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IGF-II is present in bovine corneal stroma and activates keratocytes to proliferate *in vitro*

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Abstract

Extracts of bovine corneal stroma have been shown to activate keratocytes in culture to proliferate. We fractionated stromal extract on a column of Sephacryl S-300 and tested the fractions for mitogenic activity using cell culture and for the presence of IGF-II and its binding protein IGFBP-2 by Western blot. We found that the mitogenic activity in the extract separated into major and minor peaks and that immunologically detectable IGF-II and IGFBP-2 co-eluted with the minor peak. We also compared the effects of 10 ng IGF-II/ml on keratocytes in culture to that of 2 ng TGF-β/ml over a 7-day culture period. We found that IGF-II and TGF-β, alone or combined, increased both ^{3}H-thymidine incorporation and DNA content of the cultures. The phenotype of the cells was determined by using antibodies to α-SM (smooth muscle) actin, fibronectin, SPARC, lumican and keratocan in Western blots of cell layers of media. Keratocytes cultured in IGF-II expressed no α-SM actin or fibronectin, low levels of SPARC and high levels of lumican and keratocan, indicating a native phenotype. Keratocytes in TGF-β expressed α-SM actin, fibronectin, SPARC and lumican, and expressed no or low levels of keratocan, indicating a myofibroblast phenotype. Keratocytes cultured in IGF-II plus TGF-β, however, expressed α-SM actin, fibronectin, SPARC, lumican, and keratocan by day 7 of culture. The results of this study show that IGF-II to be present in the corneal stroma, to stimulate keratocyte proliferation while maintaining native phenotype and to override the TGF-β mediated down regulation of keratocan production. The IGF-II in the stroma may serve as a mechanism to immediately activate keratocytes upon wounding and to ameliorate the scarring effects of TGF-β.

Keywords: keratocyte; myofibroblast; IGF-II; TGF-β; proliferation; activation; cell culture

1. Introduction

The corneal stroma contains an extensive extracellular matrix consisting of alternating layers of collagen lamellae interspersed with keratocytes. The matrix consists primarily of collagen types I, III, IV, V, and VI (Schmut, 1977; Birk et al., 1986; Cintron and Hong, 1988; Cintron et al., 1988; Guerriero et al., 2007), and four leucine-rich proteoglycans: decorin (Li et al., 1992), which bears chondroitin sulfate chains, and lumican (Blochberger et al., 1992), keratocan (Corpuz et al., 1996), and osteoglycin/mimecan (Funderburgh et al., 1997), which have keratan sulfate chains. These matrix components are found in other connective tissues but the corneal stroma is unusually rich in keratan sulfate proteoglycans. The keratocytes have a dendritic morphology (Muller et al., 1995) and high levels of crystalline proteins in their cytoplasm (Jester et al., 1999). During periods of homeostasis, they are relatively quiescent. When a corneal wound occurs, the keratocytes adjacent to the wounded site undergo apoptosis (Helena et al., 1998; Zieske et al., 2001), and some of the remaining keratocytes are activated to proliferate (Hanna et al., 1989; Del Pero et al., 1990; Zieske et al., 2001). The keratocytes become quiescent again when the cell void has been repopulated and the wound healed.

Keratocytes isolated from the stroma by collagenase digestion and cultured in serum free media retain their dendritic

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morphology and their quiescence (Jester et al., 1996; Beales et al., 1999) but can be activated to proliferate by a number of growth factors including, FGF (fibroblast growth factor)-2, PDGF (platelet derived growth factor), TGF (transforming growth factor)-β, IL (interleukin)-1-α, IGF (insulin like growth factor)-I and insulin (Long et al., 2000; Jester et al., 2002; Jester and Ho-Chang, 2003; Musselmann et al., 2005). Antibodies to TGF-β have been shown to reduce keratocyte activation in corneal wounds (Jester et al., 1997) and this suggests that TGF-β is present in wounds and activates keratocytes *in vivo* as well.

We previously showed an extract of corneal stroma stimulated proliferation of keratocytes in culture and proposed that growth factors may normally be present in the stroma to activate the keratocytes that, upon corneal wounding, had lost cell–cell contact inhibition due to apoptosis of adjacent keratocytes (Musselmann et al., 2003). Arnold et al. (1993) have shown IGF-II and IGFBP (insulin like growth factor binding protein)-2 to be in the aqueous humor, a fluid immediately posterior to the cornea and that extracts of the cornea exhibit a 100-fold higher binding capacity for IGF-II than that of the iris/ciliary. In this report we show that antibodies to IGF-II and IGFBP-2 react by Western blot with the stromal extract and with fractions of stromal extract containing mitogenic activity and that IGF-II stimulates keratocyte proliferation *in vitro*.

2. Materials and methods

2.1. Reagents

All chemicals and growth factors were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise indicated. ^{3}H-thymidine was obtained from Perkin Elmer (Boston, MA), polyacrylamide gels, electrophoresis solutions, nitrocellulose, CyQuant, and DMEM/F12 from Invitrogen (Carlsbad, CA), cell culture plates from Corning-Coster (Cambridge, MA), 10,000 MWCO spin concentrators from Amicon (Millipore Corp, Bedford, MA), *endo*-beta-galactosidase from Seikagaku Associates of Cape Cod (East Falmouth, MA) and Sephacryl S-300 from GE healthcare (Piscataway, NJ).

2.2. Stromal extract preparation

The extract was prepared in similar fashion to the previously described method (Musselmann et al., 2003). In brief, epithelium and endothelium were removed from the corneas and the resulting stromas were frozen in liquid nitrogen. Frozen stromas were pulverized in a Waring blender pre-cooled to liquid nitrogen temperatures. Frozen stromal powder was weighed, added to DMEM/F12 (5 ml/g powder) and extracted by stirring at 4 °C for 4 h. Insoluble material was removed by centrifugation in a Beckman 50.2 Ti rotor at 33,000 rpm at 4 °C for 1 h. The resulting extract was filter-sterilized and stored at −80 °C as 100% extract.

2.3. Extract fractionation

Eighty milliliters of 100% extract was concentrated to 20 ml using centrifugal concentrators and applied to a column (5 × 40 cm) of Sephacryl S-300 that was equilibrated and eluted with DMEM/F12 at 5 ml/min. The eluant was monitored for absorbance at 280 nm, and 25 ml fractions were collected. Each fraction was filter-sterilized, and aliquots were either diluted to 10% with DMEM/F12 for use as culture medium or spun concentrated to 1/10th volume for analysis by SDS/PAGE.

2.4. Keratocyte isolation and culture

Eyes were obtained from 1-year-old cows from Pel Freeze (Rogers, AR) and keratocytes were isolated from the corneas by using two sequential collagenase digestions as previously described (Berryhill et al., 2001). Cells were plated on day 0 in DMEM/F12 in six well plates at 20,000-cells/square cm using 2 ml media/well. The cells were allowed to attach overnight at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was changed on days 1 and 4 to DMEM/F12 or to DMEM/F12 containing IGF-II (10 ng/ml), TGF-β (2 ng/ml) or IGF-II and TGF-β combined. The DMEM/F12 was supplemented with antibiotics and 1 mM 2-phospho-L-ascorbic acid. Cultures were harvested on days 1, 4, and 7. Medium was removed from each well, cell layers were rinsed in PBS and the media and plates were stored frozen at −80 °C.

2.5. DNA measurements

The DNA content of the cells in each well was determined using a CyQuant kit according to the vendor's (Invitrogen) instructions. DNA synthesis was determined in cultures that were radiolabeled with 740,000 Bq ^{3}H-thymidine/ml of medium for 72 h beginning on day 4. The incorporation of the radiolabel into DNA was determined as previously described (Musselmann et al., 2005).

2.6. Western blot

Media harvested from four cultures was combined and spun concentrated to 1/20th of the original volume. The cells in each well were solublized in 150 µl of 1× sample buffer (from Invitrogen) and the extract from four wells were combined. Aliquots of media were digested with *endo*-beta-galactosidase according to the vendor's (Cape Cod Associates) instructions for detection of lumican and keratocan core proteins by Western blot. The volume of the aliquots taken from the media at the day 4 and day 7 harvests of each of the four different culture conditions (i.e., control, IGF-II, TGF-β, and IGF-II plus TGF-β) was normalized to the DNA content obtained for the cell layers of those cultures or in the case of extracts of cell layers, the DNA content of parallel cultures. In this way it was possible to directly compare the amount of a particular protein produced by the same number of cells. SDS/PAGE of samples from the pooled media and