Published in final edited form as:

Exp Eye Res. 2009 August ; 89(2): 218–223. doi:10.1016/j.exer.2009.03.010.

IGF-II and collagen expression by keratocytes during postnatal development[★]

Bradley P. Kane^a, James V. Jester^b, Jiying Huang^c, Andrew Wahlert^b, and John R. Hassell^{a,*}

^a Department of Molecular Medicine, College of Medicine, University of South Florida, 12901 Bruce B. Downs Blvd., MDC 7, Tampa, FL 33612-4799, USA

^b The Gavin S. Herbert Eye Institute, University of California, Irvine, CA, USA

^c Department of Ophthalmology, University of Texas Southwestern Medical Center, Dallas, TX, USA

Abstract

Keratocytes produce the extensive stromal matrix of the cornea during the late embryonic and neonatal time periods. We propose to test the hypothesis that their biosynthetic activity declines during this process. Keratocytes were isolated from corneas of 6–8-week-old rabbits and corneas of 1–2-year-old cows and their ability to proliferate and synthesize collagen in serum-free media was determined. Rabbit keratocyte cultures increased 38% in DNA content after one week and deposited collagen type I and IGF-II in the media. Bovine keratocyte cultures, in contrast, did not increase in DNA or produce detectable collagen and IGF-II. Bovine keratocytes cultured in media previously conditioned by rabbit keratocytes, however, increased 56% in DNA content, and deposited collagen type I into the media. Microarray analysis of mRNA from neonatal and adult mouse keratocytes was used to confirm these differences. Compared to adult mouse keratocytes, neonatal keratocytes showed high expression levels of IGF-I, IGF-II and collagen types III and V. Since previous studies showed that IGFs stimulate bovine keratocytes to proliferate and to synthesize procollagen type I, we therefore propose that the results of this study suggests that the IGFs may play an important role in regulating early corneal growth *in vivo*.

Keywords

keratocytes; collagen; cell culture; IGF-II; development

1. Introduction

The cornea is the transparent, anterior portion of the eye that serves to both protect the inner structures of the eye and act as the major refractive element for focusing light on the neurosensory retina. The cornea is composed of three layers: an epithelial layer, a stromal layer and an endothelial layer, but the stromal layer represents 90% of the total corneal thickness and provides the structural integrity while maintaining its transparency. The major cell type of the stroma is the keratocyte and its primary function is to make, maintain and repair its uniquely transparent extra cellular matrix [for reviews see Netto et al., 2005; West-Mays and Dwivedi, 2006; Quantock and Young, 2008].

*Supported by grants EY08104 and EY07348 from the National Institutes of Health and grants from Research to Prevent Blindness, Inc.

*Corresponding author. Tel.: +1 813 974 7881; fax: +1 813 974 7357. E-mail address: jhassell@health.usf.edu (J.R. Hassell).

Keratocytes in adult corneas are normally quiescent cells and are unusual in that they have a dendritic morphology, a high content of corneal crystallins and produce 3 keratan sulfate proteoglycans [for reviews see Chakravarti, 2006; Kao et al., 2006; Musselmann and Hassell, 2006; Jester, 2008]. Keratocytes isolated from the stroma of either rabbit or bovine corneas by collagenase digestion will retain many characteristics of their *in vivo* phenotype when cultured in serum-free medium (Jester et al., 1996, 1999; Beales et al., 1999; Berryhill et al., 2001, 2002; Funderburgh et al., 2003; Jester and Ho-Chang, 2003; Guerriero et al., 2007). This includes: quiescence, a dendritic morphology, the presence of cytoplasmic crystallins, and the synthesis and secretion of keratan sulfate proteoglycans. Most of these studies used keratocytes isolated from 6 to 8-week-old rabbits or from 1 to 2-year-old cattle, and this difference in species or in maturity may result in differences in biosynthetic activity. Therefore, in the present study, we compare the proliferative and secretory activities of native rabbit and bovine keratocytes *in vitro* and also compare the mRNA levels for collagen and growth factors in keratocytes from neonatal and adult mice.

2. Materials and methods

2.1. Reagents

Cyquant kits, polyacrylamide gels, electrophoresis solutions, nitrocellulose, RPMI vitamin mix, non-essential amino acids, anti-biotics (penicillin/streptomycin/fungizone), DMEM and DMEM/F12 were obtained from Invitrogen (Carlsbad, CA), Costar cell culture plates from Fisher Scientific (Suwanee, GA), Amicon 10,000 MWCO spin concentrators from Millipore Corporation (Bedford, MA), rabbit anti-IGF-II (PA0382) from Cell Sciences (Canton, MA) rabbit anti-IGFBP-2 from US Biologicals (Swampscot, MA), mouse anti-procollagen type I (SP1.D8, Hybridoma Bank, U. of Iowa), and all other reagents from Sigma-Aldrich (St. Louis, MO).

2.2. Isolation and culture of keratocytes

Freshly harvested eyes from 1 to 2-year-old cows and from 6 to 8-week-old rabbits were purchased from Pel Freeze (Rogers, AR) and shipped on wet ice by overnight delivery. The keratocytes were isolated from corneas by using methods previously described for cow and rabbit eyes and cultured in the media traditionally used for these species (Jester et al., 1996; Berryhill et al., 2001). Bovine keratocytes were plated in DMEM/F12 supplemented with 1% antibiotics and 1 mM 2-phospho-L-ascorbic acid (B-media) and rabbit keratocytes in DMEM supplemented with 1% antibiotics, 1% RPMI Vitamin Mix, 1 mM pyruvate, 0.1 mM non-essential amino acids, and 0.1 mg/ml ascorbic acid (R-media) on day zero at ~20,000-cells/cm² in 6-well plates using 2 ml/well. Plates were placed in a tissue culture at 37° and in a humidified atmosphere containing 5% CO₂. Media was removed and replaced with fresh or conditioned media on days 1 and 4. Cultures were harvested on days 1, 4, and 7. Medium from 4 wells was pooled and the media and plates stored at -80 °C.

2.3. DNA measurements

The DNA content per well was determined using a CyQuant kit for DNA measurement. Calf thymus DNA was used as a standard. DNA synthesis was determined in cultures incubated with media containing 5 µCi/ml of ³H-thymidine during days 4–7 of culture. The incorporation of radiolabel into DNA was determined as previously described (Musselmann et al., 2005).

2.4. Protein synthesis

Cultures were incubated with media containing 25 µCi/ml of ³H-glycine during days 4–7 of culture. The medium was harvested and incorporation into total protein deposited in the

medium and into pepsin resistant collagen was determined as previously described (Musselmann et al., 2006).

2.5. SDS/PAGE, Western blot and autoradiogram

Media samples were concentrated to 1/10th volume using spin concentrators. The size of the aliquots taken for electrophoresis on polyacrylamide gels was normalized to the DNA content of the culture. This allowed comparisons to be made by similar numbers of cells. Samples were reduced and electrophoresed according to the vendor's instructions. Gels were stained for proteins using Simply Blue. Western blots were performed using the ECL Western blotting analysis system according to the vendor's instructions. Autoradiograms were prepared by infusing the gel with NAMP-100 (Amersham), drying the gel onto a paper backing and exposing the dried gel to X-ray film.

2.6. Statistics

Statistical analysis was performed with four determined values for each point using Statview (SAS Institute, Cary, NC). Data are expressed as the mean $\pm$ standard error. Significant differences were determined by paired *t*-test.

2.7. Microarray analysis of keratocyte gene expression

Comparison of mRNA levels in keratocytes from neonatal day 4 and neonatal day 16 to adult (day 60) was determined using Spotted Array Platform at the UT Southwestern Microarray Core Facility and differences in gene expression was assessed using Genespring software. One analysis was done for each of the 3 time points. Keratocyte RNA was prepared from the corneal stroma isolated from neonatal (100 eyes per age group) and adult (40 eyes) CD-1 mice after removal of the corneal epithelium and endothelium. Isolated stromal tissue was placed in RNA later (1:10, Qiagen Cat#76106) for RNA stabilization and the tissue homogenized in TRI REAGENT (Molecular Research Center INC Cincinnati, OH, Cat#TR118). Samples were phase separated by adding 0.2 ml chloroform/ml (Sigma C-2432) followed by centrifugation at 12,000g for 15 min at 4 °C RNA was precipitated by adding 0.5 ml of isopropanol (Sigma I-9516) followed by centrifugation at 12,000g for 15 min. Separated RNA was washed three times with 1 ml of 75% ethanol and stored at -80 °C. Samples were air dried for 5–10 min and dissolved 30–50 μ l of PCR-H₂O and incubated at 55–60 °C for 10 min. Any DNA present was degraded by adding 5 μ l RNase-free DNA digestion buffer and 1.25 μ l DNase I, and incubation at room temperature for 10 min.

3. Results

Keratocytes from bovine and rabbit corneas were isolated and plated out in B-media on day zero and cultured for 7 days. Cultures were harvested on days 1 and 7 and the DNA content of the cell layer was determined (Fig. 1). The DNA content of the rabbit keratocyte cultures increased significantly ($p < 0.007$) by 38% from day 1 to day 7 of culture. The DNA content of the bovine keratocyte cultures did not significantly change during this time period. This indicates that the rabbit keratocytes may have proliferated during the 6-day culture period while the bovine keratocytes did not.

The proteins deposited into the media during days 4–7 of culture were separated by SDS/PAGE and detected by staining for protein (Fig. 2, lanes PS). The media from rabbit keratocyte cultures (lane R) contained several prominent bands; one just above the 210 kD marker and two midway between the 210 and 71 kD markers. These bands were much fainter in the media from the bovine keratocyte cultures (lane B). Western blot showed the bands between the molecular weight markers to readily react with an antibody to procollagen $\alpha 1(I)$ (lane WB).

The results in Figs. 1 and 2 suggested to us that, in contrast to bovine keratocytes, the rabbit keratocytes may be secreting growth factors into the media that induce proliferation. Therefore, we isolated R-media conditioned by rabbit keratocytes and B-media conditioned by bovine keratocytes for days 1–4 and 4–7 of culture and used this media to culture bovine keratocytes for days 1–7 of culture. ^3H -thymidine was added during days 4–7 of culture and the incorporation of the nuclide into the cells was determined (Fig. 3). The results showed that compared to unconditioned media controls, the media conditioned by bovine keratocytes for days 1–4 significantly ($p < 0.022$) inhibited ^3H -thymine incorporation while the media conditioned by rabbit keratocytes for days 1–4 and for days 4–7 significantly stimulated ^3H -thymidine incorporation by 4.6-fold ($p < 0.0005$) and 11.6-fold ($p < 0.0007$), respectively. We also determined the DNA content of the bovine keratocytes cultured in the unconditioned and conditioned R-media (Fig. 4). Compared to the DNA content of bovine keratocyte cultures in unconditioned R-media, the DNA content of bovine keratocyte cultures in media conditioned by rabbit keratocytes for days 1–4 and for days 4–7 increased significantly by 35% ($p < 0.004$) and by 56% ($p < 0.025$), respectively.

Previous studies have shown that the aqueous humor and extracts of bovine cornea stroma to contain IGF-II and IGFBP-2 (Arnold et al., 1993; Musselmann et al., 2008). Therefore, we screened the conditioned media from the bovine and rabbit keratocyte cultures using antisera to IGF-II and IGFBP-2 by Western blot (Fig. 5). The antiserum to IGF-II reacted with 2 bands in the media conditioned for days 1–4 and 4–7 by rabbit keratocytes (Fig. 5A, lanes 7 and 8) that migrated between the 28 and 38 kD markers and with 1 band that migrated just above the 6 kD marker in the media conditioned for days 4–7 by rabbit keratocytes (lane 8). This single band coincided with the migration position of the IGF-II standard (lane 1). IGF-II is normally derived from a larger precursor protein (Lelbach et al., 2005) and the bands between the 28 and 38 kD markers are likely the precursor. Antisera to IGFBP-2 reacted strongly with a single band in the media conditioned for days 1–4 and 4–7 by bovine keratocytes (Fig. 5B, lanes 4 and 5) that coincided with the migration position of the IGFBP-2 standard (lane 2). The faintly reacting bands in lanes 7 and 8 may be cross-reaction of the antisera with the IGF-II precursor protein.

Previous studies have also shown IGF-II to increase the synthesis and deposition of procollagen type I into the media (Hassell et al., 2008). Therefore we next determined if the media conditioned by rabbit keratocytes would also stimulate ^3H -glycine incorporation into proteins deposited into the media by bovine keratocytes in culture (Fig. 6). The results showed that compared to unconditioned media, the media conditioned by rabbit keratocytes stimulated ^3H -glycine incorporation by 6.3-fold while the media conditioned by bovine keratocytes had no effect on incorporation. Pepsin digests of the media showed that the synthesis of type I collagen was the major fibrillar collagen type stimulated by the rabbit conditioned media (Fig. 7).

While it is difficult to obtain corneas from cows and rabbits at different ages of development, corneas from mice of different ages can be readily obtained. Microarray analysis of gene expression during early postnatal development was determined in keratocytes isolated from mouse corneas at 3 different ages: neonatal day 4, neonatal day 16 and adult (day 60). Evaluation of the gene expression pattern identified 581 genes (419 up and 162 down) in 4-day-old neonates and 66 genes (65 up and 1 down) in 16-day-old neonates that showed at least a 3-fold change in expression compared to expression levels in adults (data not shown). Of these 581 genes, 6 collagen α genes and 7 growth factor genes were identified that showed age related differential expression (Table 1). The most abundantly expressed collagen genes in the 4-day-old and mice were collagen $\alpha 1(\text{III})$, collagen $\alpha 1(\text{V})$, collagen $\alpha 1(\text{IV})$ and collagen $\alpha 2(\text{IV})$ showing respective ratios of 21.18, 13.11, 10.56 and 9.34, compared to adult. Collagen types III and V were also abundantly expressed by the 16-day-old neonates, but at lower ratios

of 5.54 and 3.70, respectively. The most abundant growth factors expressed were IGF-II, which showed 17.43 and 3.28 ratios in the 4-day-old and 16-day-old mice and IGF-I, which showed a 6.79 ratio on day 4, compared to adult. Other growth factors showing distinct differences in the 4-day-old neonates compared to adult were elevated levels of Fallotin (5.22), BMP-1 (3.76) and Epithelium Derived Growth Factor (3.61) and reduced levels of Transforming Growth Factor (0.10).

4. Discussion

The results of these studies show that keratocytes isolated from 6 to 8-week-old rabbits have a higher level of biosynthetic activity in culture than keratocytes isolated from 1 to 2-year-old cattle. We used this difference to identify a growth factor responsible for stimulating keratocyte proliferation and suggest its possible role in corneal development. The rabbit keratocytes proliferated at low levels in culture and produced conditioned media that caused the bovine keratocytes to proliferate in culture. In contrast, the bovine keratocytes did not proliferate and did not produce mitogenic conditioned media. The rabbit keratocytes deposited IGF-II in their media while the bovine keratocytes did not deposit detectable levels of IGF-II in their media. Previous studies have shown IGF-II can stimulate bovine keratocytes to proliferate *in vitro* (Musselmann et al., 2008). This suggests that the IGF-II that was secreted into the media by the rabbit keratocytes was stimulating their own proliferation and, when this media was used to culture bovine keratocytes, it would also stimulate their proliferation as well. The gene expression study using mRNA isolated from mouse keratocytes shows that compared to adult mice, the expression of IGF-II was ~ 17.4-fold higher in day 4 mice and ~ 3.3-fold higher in day 16 mice. IGF-I, which acts on the same receptor as IGF-II, was expressed ~ 6.8-fold higher on day 4. Taken together, the similarities in IGF-II expression in these 3 different species suggest that the expression of IGF-II may be developmentally regulated.

The results of these studies also show that the bovine keratocytes did deposit IGFBP-2, a binding protein for IGF-II. IGFBPs act to modify the interaction of bound IGFs with proteases and with receptors for the IGFs (Leibach et al., 2005). Previous studies (Musselmann et al., 2008) have shown that extracts of corneal stroma from 1 to 2-year-old cattle contain IGFBP-2 as well as IGF-II. The bovine keratocytes in culture, however, did not deposit IGF-II in the media. This suggests that the IGF-II present in the stromal extract may have been made and deposited in the stroma by the keratocytes at an earlier developmental stage. The rabbit keratocytes did not make IGFBP-2 in culture. Microarray analysis of mouse keratocytes showed IGFBP-4 to be highly expressed at the 4-day time point. The expression of the IGFBPs may also be developmentally regulated.

Collagen types I and V are the major fibrillar collagens of the cornea (Birk et al., 1986) with lesser amounts of types III (Schmut, 1977; Cintron et al., 1988). Type VI collagen is a major non-fibrillar stromal collagen (Cintron and Hong, 1988). Collagen provides the cornea with its tensile strength and structural stability. The regulated assembly of collagen into fibrils is important to the growth, development, structure and function of the cornea. The collagen fibrils in the corneal stroma are hetero-fibrils, consisting of collagen types I and V and collagen type V has been shown to be essential for the initiation of collagen fibril assembly (Wenstrup et al., 2004). Our microarray analysis found mRNA levels for collagen type I to be relatively constant (i.e. less than 3-fold different) in keratocytes from neonatal and adult mouse corneas. In contrast, mRNA levels for collagen type V were 13.1-fold higher in the keratocytes from the day 4 neonatal mouse corneas and 3.7-fold higher in keratocytes from the day 16 neonatal corneas. This suggests that mRNA levels of collagen type V may play a major role in regulating collagen fibril production and stromal growth during development. The microarray analysis also showed mRNA levels for collagen type III to be elevated in neonatal corneas and this confirms a recent gene expression study using mouse corneas (Wu et al., 2008). Keratocytes

from day 4 mouse corneas also showed high expression levels of collagen type IV. Collagen type IV is found almost exclusively in basement membranes but also appears in the corneal stroma at the onset of mesenchymal invasion and its distribution correlates with the distribution of the corneal nerves (Bee et al., 1988; Fitch et al., 1991). These data suggest that the expression of different collagen types in the cornea is developmentally regulated.

The synthesis of collagen types I, III, IV is up-regulated in bovine keratocytes *in vitro* by members of the IGF-family. Pharmacological levels of insulin, a level that would activate the receptor for IGF-I and IGF-II (Lelbach et al., 2005), increases the synthesis of type IV collagen (Guerriero et al., 2007), and type I collagen (Hassell et al., 2008). IGF-I and IGF-II stimulate the synthesis of procollagen type I (Hassell et al., 2008) and IGF-I stimulates the synthesis of procollagen type III (Etheredge et al., in press). The results of the culture studies presented here show that the rabbit keratocytes deposited considerably more procollagen type I into the media than did the bovine keratocytes, and that the media conditioned by rabbit keratocytes, which contained IGF-II, stimulated bovine keratocytes to synthesize procollagen type I. This suggests that the IGF-II produced by the rabbit keratocytes stimulated their own production of procollagen type I and the production of procollagen type I by bovine keratocytes.

In conclusion, we therefore propose that IGF-I/IGF-II may act *in vivo* to maintain both keratocyte proliferation and the synthesis of the collagenous matrix needed to provide for continued corneal growth that occurs during early postnatal development.

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Fig. 1. DNA content of keratocyte cultures. Rabbit (R) and bovine (B) keratocytes were cultured in B-media, harvested on days 1 and 7 and the cells analyzed for DNA content. Compared to day 1, the DNA content of the rabbit keratocyte cultures were significantly higher on day 7.

Fig. 2. SDS/PAGE and Western blot of media from bovine (B) and rabbit (R) keratocyte cultures. Keratocytes were cultured in B-media and media was harvested from days 4–7 of culture. SDS/PAGE were either stained for protein (PS) or transferred to nitrocellulose for Western blot (WB) using antibodies to procollagen type I.

Fig. 3.

Bovine keratocytes cultured in media containing ^3H -thymidine. B-media and R-media used for the culture of bovine (BK) and rabbit keratocytes (RK), respectively, for days 1–4 and days 4–7 were then reused for the culture of bovine keratocytes for days 1–7. Unconditioned B- and R-media were used as controls. ^3H -thymidine was added to the unconditioned and the conditioned media for days 4–7 of culture of the bovine keratocytes. Legend: UB) unconditioned B-media, CB1-4) B-media conditioned by BK for days 1–4 of culture, CB4-7) B-media conditioned by BK for days 4–7 of culture, UR) unconditioned R-media, CR1-4) R-media conditioned by RK for days 1–4 of culture, CR4-7) R-media conditioned by RK for days 4–7 of culture. Incorporation of ^3H -thymidine into the cells was determined and expressed per DNA. Compared to unconditioned media, media conditioned by BK for days 1–4 significantly inhibited incorporation and media conditioned by RK for days 1–4 and 4–7 significantly stimulated incorporation.

Fig. 4. DNA content of bovine keratocyte cultures. Bovine keratocytes were cultured for days 1–7 in unconditioned R-media (UR); R-media conditioned by rabbit keratocytes for days 1–4 (CR1-4), or R-media conditioned by rabbit keratocytes for days 4–7 (CR4-7) and then analyzed for DNA content. Compared to the DNA content of the keratocytes cultured in unconditioned media, the DNA content of keratocytes cultured in conditioned media was significantly higher.

Fig. 5.

SDS/PAGE/Western blots of unconditioned media and media conditioned by bovine (BK) and rabbit (RK) keratocytes for days 1–4 and 4–7 of culture. Legend: Blot A) antibody to IGF-II, Blot B) antibody to IGFBP-2, lane 1) IGF-II standard, lane 2) IGFBP-2 standard, lane 3) unconditioned B-media, lane 4) B-media conditioned by BK for days 1–4 of culture, lane 5) B-media conditioned by BK for days 4–7 of culture, lane 6) unconditioned R-media, lane 7) R-media conditioned by RK for days 1–4 of culture, lane 8) R-media conditioned by RK for days 4–7 of culture.

Fig. 6.

Bovine keratocytes cultured in media containing ^3H -glycine. B- and R-media used to culture bovine and rabbit keratocytes for days 4–7 were then reused for days 1–7 culture of bovine keratocytes. Unconditioned B- and R-media were used as controls. ^3H -glycine was added to the media for days 4–7 of culture. Legend: UB) unconditioned B-media, CB4-7) B-media conditioned by bovine keratocytes for days 4–7, UR) unconditioned R-media, CR4-7) R-media conditioned by rabbit keratocytes for days 4–7. The incorporation of ^3H -glycine into proteins deposited into the medium was determined and expressed per DNA. Compared to unconditioned R-media, R-media conditioned by rabbit keratocytes significantly stimulated incorporation of ^3H -glycine into proteins deposited in the medium.

Fig. 7. Pepsin digests of the ^3H -glycine containing media used for the culture of bovine keratocytes. Media, from experiment described in Fig. 6, was pepsin digested, separated on SDS/PAGE and an autoradiogram of the gel was prepared.

Table 1
Comparison of neonatal to adult extracellular matrix and growth factor gene expression (greater than 3-fold increase/decrease).

Gene Description	Gene ID	Ratio
4 Day vs. Adult		
Collagen α 1 (III)	BG086357	21.18
Insulin-like Growth Factor 2	BG064815	17.43
Collagen α 1 (V)	BG067011	13.11
Collagen α 2 (IV)	BG087341	10.56
Collagen α 1 (IV)	BG085352	9.34
Collagen α 2 (VI)	BG075864	7.90
Collagen binding protein	BG086364	7.42
Insulin-like Growth Factor 1	BG074904	6.79
Collagen XIV	BG086917	5.44
Fallotein	BG073417	5.22
IGF Binding Protein-4	BG084827	3.91
BMP-1	BG085595	3.76
Epithelium Derived Growth Factor	BG078270	3.61
Transforming Growth Factor	BG072750	0.10
16 Day vs. Adult		
Collagen α 1 (III)	BG086357	5.54
Collagen α 1 (V)	BG067011	3.70
Insulin-like Growth Factor 2	BG064815	3.28