The Hyaluronan Synthesis Inhibitor 4-Methylumbelliferone Prevents Keratinocyte Activation and Epidermal Hyperproliferation Induced by Epidermal Growth Factor

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Since excessive epidermal hyaluronan is associated with hyperproliferative states and disturbed terminal differentiation of the keratinocytes, we hypothesized that 4-methylumbelliferone (4-MU), an inhibitor of hyaluronan synthesis, could counteract these phenotypic features. Cultured epidermal keratinocytes showed a concentration dependent, maximum 83% reduction of hyaluronan in the presence of 0.2–1.0 mM 4-MU, whereas less decline was seen in the synthesis of chondroitin and heparan sulfate. The reduced hyaluronan was associated with no apparent change in its molecular mass. The 4-MU-treated keratinocytes showed an accentuated epithelial morphology with a flat, round cell shape, increased stress fibers and large vinculin-positive adhesion plaques, cytoskeletal changes consistent with the markedly reduced migration rate observed in scratched monolayer cultures. High concentrations of 4-MU also caused a block in keratinocyte proliferation, reversible upon 4-MU withdrawal. In the epidermis of organotypic cultures, 4-MU prevented the hyaluronan accumulation and epidermal hypertrophy induced by epidermal growth factor. The present results concur with earlier data indicating that enhanced cell locomotion and proliferation are associated with hyaluronan synthesis in activated keratinocytes. Cell proliferation, however, was blocked more strongly than expected on the basis of the incomplete hyaluronan synthesis inhibition, and may represent a novel target of 4-MU. At any rate, 4-MU and equivalent hyaluronan synthesis inhibitors might be considered for situations where suppression of epidermal activation and hyperproliferation is warranted.

Key words: hyaluronan/keratinocyte/4-methylumbelliferone/migration/proliferation
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Hyaluronan is a unique glycosaminoglycan of very high molecular mass (up to 10^7 Da), synthesized directly into the extracellular space by an enzyme (hyaluronan synthase) associated with the cytoplasmic side of the plasma membrane (Weigel *et al*, 1997; Tammi *et al*, 2002). Hyaluronan expression is associated with tissue remodeling during morphogenesis (Camenisch *et al*, 2000; Toole, 2001), wound healing (Mack *et al*, 2003), and cancer (Toole, 2001), where it is supposed to facilitate cell division and migration by creating a soft, transient matrix and by activating intracellular signaling cascades (Turley *et al*, 2002). A direct role of hyaluronan in cell proliferation was supported by the finding that transfection of Has2 gene to fibrosarcoma cells lead to accelerated cell proliferation and cell migration (Kosaki *et al*, 1999).

Hyaluronan is actively synthesized by keratinocytes and forms a loose extracellular matrix between adjacent basal and spinous cells of human epidermis (Tuhkanen *et al*, 1998). The location of hyaluronan between the vital cell layers suggests that as a space filler it contributes to the maintenance of the free interstitial space between keratinocytes, in order to facilitate exchange of metabolites between circulation and keratinocytes, and to maintain the plasticity of the epidermis by allowing keratinocyte movements and changes of cell shape during differentiation (Tuhkanen *et al*, 1998). Furthermore, hyaluronan synthesis creates signals that control migration and proliferation of epidermal keratinocytes (Rilla *et al*, 2002). A hyperproliferative and migrating phenotype is typical of epidermal activation. Activation occurs after epidermal injury and is common in many diseases involving epidermis, psoriasis as an example (Nickoloff and Turka, 1993; Tomic-Canic *et al*, 1998). Accordingly, the proliferation rate and thickness of epidermis, regulated by growth factors like EGF (Pienimäki *et al*, 2001; Pasonen-Seppänen *et al*, 2003), KGF (Karvinen *et al*, 2003), and TGFβ (Pasonen-Seppänen *et al*, 2003), is closely correlated with the synthesis of hyaluronan by keratinocytes. The tight association of keratinocyte hyaluronan synthesis to epidermal activation implies that inhibition of hyaluronan synthesis might suppress these phenotypic features, and modify epidermal differentiation.

The coumarine derivative 4-methylumbelliferone (4-MU) has been reported to specifically inhibit hyaluronan synthesis in cultured mammalian cells (Nakamura *et al*, 1995, 1997; Kosaki *et al*, 1999; Sohara *et al*, 2001). Although the same compound has been in clinical use as a spasmolytic

Abbreviations: bHABC, biotinylated hyaluronan binding complex; EGF, epidermal growth factor; HABC, hyaluronan binding complex; KGF, Keratinocyte growth factor; 4-MU, 4-methylumbelliferone; REK, rat epidermal keratinocyte; TGF-β, transforming growth factor beta

drug (Stacchino *et al*, 1983), as far as we know, its effects on skin or skin diseases has not been studied. In this work, we show that the inhibition of hyaluronan synthesis by 4-MU in epidermal keratinocytes was associated with low cell proliferation, and cytoskeletal changes consistent with markedly reduced migratory activity. Interestingly, the suppression by 4-MU was more potent in a hyperproliferative epidermis created by epidermal growth factor, suggesting that it might be useful in normalizing such conditions. The data stress the importance of hyaluronan synthesis in epidermal activation.

Results

4-MU inhibits hyaluronan synthesis and its EGF-induced stimulation in keratinocyte cultures In the standard culture medium with 5% fetal bovine serum (FBS), slightly preconfluent rat epidermal keratinocyte (REK) cultures secreted approximately 4 ng of hyaluronan per 10,000 cells in 24 h, as measured by the ELSA assay. A concentration-dependent decrease in the amount of secreted hyaluronan was found with 0.2–1.0 mM concentrations of 4-MU (Fig 1*A*, *C*). With 0.2 mM 4-MU the relative inhibition was $\sim$ 20%, whereas at 1 mM concentration the relative inhibition was $\sim$ 35% and $\sim$ 60% after treatments of 6 and 24 h, respectively (Fig 1*A*, *C*).

A concentration-dependent decrease in the total amount of hyaluronan synthesized in the culture (medium and cell layer combined) was also found using quantitative metabolic labeling (Table I). Furthermore, it appeared that the synthesis of hyaluronan was more sensitive to 4-MU than the synthesis of sulfated glycosaminoglycans (Table I). Thus, 0.2 mM 4-MU had little effect on chondroitin sulfate (CS) or heparan sulfate synthesis. At 0.5 mM and 1 mM of 4-MU the synthesis of heparan sulfate was also decreased, but less than that of hyaluronan, whereas no constant effect was seen in CS (Table I).

Hyaluronan synthesis is strongly induced in keratinocytes by effectors like EGF (Pienimäki *et al*, 2001; Pasonen-Seppänen *et al*, 2003) and KGF (Karvinen *et al*, 2003). As compared to its effects on non-stimulated cells (Fig 1*A*, *C*), 4-MU caused a relatively more prominent, 65–85% inhibition in cells treated with EGF (Fig 1*B*, *D*), i.e., 4-MU was particularly effective in inhibiting the hyaluronan synthesis in activated keratinocytes.

A 6 h treatment with 4-MU did not change the molecular mass distribution of the secreted hyaluronan (Fig 1*E*) or that associated with the cell layer (data not shown), when analyzed with gel filtration.

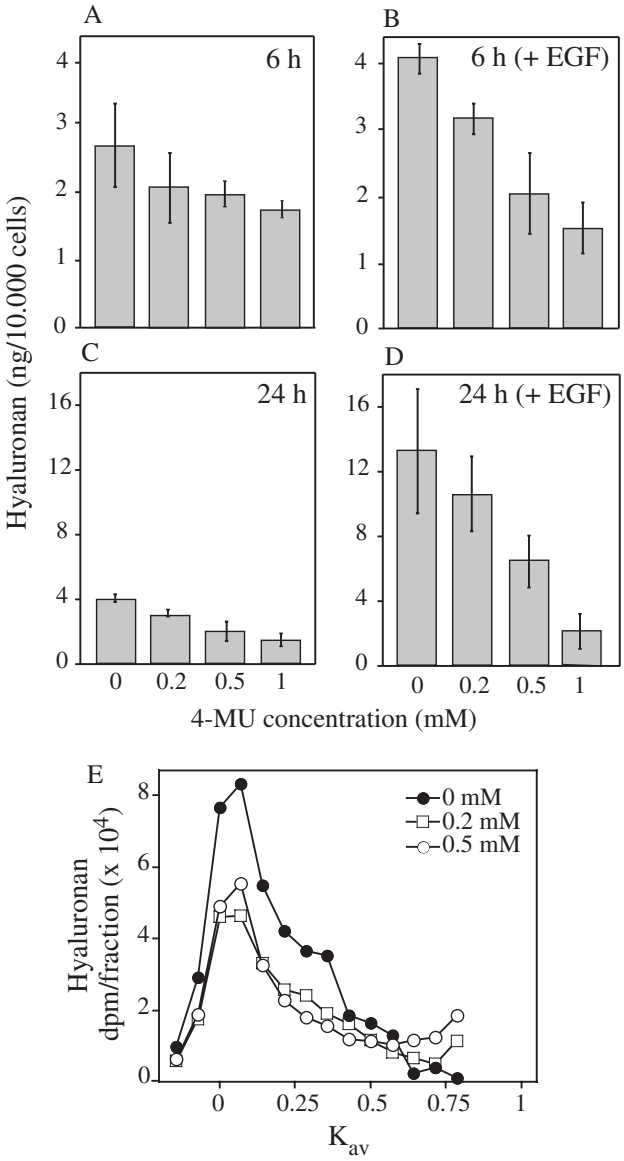

Figure 1
Effect of 4-MU on hyaluronan secreted in the growth medium of epidermal keratinocytes. Subconfluent REK cultures were subjected to the indicated concentrations of 4-MU for 6 h (*A*, *B*) and 24 h (*C*, *D*), with (*B*, *D*) and without 20 ng per mL of EGF (*A*, *C*), and the media were analyzed for hyaluronan concentration using the ELSA assays. The data represent means and ranges of two independent experiments. The molecular mass distribution of metabolically labeled hyaluronan secreted into the culture medium during 6 h incubations with different concentrations of 4-MU was analyzed with gel filtration on a 1 $\times$ 30 cm column of Sephacryl S-1000 (*E*).

Hyaluronan localization in REK is changed by 4-MU In control cultures, hyaluronan visualized with the biotinylated hyaluronan binding complex (bHABC) probe was typically localized as dense patches (Fig 2*A*), mostly on the dorsal cell surfaces, and frequently colocalized with CD44 (Fig 2*C*) (Tammi *et al*, 1998), whereas the ventral surface showed a lower hyaluronan signal, except close to the edge of the lamellipodia (Fig 2*E*). Hyaluronan in 4-MU-treated cells had a generally lower staining intensity, and it was more diffusely distributed (Fig 2*B*). Moreover, the remaining hyaluronan in the 4-MU-treated REK was mostly on the underside of the cells (Fig 2*D*), often surrounded by a circle of adhesion plaques (Fig 2*F*).

Cytoskeletal rearrangements in 4-MU-treated REK In subconfluent REK cells, the lamellipodia usually present in control cultures were largely lost by 4-MU-treatment, and the cells became flat and circular (Fig 2*B*, *D*, *F*, *H*). These changes in cell shape started already after 1 h in 1 mM 4-MU when monitored by phase contrast microscopy of live cells (data not shown). In control cells, phalloidin staining visualized filamentous actin in the cell cortex and in numerous filopodia (Fig 2*G*), whereas the filopodia were severely

Table I. REK cultures were metabolically labeled with ^{3}H-glucosamine and ^{35}S-sulfate for 6 h, and the glycosaminoglycans synthesized were analyzed using Superdex Peptide chromatography

4-MU concentration (mM)	Glycosaminoglycan (ng per 10,000 cells)		
	HA	CS	HS
0	1.08 ± 0.04	0.38 ± 0.21	5.82 ± 2.27
0.2	0.46 ± 0.09	0.54 ± 0.37	5.13 ± 2.15
0.5	0.29 ± 0.03	0.49 ± 0.33	3.59 ± 0.83
1	0.19 ± 0.01	0.46 ± 0.34	1.87 ± 0.21

The data represent the total amounts of glycosaminoglycans synthesized in the cultures (cells + medium), expressed as means and ranges from two independent experiments.

REK, rat epidermal keratinocyte; 4-MU, 4-methylumbelliferone; HA, hyaluronan; CS, chondroitin sulfate; HS, heparan sulfate.

depleted in 4-MU-treated REK. Instead, stress fibers, very rare in control cultures, appeared after a 6 h treatment with 4-MU, often forming a spindle-like structure in the middle of the cell (Fig 2*H*). The cytoskeletal changes induced by 4-MU included an increased number and size of adhesion plaques under the cells, as demonstrated by confocal microscopy of an immunohistochemical staining for vinculin (Fig 2*E*, *F*). The circular arrangement of the vinculin-positive adhesion plaques (Fig 2*H*) appeared to fix the cell periphery to the substratum and was correlated with a truncation of lamellipodia and filopodia. In addition, microscopic assays showed that all EGF-induced features in the REK phenotype (Pienimäki *et al*, 2001), including accumulation of cell surface hyaluronan, and the lifted, elongated cell shape, were efficiently blocked by 4-MU (data not shown).

4-MU inhibits REK cell migration Removing cells by scratching with a pipette tip from the bottom of the dish induces rapid migration of the REK, completely covering the cleared area in less than 2 d. In the presence of 0.1 mM 4-MU, a 25% reduction in the migration was found in a 24 h follow up, whereas 0.5 mM 4-MU produced 60% inhibition (Fig 3*A*). The inhibition was obvious already after 3 h (Fig 3*B*), excluding the possible contribution of cell proliferation in filling the cleared area.

High molecular weight hyaluronan added to the culture medium caused ~10% increase of the migration rate in both control and 4-MU-treated cultures (data not shown), indicating that 4-MU does not block the relatively small migration stimulation shown previously to occur in REK by exogenous, soluble hyaluronan (Rilla *et al*, 2002).

4-MU inhibits cell proliferation Control REK doubled their numbers approximately every 20 h until confluency slowed down the proliferation. Treatment with 0.2 mM 4-MU reduced the number of cells by 50% on the third day in culture, whereas with 0.5 mM dose, cell proliferation was completely arrested (Fig 3*C*). The cells were also counted 4 h after plating, resulting in equal numbers for control and 4-MU-treated cultures, which indicates that 4-MU does not disturb cell attachment to substratum. The number of dead, floating cells (on average 2.4% of those bound) was also similar to

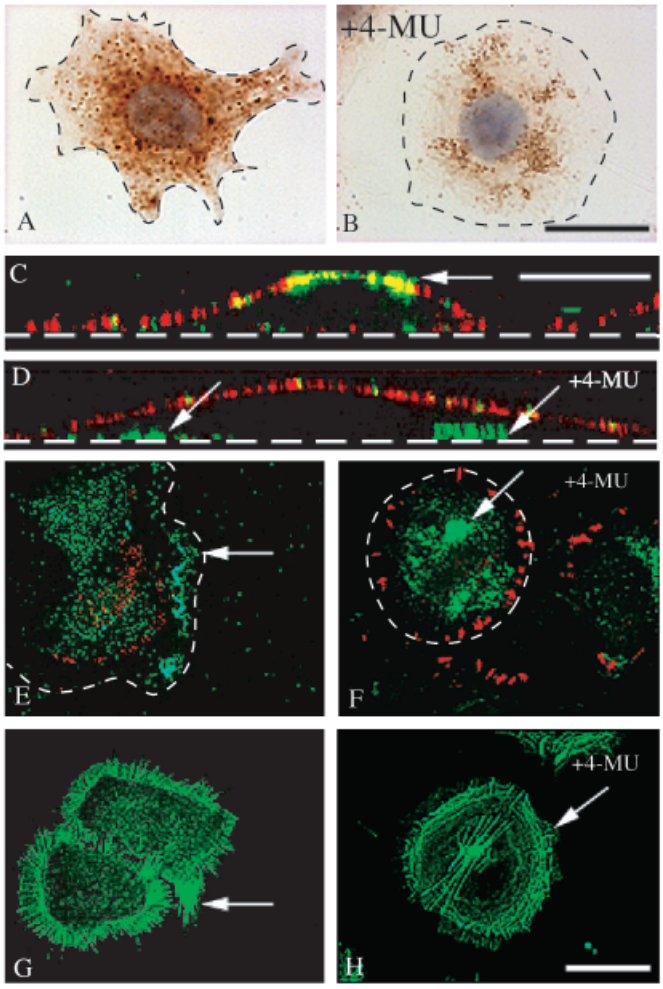

Figure 2
Distribution of cell-associated hyaluronan, CD44, actin, and vinculin in 4-MU-treated REK cells. Preconfluent cultures incubated in 0.5 mM of 4-MU for 24 h and stained with bHABC (*B*, *D*, *F*) were compared with controls (*A*, *C*, *E*) to illustrate the changed localization of hyaluronan. The vertical sections of single cells obtained by deconvolution of a stack of horizontal confocal sections (*C*, *D*) show the localization of CD44 (red) and hyaluronan (green) on the apical plasma membrane in controls (*C*), and the shift of hyaluronan to the ventral side in 4-MU-treated cells (*D*). The plate bottom is shown by the white dashed line. The arrows point the predominant localization of hyaluronan in the cells. Panels (*E*, *F*) demonstrate the increased quantity and changed distribution of vinculin-positive adhesion plaques (red) in the undersurface of cells cultured with 4-MU. Hyaluronan (green) resides under the central part of the 4-MU treated cells, surrounded by a belt of adhesion plaques. Panel (*H*) demonstrates the loss of phalloidin -positive filopodia, present in control cells (*G*, *arrow*) and reorganization of actin into stress fibers in the 4-MU-treated cells (*H*). *Scale bars* = 20 μm (*A–D*) and 10 μm (*E–H*).

that in control cultures (data not shown). Addition of high molecular weight hyaluronan (100 μg per mL) did not change the proliferation rate of 4-MU-treated cells (data not shown).

We also tested the reversibility of the proliferation block by removing 4-MU from cultures after a 24 h treatment. The effect of 4-MU on cell proliferation was fully reversible (Fig 3*D*) the cell number being duplicated 24 h after 4-MU removal.

EGF-induced hyaluronan accumulation and epidermal thickening are inhibited by 4-MU in organotypic epidermal cultures The REK cells stratify and differentiate into

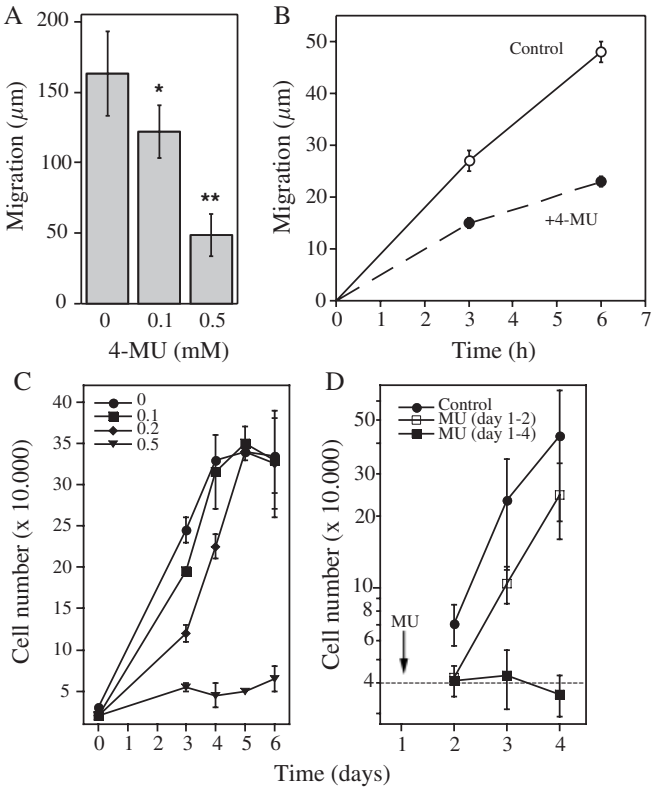

Figure 3
The effect of 4-MU on proliferation and scratch-induced migration. Cells in REK cultures were scratched off from standardized areas with a pipette tip, the cleared area recorded by videomicroscopy, the cultures incubated in the presence of 0–0.5 mM of 4-MU, and recorded again to calculate the average migration rate of the cell front. Panel (*A*) shows the migration rate as a function of 4-MU concentration during a 24-h period (means ± SE of five experiments with eight wounds in each, $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared with control, paired *t* test). Panel (*B*) shows the early time points of migration in the presence of 0.2 mM 4-MU (means and ranges of two independent cultures). For the proliferation assay, equal numbers of REK cells were plated on day 0 with the standard medium containing 0–1 mM of 4-MU. Cell numbers were counted on the days indicated, and also 4 h after plating to determine the plating efficiency (*C*). In panel (*D*) one set of cultures was grown completely without 4-MU (control), another was grown in 1 mM 4-MU for 1 d (days 1–2), then changed back to control medium, and a third set was cultured in 1 mM 4-MU through the experiment (days 1–4). The dashed line indicates the number of cells plated. The error bars represent ranges from two identical but separate experiments.

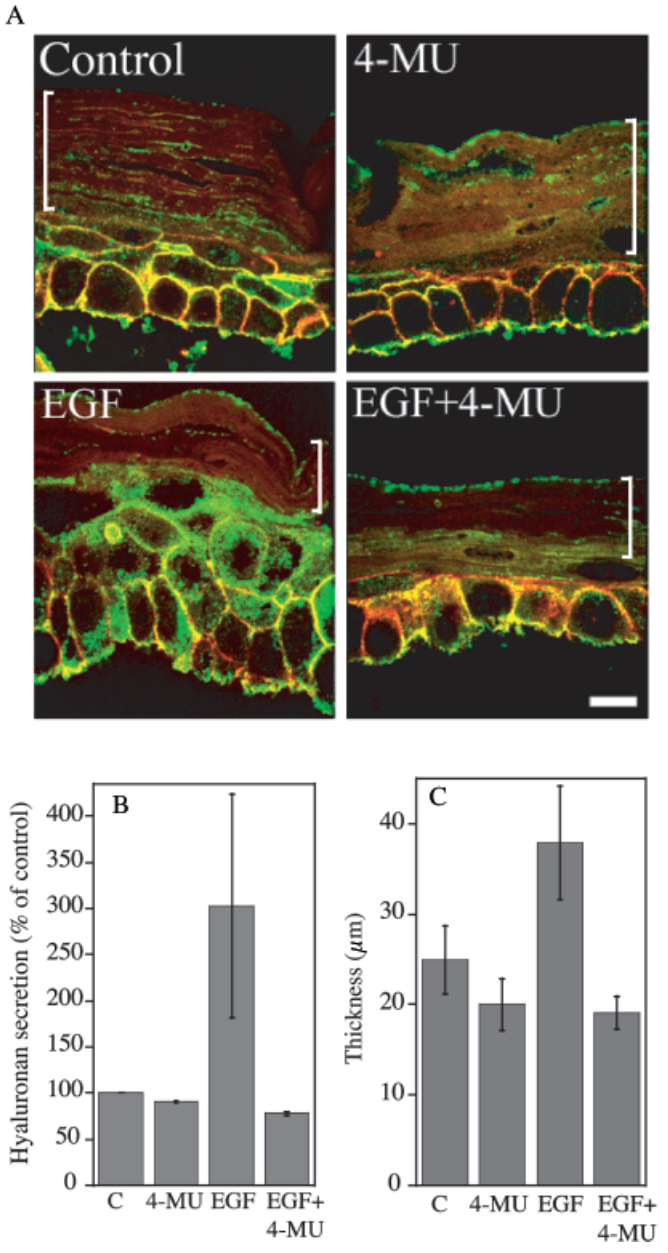

Figure 4
Effect of 4-MU on hyaluronan concentration and tissue morphology of normal and EGF-treated organotypic keratinocyte cultures. EGF (20 ng per mL) was present in the indicated 2.5-wk-old organotypic REK cultures during the whole period, and 4-MU (0.6 mM) was added at 1.5 wk. Two and half-wk-old cultures were fixed, embedded in paraffin, sectioned, and double-stained for CD44 (red) and hyaluronan (green). The yellow signal indicates colocalization of CD44 and hyaluronan. Vertical brackets indicate the stratum corneum. *Scale bar* = 10 μm (*A*). Hyaluronan secretion into growth medium during a 16-h incubation at the end of the culture period was analyzed with the ELSA assay. The error bars represent the range of two independent experiments (*B*). The thickness of the vital part of the epidermis was analyzed by morphometry. The data represent means ± SE of three separate experiments (*C*).

structurally normal epidermis when cultured on a collagen gel in the air–liquid interphase (Tammi *et al*, 2000; Pasonen-Seppänen *et al*, 2001), allowing prediction of pharmacological impacts on epidermis *in vivo*. In the organotypic cultures, the effect of 4-MU on the hyaluronan deposited in the control tissue (Fig 4*A*) or that secreted into culture medium (Fig 4*B*) was relatively small. Importantly, 4-MU did not affect the normal structure or differentiation pattern of control cultures (Fig 4*A*).

As shown previously (Pasonen-Seppänen *et al*, 2003), EGF causes a dramatic increase in epidermal hyaluronan, increases proliferation, and interferes with the normal tissue structure. 4-MU blocked the EGF-induced hyaluronan synthesis and accumulation in the epidermis (Fig 4*A*, *B*). Furthermore, 4-MU normalized the epidermal thickness and tissue architecture disturbed by EGF (Fig 4*A*, *C*).

Discussion

This study shows that 4-MU causes a marked inhibition of hyaluronan production in epidermal keratinocytes and that the inhibition is particularly effective in the epidermis activated by EGF. The decline in hyaluronan synthesis is

associated with a strong inhibition of cell proliferation, to the extent of a complete but reversible block of growth in keratinocyte monolayer cultures. Considering the potential of clinical applications, it is interesting that in the organotypic epidermis, the hyperproliferation and hypertrophy caused by EGF was normalized by 4-MU.

These results fit into an emerging pattern that keratinocyte hyaluronan synthesis is tightly connected with keratinocyte proliferation and epidermal thickness. Thus, epidermal hyaluronan synthesis is specifically upregulated by retinoic acid (Tammi *et al*, 1989), EGF (Pasonen-Seppänen *et al*, 2003), and KGF (Karvinen *et al*, 2003), factors known to stimulate keratinocyte proliferation and increase the number of vital cell layers in the epidermis. Furthermore, the effect of 4-MU on keratinocytes resembles the biological activities of TGFβ (Pasonen-Seppänen *et al*, 2003), and hydrocortisone (Ågren *et al*, 1995), both of which suppress hyaluronan synthesis, keratinocyte proliferation, and epidermal thickening. It can be concluded that 4-MU is a novel chemical effector that confirms the association between keratinocyte hyaluronan metabolism and epidermal proliferation. The inhibition levels, however, of hyaluronan synthesis and proliferation rate are not correlated in absolute units. Furthermore, since the mechanism of action of 4-MU is unknown, it is still possible that the two inhibitions are independent of each other.

The inhibition of hyaluronan synthesis by 4-MU is also associated with changes in the phenotype of single keratinocytes observed in monolayer cultures. A striking effect was inhibition of cell migration. As expected, there were distinct cytoskeletal alterations consistent with this functional transition, including the increase of stress fibers, the reduction of dynamic lamellipodia and filopodia, and the appearance of large, vinculin positive focal adhesions. Vinculin is known to be downregulated in migrating keratinocytes; during wound healing in human epidermis, vinculin is absent at the leading edge (Kubler and Watt, 1993). Similar cytoskeletal and morphological alterations are displayed by keratinocytes in which hyaluronan synthesis is inhibited by transfection of a Has2 antisense gene (Rilla *et al*, 2002). Furthermore, the Has2 antisense inhibition also reduces keratinocyte migration (Rilla *et al*, 2002).

4-MU thus suppresses migration and proliferation, two phenotypic features typical of epidermal activation, a response common in injury, irritation, inflammation, transformation, and many diseases involving epidermis, psoriasis as an example. In general, the present findings: (1) strengthen conclusions on the phenotypic features that follow changes in hyaluronan production, (2) indicate that 4-MU reproduces many of the effects of specific antisense inhibition of Has2 expression, (3) suggest that 4-MU is a readily available means to suppress hyaluronan synthesis, useful in exploring the cellular functions associated with hyaluronan, and (4) offers a potentially useful drug for the treatment of excessive epidermal activation.

Materials and Methods

Cell culture A newborn REK cell line (Baden and Kubilus, 1983) was cultured in minimum essential medium, (MEM, Life Technologies, Paisley, Scotland, UK) supplemented with 5% fetal bovine serum (FBS, HyClone, Logan, Utah), 4 mM glutamine (Sigma, St Louis, Missouri) and 50 μg per mL streptomycin sulfate and 50 U per mL penicillin (Sigma). Keratinocytes were passaged twice a week at a 1:10 split ratio using 0.05% trypsin (w/v), and 0.02% EDTA (w/v) in phosphate-buffered saline (PBS, Reagena, Kuopio, Finland).

4-MU (sodium salt, Sigma) at 10 or 100 mM stock solution in Hank's balanced salt solution (HBSS, Euroclone, Milano, Italy) was added into the culture medium to reach the concentrations indicated.

Organotypic cultures For organotypic cultures–cells were cultured on type I collagen support, and lifted to the air-liquid interface just after having reached confluence as described previously (Pasonen-Seppänen *et al*, 2001). EGF (20 ng per mL) was present in culture medium for the whole culture period (2.5 wk), and 4-MU (0.6 mM) was added after 1.5 weeks and kept until the end of the experiment (i.e., for 1 wk). The secretion of hyaluronan was assayed from a medium kept for 16 h with the cultures at the end of the whole period. A part of the cultures was fixed in Histochoice® (Amresco, Solon, Ohio), and embedded in paraffin. Hematoxylin/eosin-stained sections were morphometrically analysed to measure epidermal thickness as described previously (Pasonen-Seppänen *et al*, 2003). Antigen retrieval using TUF (Monosan, Uden, the Netherlands) was done before the double-staining for hyaluronan and CD44 as described (Pasonen-Seppänen *et al*, 2003).

Assay of hyaluronan A total of 80,000 cells were seeded on 24-well plates and cultured for 24 h. Next day, a fresh medium (5% FBS) with different concentrations of 4-MU and sometimes EGF (20 ng per mL, Sigma) was added for 6 to 24 h before counting the cells and harvesting the media for ELISA of hyaluronan. The sandwich type assay was performed as described previously (Hiltunen *et al*, 2002). Briefly, maxisorp 96-well plates (Nunc, Roskilde, Denmark) were precoated with (non-biotinylated) HABC (hyaluronan binding region of aggrecan and link protein), to catch hyaluronan from the samples and standards (range 0–50 ng per mL). After washes, biotinylated HABC was added to detect the bound hyaluronan using horseradish peroxidase streptavidin and TMB substrate solution (0.5% 3,3′,5,5′-tetramethylbenzidine in dimethyl sulfoxide (Sigma) diluted 1:50 with 0.1 M sodium acetate, 1.5 mM citric acid and 0.005% H_2O_2) for spectrophotometric quantitation.

The HABC preparations contained the hyaluronan binding regions of aggrecan and link protein purified from a trypsin digest of bovine cartilage using sequential hydroxyl apatite chromatography and Sephacryl S-1000 gel filtration (Tammi *et al*, 1994). Part of the material was biotinylated. Both HABC and bHABC were chromatographed on Sephacryl S400 gel filtration under dissociative conditions to remove hyaluronan.

Metabolic labeling and analysis of glycosaminoglycan synthesis REK cultures were incubated for 6 h in the presence of different concentrations of 4-MU and 20 μCi per mL of [^{3}H]-glucosamine and 100 μCi of [^{35}S]-sulfate (Amersham, Little Chalfont, UK). The culture media, cell trypsinization solution and cells were collected, and analyzed for labeled glycosaminoglycans with Superdex gel filtration of specific disaccharides as described previously (Tammi *et al*, 1998).

Molecular mass distribution of hyaluronan To analyze the size of secreted hyaluronan, REK cultures were incubated for 6 h in the presence of different concentrations of 4-MU and 20 μCi per mL of [^{3}H]-glucosamine (Amersham). The culture media were collected, precipitated with ethanol and dissolved in 150 mM sodium acetate buffer, pH 6.8 containing 0.1% CHAPS. The samples were chromatographed on Sephacryl S-1000 (Pharmacia, Sweden) as described previously (Karvinen *et al*, 2003). Two aliquots were taken from each chromatography fraction, one incubated at 60°C for 2 h with 0.5 turbidity reducing units of *Streptomyces* hyaluronidase (Seikagaku Kogyo, Tokyo, Japan), and the other in buffer only. 2% cetyl pyridinium chloride was added 1:1 to all

samples, and the precipitates recovered with centrifugation at 13,000 $\times$ *g* for 15 min. The content of hyaluronan in each fraction was calculated by subtracting the radiolabel in the *Streptomyces* hyaluronidase-treated samples (resistant to hyaluronidase) from those incubated in buffer only.

Proliferation assay REK cells were seeded in 24-well culture plates at 60,000 cells per well. Different concentrations of 4-MU were added immediately after plating to the culture media. Cell numbers were counted with a hemocytometer after 4 h to determine plating efficiency, and after 2–6 d to determine the proliferation rate. Cells from duplicate wells were trypsinized at each time point. The numbers of detached cells in media were also counted.

Migration analysis The REK cells were seeded at 1×10^5 cells per well in 24-well plates and grown approximately 24 h until confluence. A cell-free area was introduced by scraping the monolayer in each well crosswise with a sterile 300 μL pipette tip. The detached cells were washed off with HBSS, and fresh medium with different concentrations of 4-MU was added. The areas covered by the cells were measured immediately, and 3, 6, and 24 h later by phase contrast microscopy with a videocamera and quantitated by NIH Image software (http://rsb.info.nih.gov/nih-image/). The mean distance traveled by the cell front was calculated by converting the pixel values into micrometers (Pienimäki *et al*, 2001).

bHABC-staining Keratinocytes were seeded at 20,000 cells per well on 8-well chamber slides (Nalge Nunc, Naperville, Illinois) precoated with FBS, and grown at 37°C for 48 h. The slides were washed, fixed with 2% paraformaldehyde (vol/vol), washed, permeabilized with 0.3% Triton X-100 in 3% BSA, and probed with 5 μg per mL of bHABC in 1% BSA overnight at 4°C. The slides were washed and incubated with avidin–biotin peroxidase (ABC Standard Kit, Vector Laboratories) for 1 h, and the color was developed with 0.05% 3,3′-diaminobenzine (DAB) and 0.03% H_20_2. The nuclei were stained with hematoxylin, and the preparations were mounted in Supermount (BioGenex, San Ramon, California) and photographed using a Microphot-FXA microscope (Nikon, Tokyo, Japan). The specificity of the staining for hyaluronan was controlled by removing hyaluronan with *Streptomyces* hyaluronidase (Seikagaku, Kogyo, Tokyo, Japan), and by pretreating the probe with hyaluronan oligosaccharides (Tammi *et al*, 1998).

Immunofluorescence stainings For double staining of hyaluronan and vinculin or CD44, keratinocytes were cultured for 24 h on chamber slides, fixed, permeabilized and blocked as described above, and incubated overnight at 4°C with the anti-vinculin mAB (7.1 μg per mL, clone hvin-1, Sigma) or anti-CD44 monoclonal antibody (OX50, 1:1000, Biosource, Camarillo, California) and bHABC (5 μg per mL) in 1% BSA. After washing, the cells were incubated for 1 h with Texas Red-labeled anti-mouse secondary antibody (Vector, 1:50) and fluorescein isothiocyanate-labeled avidin (Vector, 1:500). For the visualization of the actin filaments, the cells were washed, fixed, permeabilized and blocked as described above and incubated with Bodipy FL Phalloidin (4 U per mL, Molecular Probes, Eugene, Oregon) for 20 min. After washing, the slides were mounted in Vectashield (Vector). The micrographs were obtained with an Ultraview confocal scanner (Perkin-Elmer Life Sciences, Wallac-LSR, Oxford, UK), on a Nikon Eclipse TE300 microscope with a 100 $\times$ NA 1.3 oil immersion objective (Nikon). For the three-dimensional imaging, a series of horizontal optical sections were captured through the cell at every 450 nm. The images were deconvoluted using Microtome Deconvolution 7.0 software (Vaytek, Fairfield, Iowa) and rendered with Voxblast software (Vaytek).

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