Fibroblast Growth Factor-2 Promotes Keratan Sulfate Proteoglycan Expression by Keratocytes in Vitro*

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Keratocytes of the corneal stroma produce a specialized extracellular matrix responsible for corneal transparency. Corneal keratan sulfate proteoglycans (KSPG) are unique products of keratocytes that are down-regulated in corneal wounds and in vitro. This study used cultures of primary bovine keratocytes to define factors affecting KSPG expression in vitro. KSPG metabolically labeled with [35S]sulfate decreased during the initial 2–4 days of culture in quiescent cultures with low serum concentrations (0.1%). Addition of fetal bovine serum, fibroblast growth factor-2 (FGF-2), transforming growth factor β, or platelet derived growth factor all stimulated cell division, but only FGF-2 stimulated KSPG secretion. Combined with serum, FGF-2 also prevented serum-induced KSPG down-regulation. KSPG secretion was lost during serial subculture with or without FGF-2. Expression of KSPG core proteins (lumican, mimecan, and keratocan) was stimulated by FGF-2, and steady state mRNA pools for these proteins, particularly keratocan, were significantly increased by FGF-2 treatment. KSPG expression therefore is supported by exogenous FGF-2 and eliminated by subculture of the cells in presence of serum. FGF-2 stimulates KSPG core protein expression primarily through an increase in mRNA pools.

The corneal stroma is a disc of connective tissue that constitutes about 90% of the mammalian cornea. This tissue consists of a unique transparent extracellular matrix populated by keratocytes, flattened mesenchymal cells responsible for production and maintenance of this matrix. In healing corneal wounds keratocytes become activated, begin mitosis, and migrate to the wound location, where they secrete nontransparent scar components (1, 2). Cells in the healing wound are characterized by secretion of pro-inflammatory cytokines such as interleukin-1α and proteolytic enzymes involved in tissue remodeling, collagenase, gelatinase, and stromelysin (3, 4). This remodeling (fibroblastic) phenotype is simulated in vitro when keratocytes are cultured in medium containing fetal bovine serum and subcultured by trypsinization (5).

The extracellular matrix of the normal corneal stroma is characterized by a unique class of molecules known as the corneal keratan sulfate proteoglycans (KSPG).1 These consist of three structurally related proteins modified with N-linked keratan sulfate chains (6). The three proteins (lumican, keratocan, and mimecan) are found in a number of connective tissues but are expressed at much higher levels in the cornea compared with noncorneal tissues (7–9). In noncorneal tissues, these proteins are not modified with keratan sulfate (10). The high level of expression of these three proteins combined with a specialized glycosylation is a property unique to keratocytes and constitutes an essential feature of the role of the keratocyte in maintenance of corneal transparency. This conclusion is supported by the recent demonstration that mice bearing null mutations in the lumican gene lose corneal transparency, whereas mice with a similar mutation in the gene for decorin, the major corneal chondroitin/dermatan sulfate proteoglycan, maintain clear corneas (11, 12).

Expression of KSPG appears closely linked to the stromal environment. During corneal development, secretion of KSPG commences very soon after migrating neural crest cells enter the primary corneal stroma (13). A number of early studies reported that when removed from the stroma, keratocytes rapidly lose the ability to secrete keratan sulfate (14–21). We examined this in vitro down-regulation of KSPG using cultured bovine keratocytes and reported that these cells continue to secrete KSPG proteins, but the proteins are modified with truncated under-sulfated keratan sulfate chains (22). A recent study has shown that primary bovine keratocytes in low-serum or serum-free media maintain KSPG secretion for several days in culture, whereas growth in 10% fetal bovine serum resulted in decreased KSPG synthesis (23). The apparent association between cell division and loss of KSPG secretion both in vivo and in vitro suggests that mitosis per se may be involved in KSPG down-regulation. In the current study we examined the response of freshly isolated bovine keratocytes to cytokines and serum to better define the cellular environment that determines maintenance and/or loss of expression of the KSPG differentiation marker. We report that keratocyte mitosis is not directly linked to KSPG down-regulation but that subculture after trypsin treatment, particularly in the presence of mitogen-rich fetal bovine serum, results in irreversible loss of KSPG synthesis. The presence of FGF-2, however, supports KSPG synthesis by induction of core protein biosynthesis.

MATERIALS AND METHODS

Cell Culture—Central portions of fresh corneas from slaughter-aged steers were incubated in 0.2% trypsin in a 1:1 mixture of Dulbecco’s

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1 The abbreviations used are: KSPG, keratan sulfate proteoglycan; FBS, fetal bovine serum; FGF-2, fibroblast growth factor-2; TBS, Tris-buffered saline; PAGE, polyacrylamide gel electrophoresis; DMEM/F-12, Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium, 1:1.
modified Eagle's medium and Ham's F-12 medium (DMEM/F-12; Sigma, D6905) containing antibiotics (penicillin, 100 units/ml; streptomycin 100 µg/ml; gentamicin, 50 µg/ml; amphotericin B, 2.5 µg/ml) for 16 h at 4 °C followed by 30 min at 37 °C. Epithelial and endothelial cells were removed by scraping with a plastic spatula (Cell Lifter; Fisher 08-773-2) in cold saline. The tissue was rinsed, minced into 2–3-mm cubes with razor blades, and shaken at 37 °C, 70 rpm for 2–3 h in a volume of 1 ml/corneal stoma of 1% (v/w) collagenase (Sigma C8176 L) in DMEM/F-12 with antibiotics until complete disruption of the tissue was achieved. The cell suspension was passed through a 70-µm nylon mesh (Cell Strainer, Falcon 2350), washed twice by centrifugation in DMEM/F-12 with antibiotics, and plated at 3–4 × 10³ cells/cm² in polystyrene tissue culture vessels (Costar 3506 or Corning 25100) in DMEM/F-12 with antibiotics until sufficient culture was available for experiments. The cells were rinsed twice with saline, and cell number was estimated by dye exclusion. Media were changed after 24 h of culture and subsequently at 48-h intervals. At termination of cultures, cells were rinsed twice with saline, and cell number was estimated by dye exclusion.

**Solid Phase Chondroitinase Assay**—Sulfated proteoglycans were labeled with 100 µCi/ml of carrier-free H3SO4- (ICN Radiochemicals 640405) or with 50 µCi/ml 35S-methionine/cysteine (TransLabel, ICN 51006). 48 h after labeling, the culture medium from each well was collected and combined with a rinse of the cell layer with saline. This soluble fraction contained >95% of the detectable proteoglycan and was used as the source for KSPG purification and analysis.

Analysis of total 35S-methionine-labeled secreted proteins was carried out by repeated concentration of the labeled culture medium in 0.1 ml Tris-HCl, pH 6.8, using centrifugal ultrafiltration with a 3-kDa cut-off to remove unincorporated label, followed by separation of the proteins by SDS-PAGE and autoradiography. Purification of proteoglycans from the media was achieved by passing the culture medium and rinse through SPEC-3 ml-NH2 microcolumns (Ansys, Inc., 531-07-20) preactivated with methanol and distilled water as per manufacturer's instructions. The columns were rinsed with 3 ml of 6 M urea, 0.2 M NaCl, 0.02 M Tris-HCl, pH 8. Proteoglycans were eluted from the columns with 0.4 ml of 4 M guanidine HCl, 0.02 M Tris-HCl, pH 8.

**SDS-PAGE Analysis**—Column eluates were dialyzed against 0.1 M Tris-acetate, pH 8, followed by digestion with chondroitinase ABC 0.1 unit/ml or keratanase II (Seikagaku) and/or endo-β-galactosidase (Sigma) each at 0.02 unit/ml for 1 h at 37 °C, followed by separation on a 3–12% acrylamide gel as described previously (24). The separated proteoglycans were electrophoretically transferred to Millipore Immobilon polyvinylidene difluoride membranes, standards were detected by staining with Ponceau S, and radioactivity was detected on x-ray film using BioMax low energy transcreen (Millipore Corp.) at −70 °C.

**Immune Precipitation**—Immune precipitation of labeled KSPG was carried out using a minor modification of our previously published procedure (25, 26). Affinity purified polyclonal anti-KSPG antibody reactive with all the purified proteoglycans to polyvinylidene difluoride membranes, standards were detected by reactivity with Ponceau S, and radioactivity was detected on x-ray film using BioMax low energy transcreen (Millipore Corp.) at −70 °C.

**RESULTS**

In an effort to define factors involved in KSPG regulation we compared KSPG secretion by primary keratocytes isolated by collagenase (23) to that from cells obtained by outgrowth corneal explants (22) using SDS-PAGE and immunodetection by monoclonal antibody I22, which recognizes sulfated regions of keratan sulfate chains (25). Fig. IA (lane 1) shows that primary cells secreted a heterogeneous high molecular weight material reacting with the antibody, similar in size to purified bovine corneal KSPG (lane 3). Culture medium from the outgrowth cells (lane 2) had virtually no KSPG reacting with this antibody. Active secretion of a high molecular weight KSPG by the primary cultures was confirmed by metabolically labeling with [35S]sulfate (Fig. 1B). Approximately 30–50% of the labeled proteoglycan was resistant to digestion with chondroitinase ABC (Fig. 1B, lane 2). This chondroitinase-resistant proteoglycan had a size distribution similar to the KSPG identified with antibody I22 and was >90% sensitive to keratanase digestion (Fig. 1B, lane 3). Molecules with properties similar to normal corneal KSPG, therefore, appear to make up a significant proportion of the proteoglycans secreted by these cells in culture. Confirmation of the chondroitinase-resistant 35S-labeled proteoglycans as KSPG was demonstrated by immunoprecipitation using antibody I22. As shown in Fig. 1C, the macromolecular components of the immunoprecipitated proteoglycans were sensitive to endo-β-galactosidase and specifically competed with unlabeled purified KSPG. A 50 kDa band generated by endo-β-galactosidase (Fig. 1C, lane 2) is the previously detected size of KSPG core protein. Labeling of this band may result from incomplete sulfation of these proteins (7). KSPG secreted by quiescent primary keratocytes is larger and more highly sulfated than that we described previously in cells that had undergone extensive cell division (22), suggesting the possibility that active cell division may be involved in modulation of KSPG expression. We examined this hypothesis by treatment of the primary cells with mitogens. The primary
keratocytes divided rapidly in the presence of several cytokines and sera (Fig. 2A), but KSPG secretion exhibited a mixed response to these agents, Fig. 2B. As we found previously (23), FBS reduced the amount of sulfated KSPG, however other mitotic agents such as platelet-derived growth factor and interleukin 1a had little effect. One mitogen, FGF-2 (also known as basic fibroblast growth factor) clearly increased KSPG secretion in comparison with the other treatments. Immunoprecipitation of the KSPG from these cultures with anti-keratan sulfate antibodies (Fig. 2C) provided results similar to the solid phase assay of keratan sulfate and also showed that KSPG secreted in the presence of transforming growth factor beta or FBS is somewhat smaller in size than that from control cultures.

Stimulation of KSPG by FGF-2, as shown in Fig. 3A, required a minimum of 2–4 days of exposure. Without FGF-2, KSPG secretion dropped about 50–70% during a 6-day culture period, but in the presence of FGF-2, KSPG secretion was stimulated after a 2-day lag period. At the end of the 6-day period sulfated KSPG secretion was as much as 10-fold greater than cultures without FGF-2. The amount of stimulation was a function of FGF-2 concentration and below 1 ng/ml closely matched the increase in cell number (Fig. 3B). At FGF-2 concentrations above 1 ng/ml, however, the KSPG per cell secretion increased slightly in comparison to control. Concentrations greater than 10 ng/ml FGF-2 were difficult to assay because these resulted in loss of cell attachment (not shown).

Previously, culture of primary cells in 10% FBS was found to result in a significant reduction in KSPG secretion (23). When FGF-2 was combined with FBS, however, KSPG secretion on a per cell basis was maintained at the level of the cultures in 0.1% horse serum (Fig. 4A). We showed earlier that keratocytes subcultured using trypsin and grown in FBS secrete very little KSPG (22). This conclusion was confirmed in Fig. 1A (lane 2).

Sulfate is incorporated primarily into the keratan sulfate chains of the KSPG, and previously we found that in passaged cells, keratan sulfate chains were severely truncated (22). Keratan sulfate chain length, examined by gel filtration chromatography, of protease-treated KSPG showed that keratan...
sulfate in untreated primary cultures (Fig. 5B) eluted later, indicating a somewhat smaller molecular mass compared with keratan sulfate made by intact bovine corneas (Fig. 5A). The most abundant keratan sulfate chains after FGF-2 treatment (Fig. 5C) were similar in size to those in untreated cultures; however, more label eluted in earlier (high molecular weight) fractions after FGF-2 treatment than in the control cultures, indicating an increased proportion of longer keratan sulfate chains after FGF-2 treatment. Keratan sulfate from cells maintained in 10% FBS (Fig. 5D) were clearly smaller than those from untreated cultures.

The moderate changes in keratan sulfate chain length shown in Fig. 5 indicate that the dramatic alterations in sulfate-labeled KSPG in response to FGF-2 seen in Figs. 2 and 4 cannot be explained completely in terms of keratan sulfate chain length. These data suggest that an increase in the secretion of the proteins to which the keratan sulfate chains are attached may be involved. This hypothesis was supported by the observation that FGF-2 clearly stimulated incorporation of [35S]methionine into secreted KSPG (Fig. 6A). To assess whether these KSPG core proteins were stimulated on a per cell basis, equal amounts of nonfractionated methionine-labeled proteins from FGF-2-treated and control cultures were separated before or after treatment with endo-β-galactosidase to release proteins bound to keratan sulfate chains (Fig. 6B). KSPG core proteins released by endo-β-galactosidase exhibiting an apparent molecular mass of 48–50 kDa could be clearly differentiated from other labeled proteins in the culture media (Fig. 6B, arrow). Densitometric analysis of the autoradiograms indicated a 4.5-fold increase in KSPG proteins in FGF-2 treated cultures on a per cell basis (Fig. 6C).

Pool sizes of mRNA of the KSPG proteins were examined with a ribonuclease protection assay. As seen in Fig. 7A, levels of all three mRNAs increased in the presence of FGF-2, but levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA did not change. In Fig. 7B, densitometric analysis of the individual protected bands found that lumican transcripts increased 2–3-fold, mimecan showed only a slight increase, and the keratocan mRNA increased 8–10-fold during 6 days of treatment in FGF-2. Summing all three KSPG mRNAs showed an approximate 3-fold increase as a result of FGF-2 treatment.

**DISCUSSION**

This report examines factors that affect in vitro secretion of corneal KSPG proteins bearing macromolecular keratan sulfate chains. The lability of corneal keratan sulfate synthesis has been well established in the literature (14, 16, 18–21, 28), yet it is only recently that the mechanism of this down-regulation has begun to be investigated in detail. A report from our laboratory showed that passaged bovine keratocytes express the KSPG proteins in culture but observed these to be modified with oligosaccharide chains bearing minimal sulfation, rather than the fully sulfated keratan sulfate chains typical of normal corneas (22). In 1996 Jester et al. (29) found that primary
keratocytes under serum-free conditions maintained a dedifferentiated morphology typical of keratocytes in the normal cornea in vivo. More recently, Beales et al. (23) reported that primary keratocytes in low serum and serum-free conditions secrete KSPG similar to that made in vivo, providing an initiation point for the current investigation. Our current findings, however, demonstrate a useful experimental system with which we can begin the unraveling of the long-standing enigma of KSPG biosynthesis.

The current study is not the first to examine the effects of FGF on keratocytes. Nakazawa et al. (30) reported a modest increase of both keratan sulfate and dermatan sulfate as a result of a 16-h FGF-2 treatment of primary chick keratocytes in collagen gels. These results are not inconsistent with our findings (Fig. 3) that 2–4 days is required before the most significant effects on KSPG can be observed in primary bovine keratocytes. A report by Jester et al. (29) found that 7 days of FGF-2 treatment of primary keratocytes reversed the induction of α-smooth muscle actin, a myofibroblastic marker, in response to serum or transforming growth factor β. These results suggest that FGF may alter a range of keratocyte cellular functions. That FGF-2 may be a generalized differentiation factor for corneal stroma is not unlikely. Action of FGF-2 during embryonic development is important in the differentiation of numerous tissues including lens and retina (31, 32). FGF-2 has also been implicated as a survival factor for a variety of quiescent or terminally differentiated cells (33). Smooth muscle cells in culture maintain differentiated characteristics via autocrine expression of FGF-2 and enter apoptosis when this secretion is blocked (34). Keratocytes express both mRNA for FGF-2 and for its receptor (35). It seems reasonable to hypothesize, therefore, based on our current data, that autocrine FGF-2 production by keratocytes may be involved in the maintenance of the differentiated keratocyte phenotype in vivo.

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