Maintenance of the Keratocyte Phenotype during Cell Proliferation Stimulated by Insulin*

Kurt Musselmann‡, Bridgette Alexandrou‡, Bradley Kane§, and John R. Hassell§

From the ‡Department of Biochemistry and Molecular Biology, University of South Florida College of Medicine and §Shriners Hospitals for Children Tampa, Tampa, Florida, 33612

Keratocytes normally express high levels of aldehyde dehydrogenase and keratocan. They proliferate and lose their keratocyte markers when they become fibroblastic during corneal wound healing. Keratocytes cultured in fetal bovine serum also become fibroblastic, proliferate, and lose these markers. In this report, we studied the effects of three serum growth factors, fibroblast growth factor-2, insulin, and platelet-derived growth factor-BB, on keratocyte proliferation and the maintenance of the keratocyte markers in 7-day cultures in cells plated at low (5,000 cells/cm²) and high (20,000 cells/cm²) density in serum-free medium. Keratocyte proliferation was measured by [³H]thymidine incorporation and by DNA content of the cultures. Cytosolic aldehyde dehydrogenase and keratocan accumulated in the medium were quantified by Western blot. The results showed that all the growth factors stimulated proliferation, but insulin stimulated proliferation more consistently. The keratocyte markers aldehyde dehydrogenase and keratocan were maintained after 7 days in culture in all growth factors, but keratocyte cell morphology was only maintained in medium containing insulin. Most of the proteoglycans were degraded in cultures of keratocytes plated at low density and cultured in the absence of growth factors. This degradation was prevented when keratocytes were cultured in the presence of the growth factors or when keratocytes were plated at high density. The results of this study show that insulin can expand keratocytes in vitro, maintain their phenotype, and prevent proteoglycan degradation.

Keratocytes, the cells of the mature corneal stroma, have a dendritic morphology and are responsible for the maintenance of the extracellular matrix of the stroma. These cells produce lumican and keratocan, two keratan sulfate proteoglycans (1, 2) that are necessary to maintain the transparency and shape of the stroma. Lumican and keratocan are members of the small leucine-rich protein family and are the most abundant keratan sulfate proteoglycans in the corneal stroma (3). Lumican is also present in many tissues (1) as a glycosylated protein. Keratocan is exclusively found as a proteoglycan in the cornea (1, 4). Both keratocan and lumican (3, 5, 6) interact with collagen fibrils and modulate their size and spacing (3, 5, 7, 8). The collagen fibrils of both lumican (3, 5, 7, 9) and keratocan (3, 10) knock-out mice are larger and less densely packed than the collagen heterofibrils in the normal stroma, and in the lumican null mouse the corneas lose transparency (3, 7). The corneas in the keratocan null mouse were reduced in thickness, most likely because of reduced hydration of the corneal stroma. Mutations in the human keratocan gene have been identified as a cause of cornea plana (10–12). This suggests that keratocan plays a fundamental role in maintenance of the corneal structure and that its function cannot be rescued by other members of the small leucine-rich protein family (13).

Keratocytes normally exhibit a dendritic morphology but become fibroblastic in appearance during wound healing. Both keratan sulfate synthesis and the expression of keratocan substantially decrease during the fibroblast/myofibroblast transformation that occurs during wound healing (14–16). Keratocytes isolated from the stroma and cultured in medium supplemented with fetal bovine serum exhibit fibroblastic or myofibroblastic phenotypes (17, 18). Keratan sulfate synthesis and keratocan expression decrease (18, 19) in a manner reminiscent of in vivo wound healing (15, 18). This indicates that keratocan can be considered a marker for the native keratocyte phenotype (18, 20).

Keratocytes contain unusually large amounts of corneal crystallins such as aldehyde dehydrogenase (ALDH4) (18, 20, 21). It has been speculated that crystallins may play a role in matching the refractive index of the intracellular space to the refractive index of the stroma (21), or they may help organize the intracellular space and thus reduce keratocyte reflectivity (22). ALDH may also protect the cornea from UV light irradiation (23). Corneal crystallins decrease markedly in wound-healing keratocytes in vivo, and this would partially explain the increased haziness observed during the healing of corneal wounds (18, 20, 21). ALDH is a 52-kDa protein and is lost from the cytoplasm during culture in serum (20). This indicates that a high level of cytosolic ALDH is also a marker of the native keratocyte phenotype.

Keratocytes isolated from the stroma and cultured in serum-free medium retain a dendritic morphology and maintain the expression of ALDH and keratocan (15, 18, 20, 24). The loss of keratocan and ALDH expression by culture in fetal bovine serum is likely because of factors in serum that change the keratocyte phenotype. Serum is a complex mixture of salts, proteins, lipids, growth factors, and hormones. One of the growth factors in serum is PDGF-BB (25), which is released from the platelets during the clotting process (26). Removal of the platelets from blood prior to the clotting process produces serum with substantially less growth-promoting properties (27). In keratocyte cultures, the inclusion of PDGF-BB in culture medium causes cell migration and proliferation, as well as collagen gel contraction and fibroblastic differentiation (24). Insulin is also present in serum (28). Insulin interacts with its receptor and modulates metabolic processes but also acts as a mitogen for some cell types (29, 30). Studies have shown that insulin receptors

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‡ To whom correspondence should be addressed: Shriners Hospital for Children, Research Molecular Biology, 12502 Pine Dr., Tampa, FL 33612. Tel.: 813-975-7144; Fax: 813-975-7127; E-mail: jhassell@shctampa.usf.edu.

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1 The abbreviations used are: ALDH, aldehyde dehydrogenase; FGF-2, fibroblast growth factor-2; PDGF-BB, platelet-derived growth factor-BB; DMEM/F-12, Dulbecco’s modified Eagle’s medium and Ham’s F-12 Medium; 1:1; ITS, insulin, transferrin, and selenium medium supplement, used at 1× from 100× concentrate; PBS, phosphate-buffered saline; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-[hydroxyethyl]propane-1,3-diol.

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are present in the anterior segment of the eye (31), and insulin has been identified as a component of tear film (32). Patients with type I and II diabetes mellitus have abnormal changes that occur at the anterior segment of the eye (33). Patients with diabetes show a thickening of the corneal stroma (33, 34) due to accumulation of water and elevated stromal mRNA and protein levels of MMP3 and MMP10 (stromelysin-1 and 2, respectively) (34–36). Fetal bovine serum also contains FGF-2 (37, 38), and FGF-2 has been shown to promote keratocyte proliferation as well as keratan sulfate proteoglycan secretion into the medium when cells were cultured in 0.1% platelet-poor horse serum (39). Here we characterize the effects of FGF-2, insulin, and PDGF-BB in defined cell culture medium on keratocyte proliferation and morphology as well as on proteoglycan synthesis and ALDH expression, two known markers of the native keratocyte phenotype. We found that all growth factors caused cell proliferation and maintained keratocan and ALDH expression, but only insulin maintained the dendritic morphology of kerocytes. The results of this study suggest that insulin may play a role in the maintenance of the corneal stroma after wounding.

MATERIALS AND METHODS

Chemicals—Chemicals were from Sigma-Aldrich unless otherwise indicated.

Cell Culture—Bovine keratocytes were isolated using two sequential collagenase digestions as described previously (40, 41). Briefly, corneas were obtained from freshly harvested, 12–18-month-old calf eyes (Pel-Freez, Rogers, AR). The central portion of the cornea was minced and digested with 3.3 mg/ml SigmaBlend Collagenase Type L at 37 °C under shaking for 45 min to remove the epithelium and the endothelium. The remaining stromal fragments were further digested with fresh collagenase for an additional 150 min to disrupt the matrix and release the keratocytes. The cells were pelleted by low speed centrifugation (400 × g for 10 min) and resuspended in DMEM/F12 (Invitrogen). Cell viability and number were determined using trypan blue exclusion. The cells were then plated in serum-free DMEM/F12 into 6-well plates (Costar, Cambridge, MA) at low density (5,000 cells/cm²) or high density (20,000 cells/cm²) and allowed to attach overnight at 37 °C in 5% CO₂. The medium was changed the next day and every third day afterward with fresh DMEM/F12 or DMEM/F12 supplemented with FGF-2 (10 ng/ml), insulin (10 μg/ml), PDGF-BB (50 ng/ml), or ITS (contains insulin (10 μg/ml), transferrin (5.5 μg/ml), and selenium (0.67 μg/ml) (Invitrogen)). FGF-2 and PDGF-BB were used at concentrations previously shown to stimulate maximum or near maximum proliferation of kerocytes (24). The FGF-2 and PDGF-BB were dissolved in 1 mg/ml bovine serum albumin as per the manufacturer’s instructions that resulted in a final concentration 400 and 1,000 ng of bovine serum albumin/ml medium, respectively. The presence of these low levels of bovine serum albumin in the medium did not result in an increase in DNA content, ALDH, keratocan levels, or 35SO₄ incorporation into proteoglycans (data not shown). 35SO₄ Incorporation into Proteoglycans—Cultures were supplemented with 50 μCi/ml 35SO₄ (PerkinElmer Life Sciences) on day 1 for 72 h, and the medium was collected, frozen, and lyophilized. The dry medium was then reconstituted in 4 M GuHCl and unincorporated 35SO₄ was removed using PD10 columns (Amersham Biosciences). The fractions containing incorporated 35SO₄ were combined, concentrated using Amicon Ultra spin-concentrators (Millipore Corp., Millford, MA), fractionated using a Superose 6 12/30 HR column (Amersham Biosciences), equilibrated, and eluted with 4 M GuHCl containing 0.05 M sodium acetate, pH 6.5, at a flow rate of 0.3 ml/min. Fractions (0.6 ml) were collected, and incorporation was measured by liquid scintillation counting.

DNA Quantitation and [3H]Thymidine Incorporation—Cell cultures were labeled with 20 μCi/ml of [3H]thymidine (PerkinElmer Life Sciences) for 72-h periods on days 1 and 4. The cell layers were harvested on days 1, 4, and 7 to measure DNA content and [3H]thymidine incorporation. Briefly, the cell layers were rinsed with PBS, frozen, thawed, and then solubilized in a lysis buffer supplemented with a DNA-binding dye (Cyquant, Molecular Probes, Invitrogen). Three aliquots from each well were used to determine the DNA content/well using fluorimetry at 480/535 nm. [3H]Thymidine incorporation was determined by precipitating an aliquot of the solubilized cells overnight at 4 °C in 10% trichloroacetic acid using bovine serum albumin as a carrier protein. The precipitated DNA was collected onto glass fiber prefilter filters (Millipore catalog number APFC02500) by vacuum filtration. The filters were washed three times with 5 ml of ice-cold 5% trichloroacetic acid and placed into vials, and incorporation was measured by liquid scintillation counting.

Immunoblotting—Cell layers were solubilized in 2% SDS and examined for ALDH levels by SDS-PAGE Western blot. The amount of cell layer extract loaded on the gel from different culture conditions was adjusted to give a similar level of signal. The keratocan content in the medium was also measured by Western blot. The medium was collected, dialyzed, and lyophilized. The powder was reconstituted in water at either 1/150th of the original volume from keratocytes plated at low density or in 1/50th of the original volume from medium from keratocytes plated at high density. Medium equivalent to 3 wells for keratocytes at low density and medium equivalent to half a well for keratocytes at high density were digested with endo-β-galactosidase (Seikagaku, Associates of Cape Cod, E. Falmouth, MA), and keratocan core protein levels were determined by SDS-PAGE Western blot. The proteins were loaded and separated in 4–10% bis-tris gels (Novex, Invitrogen). The separated proteins were electrotransferred to nitrocellulose membranes (Bio-Rad) at room temperature, blocked with 0.2% I-Block and 0.3% Tween 20 in PBS (0.2% I-PBS-T), and incubated overnight at 4 °C with either 1:5,000 anti-ALDH antibody or 1:1,000 rabbit anti-bovine keratocan antibody. Rabbit polyclonal antiserum to bovine ALDH was a generous gift of Dr. R. Lindahl (University of South Dakota), and the rabbit antiserum to bovine keratocan was previously described (41). Membranes were rinsed in 0.1% I-PBS-T, incubated at
room temperature for 1 h in 1:10,000 horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Biosciences), and rinsed four times with 0.1% I-PBS-T. Protein bands were visualized using chemiluminescence (ECL Western blotting analysis system, Amersham Biosciences) on Kodak Bio-Max XAR x-ray film. Films were scanned, and the band density for ALDH and keratocan core protein was measured using a Bio-Rad GS-710 Calibrated Imaging Densitometer. The resulting pixel density was divided by the DNA content of the culture and expressed in pixel density/µg of DNA.

Cell Morphology—Cell morphology was studied with a Nikon Diaphot phase contrast microscope and recorded using a Nikon D100 digital camera. Images were resized using Adobe Photoshop, and image levels and contrast were adjusted after the images were converted to grayscale.

Statistical Analysis—Statistical analysis was performed using Statview (SAS Institute, Cary, NC). Samples were analyzed using a paired t test. S.E. was used when n > 3, and S.D. used when n = 3.

RESULTS

Keratocytes plated at low density were radiolabeled with [3H]thymidine from days 1 to 4 and days 4 to 7 to determine the effect of growth factors on DNA. Keratocytes cultured in all tested growth factors showed a significant increase in [3H]thymidine incorporation during both labeling periods (Fig. 1) when compared with control. FGF-2 and PDGF-BB, however, stimulated significantly more incorporation during the second time period than during the first time period.

The DNA content of the cultures was measured on days 4 and 7 to determine whether the number of cells in the culture increased. By day 7, the DNA content/well of cells cultured in ITS and insulin increased 3.1-fold (to 330 and 323 ng/well), and cells in PDGF-BB increased 2.3-fold (to 241 ng/well), whereas culture in FGF-2 resulted in a 36% increase (to 142 ng/well), and the control cultures had a 20% increase (to 125 ng/well) (Fig. 2A). The DNA content of cultures plated at high density (Fig. 2B) also increased over the 7-day study period, but the maximum increase in DNA content was 59% for cells cultured in insulin, followed by ITS, FGF-2, and PDGF-BB (52, 47, and 27% respectively), but the DNA levels of the control cultures declined by 31%. The lower growth rate of the cells plated at high density is likely because of contact inhibition.

ALDH is a 52-kDa cytoplasmic protein detected in Western blots of cell layer extracts (Fig. 3, lane 1). The pixel densities of this protein in each culture condition were determined and expressed per µg of DNA of the cultures. Keratocytes plated at high density and cultured for 7 days contained 1.8× more ALDH/µg of DNA than keratocytes seeded at low density (Fig. 4). The levels of ALDH in keratocytes plated at low density and cultured in the presence of growth factors was not signifi-
Keratocyte Culture in Insulin

The results of this study show that all the growth factors tested stimulated [³H]thymidine incorporation and increased the DNA content of the cultures over the 6-day culture period. Comparing the response to each of the different growth factors, however, shows that only insulin stimulated thymidine incorporation similarly during the first 3-day and the second 3-day time period. Furthermore, only the cultures in insulin consistently increased in DNA content during the 6-day culture period and attained the highest DNA content at the end of the 6-day culture period for both low and high initial plating densities. Even with this extensive proliferation over the 6-day time period, keratocytes in insulin maintained ALDH content and keratocan production, the two markers of the keratocyte phenotype, as well as their dendritic morphology.

**DISCUSSION**

Phase-contrast microscopy was used to examine the morphology of the cultured cells prior to harvesting on day 7 (Fig. 6). Cells in DMEM/F12 retain the characteristic dendritic morphology of in situ keratocytes (Fig. 6A). Cells cultured in FGF-2, however, appear fibroblastic, with flattened, spindle-shaped cell bodies and lack the dendritic processes (Fig. 6B) characteristic of keratocytes. Cells cultured in insulin and ITS appear flattened but still retain their dendritic processes (Fig. 6, C and D, respectively), yet the dendrites of cells cultured in ITS appear longer and join with other cells. Cells cultured in PDGF-BB appear fibroblastic, have flat cell bodies, and are spindle-shaped (Fig. 6F).

**FIGURE 3.** Representative Western blots of keratocytes for ALDH and medium for keratocan. All samples obtained from control cultures at high density. Lane 1, cytosolic ALDH is detected as a 53-kDa band. Lane 2, keratocan in the medium is detected by the antibody as a smear of 80–160-kDa size. Lane 3, the medium is digested with endo-β-galactosidase, and the core protein of keratocan is detected as a band at 51 kDa.

**FIGURE 4.** Pixel densities of ALDH content of the cell layers (A) and keratocan in the medium (B). A, the Western blots using antibodies against ALDH were scanned, and the pixel density of each band was determined. The pixel density value was divided by the micrograms of DNA in the culture. Keratocytes plated at low (open bars) and high (filled bars) density and cultured for 7 days. Keratocytes plated at high density cultured in FGF-2 showed a significant decrease in cytosolic ALDH (p < 0.02). For all samples, 3.8, cell media for keratocytes at low and high density were collected, concentrated 150- or 50-fold (low and high density, respectively), the medium equivalent to 3 wells (low density) or half a well (high density) was digested with endo-β-galactosidase, separated by SDS-PAGE, and plotted against keratocan core protein. The bands were scanned, and the pixel density was determined. The pixel density was then divided by the DNA content of each culture. At low density (open bars) FGF-2, insulin, ITS, and PDGF-BB stimulate keratocan secretion, whereas at high density (filled bars) keratocan accumulated in the medium of all culture conditions. Bars represent mean of three determinations ± S.D. (for both A and B). At low density, only insulin significantly stimulated keratocan accumulation in the medium (p < 0.03). Keratocytes plated in the absence of growth factors accumulated significantly more keratocan in the medium than keratocytes plated at low density (p < 0.02). For all samples was 3.

**FIGURE 6.**}
These results suggest that insulin may be useful in expanding a population of keratocytes in culture without changing their phenotype.

Keratocytes cultured in FGF-2 and PDGF-BB also showed increased incorporation of $[^3H]$thymidine in keratocytes plated at low density. The rate of incorporation, however, was significantly higher during the second 3-day time period than during the first time period, but the DNA content of the cultures did not increase from day 4 to day 7. This suggests that in addition to increased cell proliferation, FGF-2 and PDGF-BB may also be causing cell death. Like the culture in insulin, ALDH and keratocan production were maintained by culture in FGF-2 and PDGF-BB. Unlike insulin, however, culture in FGF-2 and PDGF-BB caused the keratocytes to become spindle-shaped or fibroblastic. This suggests that cell morphology and ALDH/keratocan production are not linked. Overall, these results would suggest that the loss of ALDH and keratocan by keratocytes cultured in medium containing fetal bovine serum (17, 18, 20, 21) is because of factors other than the insulin, FGF-2, and PDGF-BB present in the serum.

The results of this study also show that plating keratocytes at low density and in the absence of growth factors results in the degradation of the proteoglycans secreted into the medium. The incorporated $^{35}$SO$_4$ from keratocytes at low density and in the absence of growth factors eluted between fractions 27 and 32, close to the elution position of the keratan sulfate chains released by protease digestion from human and rabbit corneas (14, 42). In contrast, the $^{35}$SO$_4$ incorporated by keratocytes cultured with growth factors eluted in one major peak between fractions 17 and 21, in the elution position of keratocan (fractions 18–20) and decorin (fractions 17–21). Keratocan and decorin are members of the leucine-rich proteoglycan gene family (3). These proteoglycans have a core protein of $\sim$40 kDa and contain multiple leucine-rich repeats. Keratocan has five N-linked glycosylation sites, but only three contain keratan sulfate chains (43). Decorin has one O-linked glycosylation site that contains the single chondroitin sulfate chain (44). At high density, most of the $^{35}$SO$_4$ incorporated by keratocytes cultured in DMEM/F12 alone eluted between fractions 16 and 21, with only some of the incorporated $^{35}$SO$_4$ eluting between fractions 26–31. This suggests that the presence of growth factors in the medium for cells plated at low density or plating cells at high density is necessary to prevent the degradation of proteoglycans. This may explain why the keratocan signal was lower in low density control-plated keratocytes than keratocytes plated at high density. The core proteins of the proteoglycans may be degraded and not detected by Western blot, but the $^{35}$SO$_4$ incorporated in the residual GAG chains is detectable. The growth factors may be playing a role in protecting the secreted proteoglycans from proteolysis or stimulating the production of a cell product that inhibits proteolysis. The proteoglycans produced by keratocytes plated at high density are not degraded in the absence of growth factors. This suggests that plating density may play an important role in proteoglycan stability and accumulation. It is possible that keratocytes secrete other extracellular matrix components that interact with the proteoglycans in the medium and that at low density the unstimulated keratocytes do not secrete enough extracellular matrix components to prevent the degradation.

Insulin acts through its transmembrane receptor and modulates several metabolic processes with respect to glucose, lipids, and proteins (29). The interaction of insulin with its receptor also activates members of the mitogen-activated protein kinase family (30). We have shown that insulin stimulates keratocytes to proliferate in culture. This suggests that keratocytes in culture express the insulin receptor. Keratocytes cultured in insulin maintain their dendritic morphology and the expression of keratocan and ALDH. Insulin is also required for the maintenance of the adipocyte phenotype.
(45), and the addition of insulin to the medium of activated hepatic stellate cells results in the up-regulation of putative adipogenic transcription factors resulting in the cells returning to their quiescent, pre-myofibroblastic state (46). Insulin may be an important growth factor needed to maintain the proliferation and the phenotype of a number of different cell types.

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