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ALTERED GELATINOLYTIC ACTIVITY BY KERATOCONUS CORNEAL CELLS

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SUMMARY: Keratoconus involves thinning and central protuberance of the cornea, scarring and significantly decreased vision. It is one of the major causes of corneal transplantation in this country, but the etiology of this disorder is unclear. In the present study stromal keratocytes were isolated and cultured from normal and keratoconus human corneas. Consistent with the phenotype of cornea thinning, we observed an increased gelatinolytic activity in keratoconus cultures. Characterization of enzyme properties in these cells suggested that gelatinase (type IV collagenase) was responsible for the majority of proteolytic activity found in this system. This elevated gelatinolytic activity was present in spite of lower amounts of total protein being produced by the keratoconus cultures.

Keratoconus is a non-inflammatory, bilateral corneal disorder characterized by central thinning and ectasia leading to scarring and decreased visual acuity. While the cause of keratoconus is unknown for the majority of cases, it has been associated with inherited systemic diseases such as Ehlers-Danlos syndrome, osteogenesis imperfecta, and trisomy 21 (see 1 for review). Recent studies have suggested that increased collagenolytic activity may play a significant role (2-4).

It has become increasingly apparent that the group of enzymes known as metalloproteinases are important in connective tissue remodeling. A recently described metalloproteinase, gelatinase (type IV collagenase), shows marked specificity for gelatin (denatured collagen), types IV, V, and VII collagen and fibronectin (5-8). It is secreted by polymorphonuclear leukocytes/monocytes (9-11), endothelial cells (12), fibroblasts (6,8), metastatic tumor cells (13-15), and H-ras oncogene transformed human bronchial epithelial cells (6). The progelatinase molecule demonstrates considerable amino acid homology to human procollagenase and prostromelysin and rat prostromelysin suggesting parallel evolutionary development (6). However, the regulation of these enzymes is independent as suggested by various studies which showed that progelatinase synthesis was selectively increased while procollagenase production declined in response to transforming growth factor-Beta (8). Unlike collagenase and stromelysin, levels of gelatinase expression are not enhanced by exposure of cells to tumor promoter 12-O-tetradecanolyphorbol 13-acetate (TPA) but could be

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increased by transformation with H-ras oncogene and treatment with transforming growth factor-Beta (6,8).

Since it had been suggested that keratoconus may be secondary to an abnormality of the enzymatic degradation of corneal cells it seemed reasonable to compare the gelatinase digestion of normal and keratoconus keratocytes. Indeed, it was observed that the normal keratocytes produced a significantly greater amount of[^{35}S] methionine containing gelatinase but the gelatinolytic activity of the enzyme was diminished in comparison to the keratoconus keratocytes.

MATERIALS AND METHODS

Keratoconus corneas were obtained within 24 hours after penetrating keratoplasty. Age matched normal human eyes were obtained from the National Disease Research Interchange (NDRI) within 48 hours of death. After removal of the epithelium and Descemet's membrane/endothelial cells, the keratocytes were enzymatically dissociated from the stromal lamellae and cultured in minimal essential media (MEM) with 10% fetal calf serum (FCS), penicillin (100u/ml), streptomycin (100ug/ml), gentamicin (10ug/ml), and fungizone (50ug/ml) (16). Cultures were passaged (1:4) with trypsin(0.25%)/versene (0.3%). Confluent cultures were rinsed thoroughly with balanced salt solution and then incubated for 24-48 hours in MEM with 0.2% bovine serum albumin and 50 uCi/ml [^{35}S] methionine. Aliquots of the media from the normal and keratoconus cultures were fractionated by modifications of the methods of O'Farrell (17), Unemori and Werb (18), and Herron et al (19). The first dimension for isoelectric focusing contained 4% pH 4-6 and 2% pH 3.5-10 ampholytes (LKB) and were run at 4^{o}C at 300 volts overnight and then another 2 hours at 500 volts. The second dimension was run at constant current (20 milliamps) for 4 hours in a Laemmli buffer system (20) with a 10% acrylamide slab gel containing gelatin (1mg/ml). The slab gels were then incubated 30 minutes in 2.5% Triton X-100 and then overnight at 37^{o}C in Tris (50mM)/$CaCl_2$ (5mM),0.2% NaN_3 pH7.4. Gels were stained with Coomassie blue and then destained with methanol(10%)/acetic acid(10%). To visualize the radioactively labeled proteins, the same two dimensional gelatin substrate gel was impregnated with Fluor-Hance, dried and then exposed to Kodak XAR-5 film.

RESULTS AND DISCUSSION

Cell layers of normal and keratoconus cultures were closely matched with regards to the total protein, 800 and 750 ug/24 hours, respectively, and total recovered DNA, normals with 3 ug and keratoconus with 2.4 ug. However, when 10 ul aliquots of the conditioned media were fractionated by two dimensional gel electrophoresis it became apparent that the normal cultures contained significantly increased [^{35}S] methionine containing proteins as compared to the keratoconus media (Figure 1). In addition, there was a qualitative disparity between the normal and diseased samples with regards to 2 distinct proteins that were present in the normal but not the keratoconus or vice versa (see a and b, Figure 1). Overlapping comparison of the radioactively labeled proteins (Figure 1) with the gelatin substrate gel (Figure 2) demonstrated that protein C corresponded with the gelatinolytic activity. While comparison of spot intensity and size strongly suggested that the normal media contained a greater quantity of protein C than did the keratoconus cultures (Figure 1), it was the keratoconus media that displayed increased gelatinolytic activity in the substrate gels

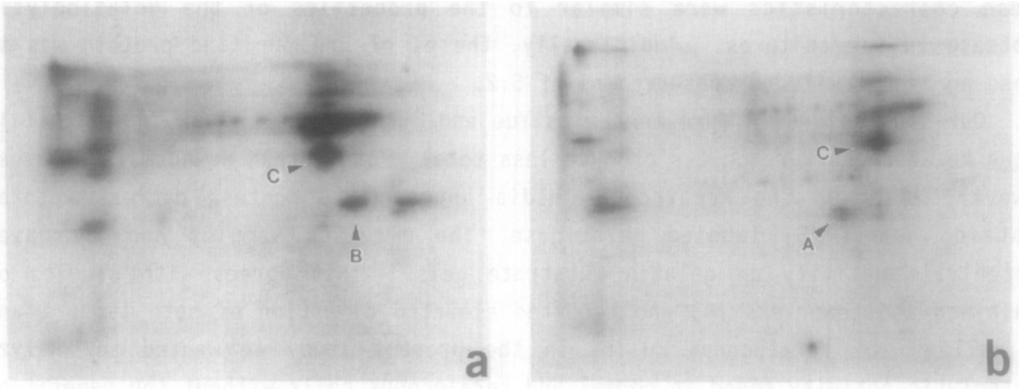

Figure 1: Fluorogram of the same two dimensional gelatin substrate electrophoresis gels seen in Figure 2 from normal and keratoconus keratocyte cultures. Normal (1a) shows greater size and intensity of spots than seen in keratoconus (1b). Qualitatively most proteins are found in both normal and keratoconus cultures except for proteins A and B. Protein C corresponds to the gelatinolytic activity seen in Figure 2.

(Figure 2). Further characterization showed that the gelatinolytic activity was inactivated by chelators, dithiothreitol (DTT) and B-mercaptoethanol but was not effected by 1mM phenylmethanesulfonyl fluoride (PMSF). Moreover, it was a Ca-dependent gelatinolytic protein, with a molecular weight of approximately 68kDa and a pI of approximately 5, which showed markedly decreased affinity for native type I collagen and casein as substrates.

Our data clearly implicated protein C to be the neutral metalloproteinase, type IV collagenase. Recent studies revealed a molecular weight of 72kDa for the zymogen form and 66kDa for the active form (6,8). It had a selected preference for gelatin as a substrate, was inhibited by EDTA and DTT but was not effected by serine proteinase inhibitors, and showed optimum activity at pH 7-7.5 (5,6,8).

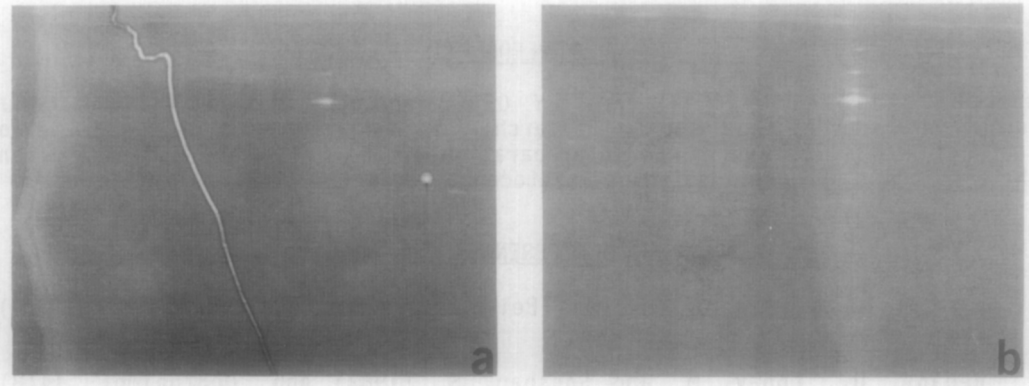

Figure 2: Two dimensional gelatin substrate electrophoresis gel of 10 uls of culture media from normal (2a) and keratoconus (2b) cultures. Background staining is of Coomassie blue stained gelatin. Areas of clearing are sites of enzymatic digestion.

These characteristics were similar to the properties of the gelatinolytic protease in our cultures. Additionally, the pI of the purified protein was in close agreement with our observed pI of 5.2.

Our studies are in agreement with Yue and coworkers (21) who showed that in vitro keratoconus keratocytes produce less total protein than do normal cultures. However, although the keratoconus media appeared to contain decreased total protein and [^{35}S] labeled gelatinase, the diseased samples had increased proteolytic activity on gelatin substrate gels. This agrees with studies of Ihalainen and coworkers (4) who reported elevated digestion of both types I and IV collagen by keratoconus cells. In the present study we wanted to analyze proteolytic activity found in normal and keratoconus cells without the benefit of phorbol ester, oncogene, or exogenous (i.e., TGF-B) induction. In addition, we initially chose not to activate the metalloproteinases with trypsin or organomercurials because we wanted to study the unaltered forms of the enzymes produced by the cells since it may more closely reflect the pathological state found in this disease. We found that under these conditions for both normal and keratoconus cells, the major metalloproteinase produced was gelatinase and not interstitial collagenase or stromelysin. Even when cultures were exposed to phorbol ester induction, the gelatinase activity predominated over other metalloproteinases (data not shown). It may be that normal media contained latent gelatinase that once properly activated would have significantly increased digestion. However, it seems unlikely because when we activated normal and keratoconus media with either trypsin or organomercurials, the level of digestion appeared to be equivalent. It is intriguing to speculate that the thinning and abnormal stromal tissue seen in keratoconus corneas are due to elevated proteolytic activity. There may be a loss of the some regulatory control of the gelatinase molecule or perhaps an inherent difference between the diseased and normal enzyme (i.e., post-translational modifications, amino acid differences leading to increased susceptibility to activation, etc.). Further studies are presently underway to investigate this exciting hypothesis.

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