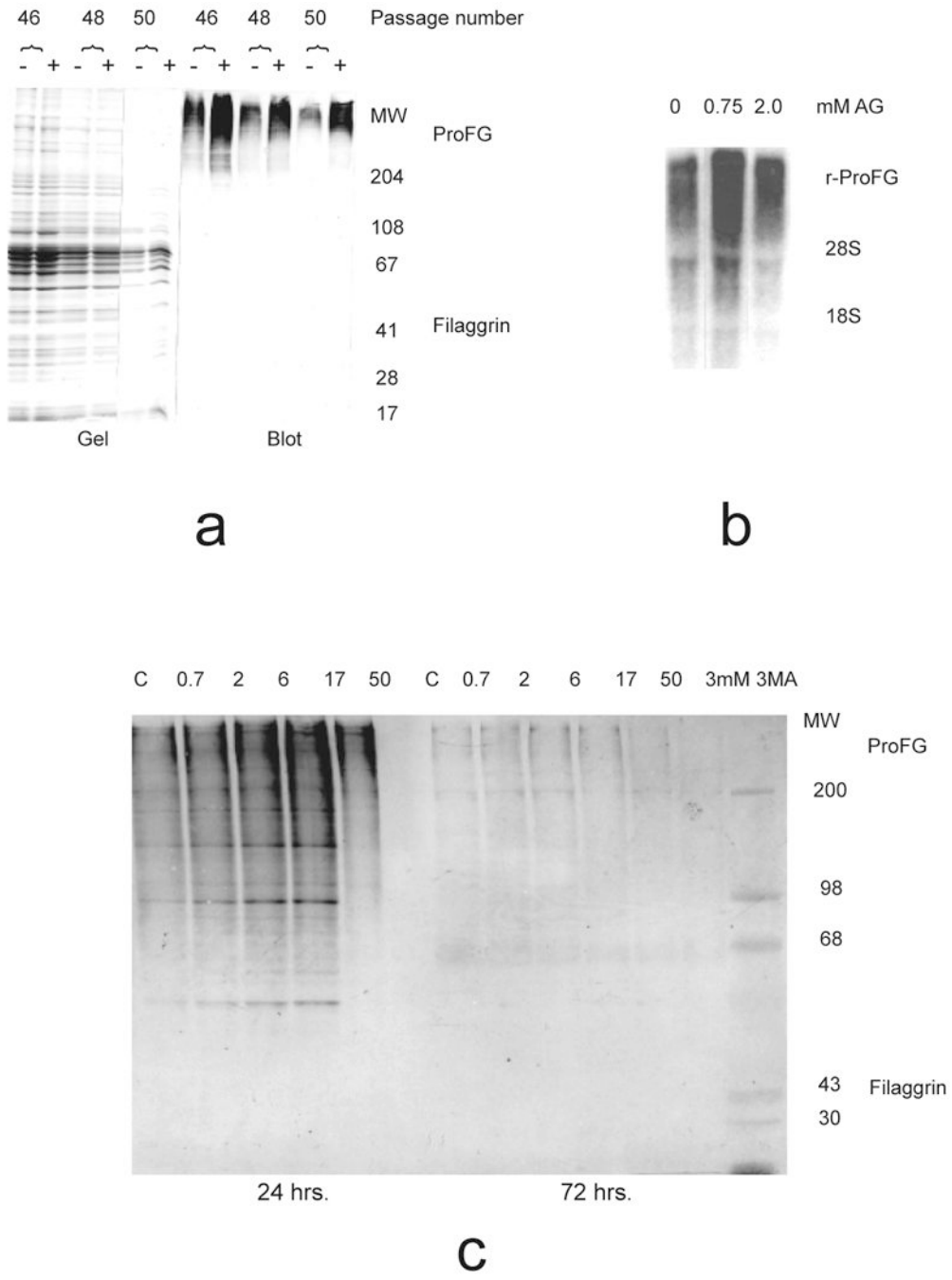


Figure 2.

Role of aminoguanidine and 3-methyl adenine in profilaggrin/filaggrin expression. ProFG and Filaggrin are marked where they normally appear. a. Gel and Western blot of equal protein loadings of extracts from cultures grown and passaged as separate cell lines in medium without (-) and with (+) 1mM AG and analyzed for profilaggrin. Cultures were grown in medium with or without aminoguanidine and studied beginning with passage 46. b. Northern blot analysis of cultures (passage 39) grown in 0, 0.75, and 2 mM AG showed that profilaggrin mRNA levels were highest in cultures in 0.75 mM AG, paralleling protein levels. c. Enhancement of profilaggrin expression by 3-methyl adenine. Various concentrations of 3-MA were added to the cells on the day they became confluent. Cells

were harvested after 24 and 72 hr., extracted, 5 ul of protein loaded in each lane, and analyzed by Western blotting with antibody to profilaggrin. The different concentrations of 3-MA added are indicated on the top.

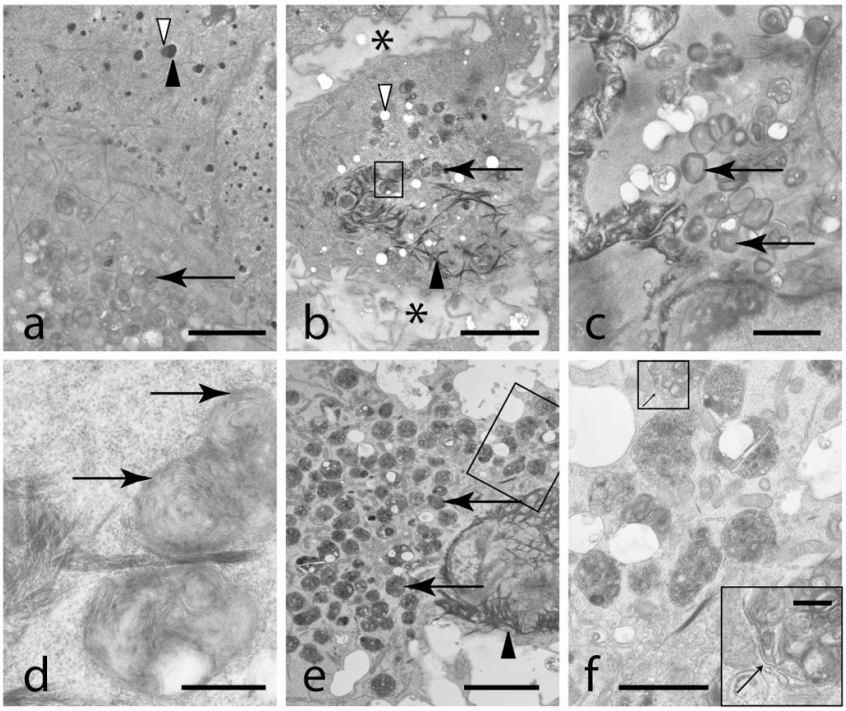


Figure 3.

Electron micrographs of rat keratinocytes at day 5 post-confluence. a. Control cells, in absence of 3-MA. An area of the dish with two adjacent cells. The upper cell has intact keratohyalin and is similar in morphology to those seen in (25). The L-granules are shown with open arrowhead, and P-granules with closed arrowhead. More commonly the cells in this sample resembled the lower cell, with no keratohyalin. Instead, many round lipid aggregates (arrow) were seen in the cytoplasm. Magnification bar = 3 μm. b. A second example of the disrupted architecture of the epidermal cells showing lipid aggregates (arrow). Note the perinuclear intermediate filament cage (closed arrowhead), the fact that many of the aggregates have solubilized in the fixative leaving round “holes” (open arrowhead), and the large interdigitated intercellular space (*). Rectangle delineates area expanded in d. Magnification bar = 5 μm. c. A magnified view of lipid aggregates (arrows), with some showing partial solubilization. Magnification bar = 2 μm. d. Shows a higher magnification of the aggregates (arrows) from panel b in the area adjacent to the perinuclear intermediate filament cage, showing appearance of lipid bilayers. Magnification bar = 0.5 μm. e. A typical cell from the 3-MA treated cultures showing accumulation of many vesicles (arrows), absence of intermediate filaments except in the perinuclear cage (closed arrowhead), and large intercellular spaces. Rectangle delineates area expanded in f. Magnification bar = 4 μm. f. Higher magnification of the area delineated in e, showing small lipid aggregates, similar to the large ones seen in panels a-d. Rectangle delineates area expanded in insert. The vesicles appear to contain condensed cytoplasm. Arrow identifies a double bilayer area where the vesicles appear to be forming from a flattened lipid vesicle. Magnification bar = 1.5 μm. Insert shows double layered vesicle more clearly. Magnification bar = 0.25 μm.

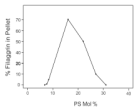


Figure 4.
Effect of phosphatidylserine (PS) on filaggrin solubility. Filaggrin and phospholipid vesicles were mixed as described in methods, centrifuged, and supernatants and pellets were analyzed separately by SDS-PAGE. The filaggrin in the pellets was determined by scanning a stained gel at different PS mole % in the mixed vesicles.

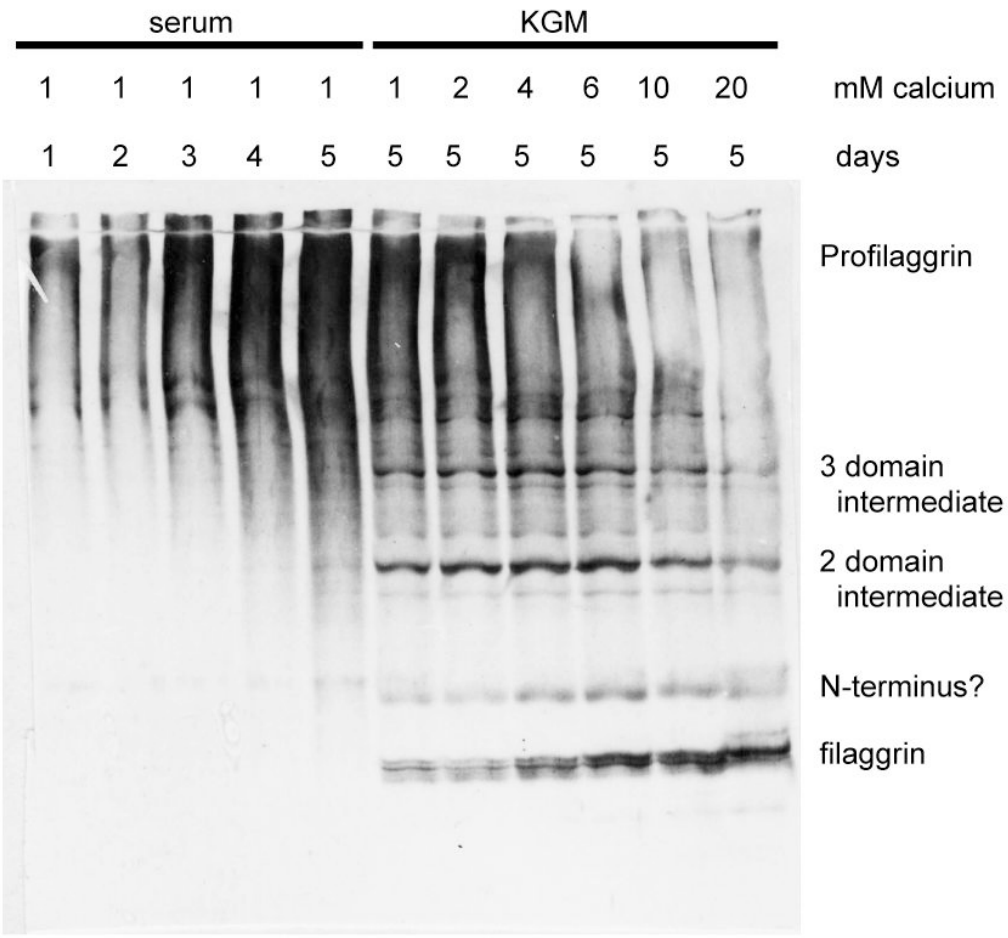
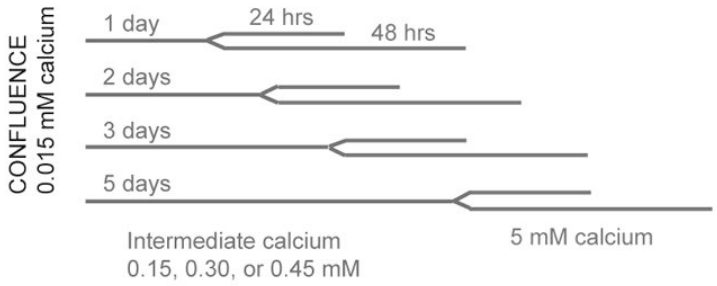


Figure 5.
Suppression of profilaggrin processing to filaggrin by serum. Cells were cultured +/- serum, with varying amounts of calcium. Processing of profilaggrin to filaggrin or intermediates in the presence of serum was variable; in this particular experiment none was seen (but see Fig. 1). The calcium dependence of processing was apparent, in that increasing calcium produced increasing filaggrin yields.



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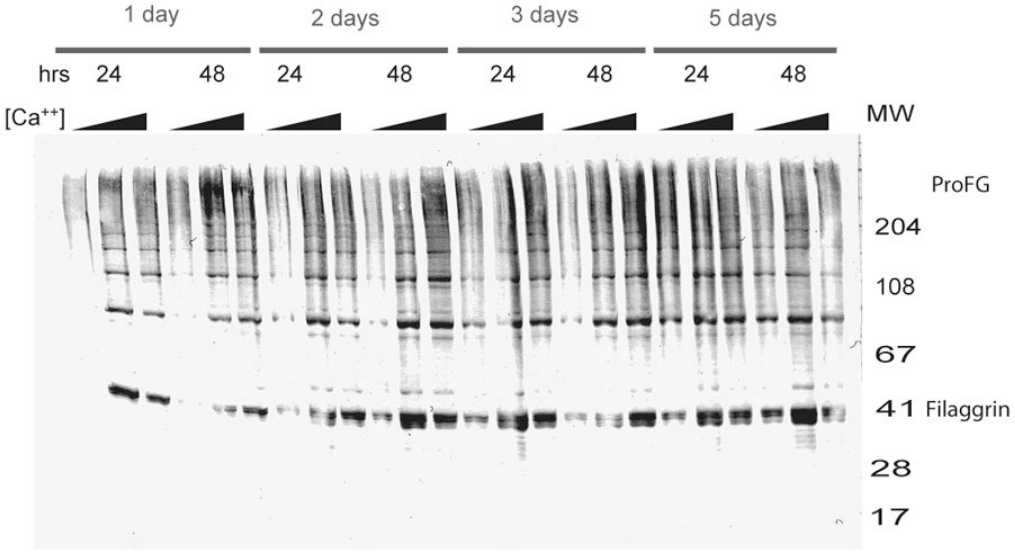

b

Figure 6.

Effect of culturing keratinocytes at an intermediate calcium concentration on processing of profilaggrin after shifting to 5 mM calcium. Cells were plated in serum containing medium, and transferred to 15 μM [Ca^{2+}] at confluence (sufficient to induce maximum profilaggrin synthesis). On the next day, cells were transferred into 0.15, 0.30, or 0.45 mM [Ca^{2+}] for 1,2,3, or 5 days, then transferred to 5 mM [Ca^{2+}] which induces near maximum profilaggrin processing (see Fig. 5) and harvested 1 or 2 days later.