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Hyaluronidase Expression in Human Skin Fibroblasts

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Hyaluronidase activity has been detected for the first time in normal human dermal fibroblasts (HS27), as well as in fetal fibroblasts (FF24) and fibrosarcoma cells (HT1080). Enzymatic activity was secreted predominantly into the culture media, with minor amounts of activity associated with the cell layer. In both classes of fibroblasts, hyaluronidase expression was confluence-dependent, with highest levels of activity occurring in quiescent, post-confluent cells. However, in the fibrosarcoma cell cultures, expression was independent of cell density. The enzyme had a pH optimum of 3.7 and on hyaluronan substrate gel zymography, activity occurred as a single band corresponding to an approximate molecular size of 57 kDa. The enzyme could be immunoprecipitated in its entirety using monoclonal antibodies raised against Hyal-1, human plasma hyaluronidase. PCR confirmed that fibroblast hyaluronidase was identical to Hyal-1. The conclusion by previous investigators using earlier technologies that fibroblasts do not contain hyaluronidase activity should be reevaluated. © 1999 Academic Press

Hyaluronan (hyaluronic acid, HA)[2] is a glycosaminoglycan (GAG) of the extracellular matrix (ECM) prominent whenever rapid tissue growth occurs, particularly during embryogenesis, wound healing and tumorigenesis (1–3). HA is unique among GAG's in that it lacks both sulfation and covalent linkage to a core protein. Structurally, HA is a straight chain polymer consisting of disaccharide repeats of glucuronate and N-acetylglucosamine connected by β-linkages. *In vivo*, degradation of HA to monosaccharides is accomplished by the coordinated action of hyaluronidases, a family of endoglycosidases that cleave internal β(1,4) linkages, plus two exoglycosidases, β-glucuronidase (EC 3.2.1.31) and β-*N*-acetylhexosaminidase (EC 3.2.1.30) that hydrolyze linkages at the non-reducing termini.

Hyaluronidases have been categorized by substrate specificity. These include bacterial hyaluronidase (EC 4.2.99.1) with absolute specificity for HA, the leech-type hyaluronidase (EC 3.2.1.36) that are endo-β-glucuronidases rather than endo-β-*N*-acetylglucosaminidases, and various vertebrate hyaluronidases such as the testicular-type hyaluronidase (EC3.2.1.35) that can also depolymerize chondroitin sulfate, albeit at a slower rate (4, 5). Hyaluronidases of the latter type have also been isolated and characterized from somatic sources such as from human plasma and urine. The enzyme from each of these two sources is coded for by the same gene, HYAL1 (6, 7). This gene constitutes one of the family of six paralogous hyaluronidase-like sequences present, three each, on chromosomes 3p21.3 and 7p31.3 (8).

More than 50% of total body HA is sequestered within the dermal layer of skin (9). Much of this HA, synthesized by dermal fibroblasts, is degraded as it is being removed by the lymphatics. The remainder is catabolized by liver and kidneys (10, 11). There has long been speculation, however, regarding the existence of an additional local mode of HA turnover, by a hyaluronidase activity associated with dermal fibroblasts. Historically, efforts to identify such an activity in cultured human dermal fibroblasts have been unsuccessful (10–12). More recently, an extracellular depolymerization of HA was documented by an uncharacterized endo-β-*N*-acetylglucosaminidase in the conditioned media from cultured human skin fibroblasts (13). However, an exogenous source for this activity can be invoked, such as from the fetal bovine serum used in these cultures (14, 15). Other types of fibroblasts, such as from embryonic (16, 17) and adult chickens (18), and those derived from healing wounds (19), have been documented to contain hyaluronidase activity. However, the identification of a hyaluronidase

Abbreviations used: HA, hyaluronan, hyaluronic acid; ECM, extracellular matrix; GAG, glycosaminoglycan; rTRU, relative turbidity-reducing units; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; cDNA, complementary DNA; and PMA, phorbol 12-myristate 13-acetate.

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[2] All sugars described herein have the D configuration.

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activity associated with normal mammalian dermal fibroblasts has remained elusive. At one time, it was concluded that no such enzyme exists (10, 11). A decision was made therefore to reexamine this problem, using the newer technologies (20, 21) not available during that earlier period.

MATERIALS AND METHODS

Generation of samples from cell cultures. Cultures of human dermal fibroblasts (HS27) and human fibrosarcoma cells (HT1080) were obtained from the American Type Culture Collection (ATCC). Skin fibroblasts were used routinely between passage numbers 9 and 20. Primary cultures of human fetal fibroblasts (FF24) obtained by the UCSF Department of Pathology were derived from a spontaneous abortion at week 24 of gestation. All cells were cultured at 37°C in an atmosphere of humidified air with 5% CO_2. Both the HS27 and FF24 cells were cultured in Dulbecco's Modified Essential (DME H16, 1g/L glucose) medium (GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (UCSF Cell Culture Facility, San Francisco, CA). HT1080 cells were cultured in Roswell Park Memorial Institute (RPMI 1640) medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Upon reaching confluence in 75 cm^2 cell culture flasks (Costar, Cambridge, MA), conditioned media was removed and kept on ice while cell layers were harvested using 0.05% trypsin (UCSF Cell Culture Facility). Cell layer extracts were spun at 10,000g for 1 min at 4°C. Protein was extracted from samples in phosphate-buffered saline (PBS, Sigma, St. Louis, MO) containing 46 mM n-octylglucoside (Boehringer Mannheim, Indianapolis, IN) plus 1 tablet of Complete protease inhibitor cocktail (Boehringer Mannheim) for 30 min at 4°C.

Assays for hyaluronidase activity and protein. Hyaluronidase activity was determined in two ways. A microtiter-based assay (20) was employed, and activity expressed in rTRU (relative turbidity-reducing units). Bovine testicular hyaluronidase (Sigma VI-S, 3,000 rTRU/mg, St. Louis, MO) was used as an enzyme standard. The HA substrate gel zymography was performed as described previously (21). Recombinant Hyal-1, human plasma hyaluronidase (6) was used as a control for the HA substrate gel zymography experiments. Protein concentrations were measured using the Lowry procedure (Pierce, Rockford IL).

Immunoaffinity isolation of fibroblast hyaluronidase. Purified IgG from the hybridoma clones 4D5 and 17E9 was used for immunoprecipitation. Three mg of purified IgG from the hybridoma clone was coupled to 1 ml of Protein A Sepharose 4B (Zymed, South San Francisco, CA) to produce anti-HYAL1 beads as previously described (6). One mg of cell extract was immunoprecipitated with 10ul of anti-HYAL1beads overnight at 4°C. After incubation, the anti-HYAL1 beads were washed three times with PBS. For the HA gel zymography, 10 μl of Laemmli buffer was added to the anti-HYAL1 beads to elute the hyaluronidase and loaded directly into the gel.

RT-PCR analysis of fibroblasts. Briefly, the cells were lysed in 600 μl lysis buffer and passed through a QIAshredder minicolumn (Qiagen). Total RNA was isolated from 5×10^6 fibroblasts using the RNeasy (Qiagen) RNA isolation kit according to the manufacturer's protocol. Complementary DNA was then generated using the Thermoscript system (LifeTechnologies) using the manufacturer's protocol. For the PCR reaction, a 4 μl sample of cDNA was amplified in a 50 μl reaction with the Advantage-2Taq polymerase (Clontech) in standard conditions with the following primers (5′-TCAGCCCCAAGG TTGTCCTCGACCA-3′ and 5′-CTGCCAGCCAGGGTAGCATCGACAT-3′) at a hybridization temperature of 69°C. Amplification products were detected in a 1% agarose gel, into which 10 μl of sample per lane was injected. DNA Molecular Weight Markers III (Boehringer Mannheim) were included on each gel for sizing.

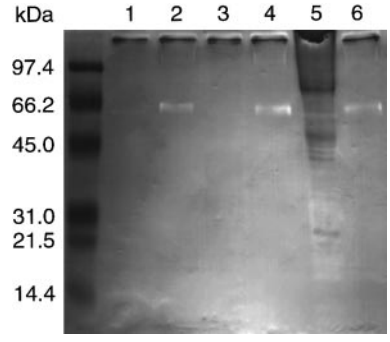

FIG. 1. Hyaluronan substrate gel zymography demonstrating hyaluronidase activity at pH 3.7 from cell-layer extracts and culture media of normal human dermal fibroblasts obtained from an adult circumcision, HS-27 (lanes 1 and 2), fetal dermal fibroblasts FF24, obtained from a 24 week abortus (lanes 2 and 3), and of human fibrosarcoma cells, HT1080 (lanes 5 and 6). Molecular weight markers are in the farthest left lane with molecular weights indicated in the ordinate. A single band of approximately 59 kDa can be seen in each lane, with the predominant band in the respective culture media (lanes 2, 4, and 6). The lane containing the fibrosarcoma cell extract was purposely overloaded to document existence of a band, albeit faint.

RESULTS

Hyaluronidase activity was examined at both neutral (pH7.4) and acid (pH3.7) conditions. No activity was detected in any of the cell extracts or their respective conditioned media when assays were performed at neutral pH. However, activity could be detected under acid conditions in all cells examined. Hyaluronan substrate gel zymography at acid pH revealed a single band of activity in the conditioned media of all cells, and a very minor band of activity in the cell-associated samples (Fig. 1). In both adult HS27 and fetal FF24 dermal fibroblasts, as well as in the fibrosarcoma cell conditioned media (lanes 2, 4, and 6 respectively), a single major band of activity was observed at 57 kDa. In the cell-layer associated extracts, a very minor band of activity could be seen that also had a size of 57 kDa (lanes 1, 3, and 5, respectively). Lane 5 containing the fibrosarcoma cell-layer extract was purposely overloaded, so that unequivocal activity could be documented. Under neutral conditions, substrate gel zymography failed to detect activity in any of the fibroblast or fibrosarcoma samples in either cell-associated or conditioned media samples. Since most enzyme was in a secreted form, only activity from conditioned media was examined in subsequent experiments.

The state of confluence was important in the production of hyaluronidase by fibroblasts. Both adult HS27 and fetal FF24 dermal fibroblasts made significantly more

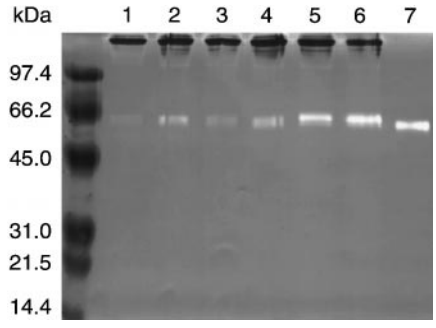

FIG. 2. Hyaluronan substrate gel zymography demonstrating hyaluronidase activity at pH 3.7 from the media of cells comparing preconfluent and confluent cultures of human dermal fibroblasts, HS-27 (lanes 1 and 2), fetal fibroblasts, FF24 (lanes 3 and 4), and fibrosarcoma cells (lanes 5 and 6). The recombinant human plasma hyaluronidase, Hyal-1 (6), is shown in lane 7. Molecular weight markers are in the farthest left lane with molecular weights indicated in the ordinate. The adult and fetal fibroblast hyaluronidases have greater activities in confluent cultures (lanes 2 and 4) than in their respective preconfluent cultures (lanes 1 and 3). State of confluence had little influence in the fibrosarcoma cell cultures (lanes 5 and 6). Equal amounts of protein were loaded onto each lane. The adult and fetal fibroblast hyaluronidases have a motility between that of the fibrosarcoma-derived enzymes and recombinant enzyme.

hyaluronidase when post-confluent (Fig. 2, lanes 2 and 4) than when pre-confluent (lanes 1 and 3). On the other hand, the HT1080 fibrosarcoma cells produced an abundance of hyaluronidase regardless of culture conditions, whether preconfluent or postconfluent (Fig. 2, lanes 5 and 6 respectively). Of interest is the observation that the marker Hyal-1, purified plasma hyaluronidase (lane 7), had a slightly lower molecular size than any of the cell-derived samples. The fibrosarcoma samples were also slightly lower in molecular size compared to the fibroblast-derived samples.

The pH profile of each sample was examined in detail. The profile of activity as a function of pH in the conditioned media from each sample was identical to that of recombinant human plasma enzyme (6) (data not shown).

Hyaluronidase activity from both the cell layers and conditioned media of all fibroblast cultures were completely immunoprecipitated in their entirety by the 17E9 monoclonal antibody (data not shown), an antibody specific for the human plasma enzyme.

Gene-specific RT-PCR was performed using primers specific for HYAL1. The mRNAs from HS27, FF24, and HT1080 fibroblast were examined. As demonstrated in Fig. 3, each of the three cell types contained HYAL1 mRNA.

Cytokines and growth factors are known to stimulate HA synthesis, and others are thought to control levels of HA deposition. Some of these were investigated, to establish whether such modulation could take place at the level of hyaluronidase activity. Preliminary results indicated that IL-1 stimulated Hyal-1 activity, as did insulin. The phorbol ester PMA (phorbol 12-myristate 13-acetate) had a modest stimulatory effect, while TGFβ exerted no effect. A systematic survey of the effect of growth factors and cytokines on Hyal-1 expression is underway.

DISCUSSION

Isolation of human plasma hyaluronidase, Hyal-1 (6, 7) and production of specific monoclonal antibodies facilitated the present studies. As shown, adult and fetal fibroblasts as well as fibrosarcoma cells express an acid-active hyaluronidase activity similar to Hyal-1. While plasma and fibroblast hyaluronidases are products of the same HYAL1 gene, as documented by PCR analyses, minor modifications were observed. Post-translational glycosylation may account for differences in motility on HA-substrate gels (Fig. 2) that distinguished fibroblast, fibrosarcoma, and plasma forms of Hyal-1.

The human dermal fibroblast hyaluronidase had a pH optima of 3.7, similar to that described for other somatic hyaluronidase activities, including liver (22), kidney (23), lung (24), brain (25), and placenta (26). The fibroblasts also had a single band of enzymatic activity at 57kDa, evident in both the cell layer and conditioned media fractions, similar in size to the activity described for other fibroblast hyaluronidases derived from the skin of chick embryos (17) and from rabbit skin wound tissue (27).

The PCR studies confirmed that transcripts for the other hyaluronidases coded for on chromosome 3p21.3, Hyal-2 and Hyal-3, are also present in fibroblasts (not shown). Hyal-2 is an acid-active enzyme that degrades high molecular weight HA to a 20 kDa intermediate-sized product (28, 29). Hyal-3, also found in chondrocytes (30), is an activity that has not yet been characterized. Investigation of these additional fibroblast hyaluronidases is currently underway.

A patient with congenital absence of Hyal-1 activity in plasma and tissues was identified recently (31, 32). It

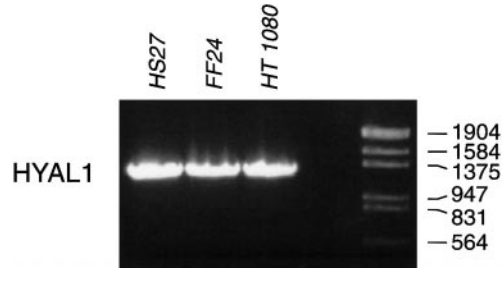

FIG. 3. A gene-specific RT-PCR survey of hyaluronidases using primers specific for HYAL1. The mRNA's from the three cell types, HS27, FF24, and HT1080, were examined. All three cell types contained HYAL1 mRNA. The bp markers are in the far right lane.

was assumed that this was an absence of an acid-active lysosomal enzyme, and therefore constituted a lysosomal storage disease. The syndrome was termed Mucopolysaccharidosis IX (MPS IX). The patient did have macrophages filled with numerous large membrane-bound clear vacuoles that stained with Alcian blue and colloidal iron. The degree of staining was reduced by pretreatment with bacterial hyaluronidase. Fibroblasts in this patient also contained such vacuoles, however, less numerous than in macrophages. Absence of Hyal-1 may have been compensated for by increased expression of Hyal-2 or Hyal-3, accounting for the relatively normal phenotype found in this patient.

The monoclonal antibody against the human plasma hyaluronidase form of Hyal-1 was used to obtain fibroblast hyaluronidase from both cell-layer and conditioned media fractions. Neither structural nor functional differences between these two forms have been established. Slight modifications may be considered that perhaps parallel differences found between the membrane-associated and soluble forms of sperm hyaluronidase, PH-20 (33–35). Excision of a carboxyl-terminal peptide comprises a portion of the difference between membrane-bound and soluble forms of PH-20. Removal of a carboxyl fragment also occurs in the urine form of Hyal-1 (36). The functional differences between circulating plasma Hyal-1 and the processed form of Hyal-1 found in urine are not known. No changes in molecular size of the cell-layer and the culture media forms of the fibroblast enzyme were detected. But other more subtle differences in the two forms of Hyal-1 may be occurring.

Functional differences between media and cell-layer forms of fibroblast hyaluronidase may be invoked. The HA substrate and the secreted form of the enzyme are extracellular.

There has been long standing evidence that HA is taken up by cells for degradation (37, 38). This uptake of HA by cells is thought to involve the HA receptor, CD44 (39–43). The HA is presumably delivered to the lysosomal compartment for catabolism.

It has been established that the ability of cultured fibroblasts to bind and internalize HA is dependent upon their state of confluence. At confluence, cells internalize and degrade HA three- to four-times more rapidly than do fibroblasts from corresponding subconfluent cultures (38). We have confirmed here that production of hyaluronidase by human fibroblasts, with the exception of the transformed cells, increases with confluence, suggesting there may be cause and effect in the internalization and degradation of HA and the levels of Hyal-1 expression. An earlier publication from this laboratory documented that HA synthesis is maximum when cells are sparse, and decreases at confluence (44). Preconfluent fibroblasts incorporate six-times more radiolabeled glucosamine precursor into HA at preconfluence than they do in the confluent or postconfluent state. HA synthesis is most abundant when cells are rapidly dividing and moving as they fill the culture dish. Other investigators have already shown an association between increased HA synthesis with both mitosis (45–47) and cell movement (48, 49). At confluence, hyaluronidase production is increased perhaps to remove preexisting HA, at the same time that HA production is being slowed down. HA, the hallmark of the ECM of rapidly growing and moving cells must be removed in order for cessation of growth to occur. There is a parallel in preconfluent and postconfluent fibroblasts with the early stem cell proliferation and movement in embryology and the onset of differentiation (2), and between tissues undergoing regenerative process followed by the cessation of repair (50).

The controls that modulate Hyal-1 expression are unknown, as well as the controls for regulating other members of this parologous family of enzymes. Calcium can modulate Hyal-1 expression in epidermal cells of skin. Cultured human skin keratinocytes increase Hyal-1 activity 25-fold when the calcium of the culture medium is increased from 0.05 to 1.50 mM (20). Levels of fibroblast Hyal-1 activity did not change with modulation of calcium levels in the culture medium, indicating that dermal and epidermal Hyal-1 are under entirely separate controls (data not shown).

The two compartments of skin, dermis and epidermis, in reality function as two separate organs. Over 50% of total body HA is present in skin (51, 52). Both compartments contain high levels of HA (53, 54). The HA and its turnover in epidermis is separate from that of the dermis. The half-life of HA synthesized in the dermis from labeled precursors is approximately 12-19 hours. Dermal HA has access to the lymphatic and cardiovascular systems, where most of it becomes degraded. From the present studies, it is assumed that local degradation also occurs.

The content of HA in the papillary dermis is far greater than that of the reticular dermis. It is not known what differences exist between fibroblasts in these two dermal compartments, whether they vary in their rates of HA turnover, or whether they have separate Hyal-1 control mechanisms.

We recently identified a post-transcriptional, pretranslational throttle for HYAL1 (55). A retained intron in the 5′ region of HYAL1 mRNA occurs in certain oral squamous cell carcinoma cell lines, preventing translation. This is the basis for the loss of Hyal-1 activity in such cells. Whether this mechanism is operative in the modulation of Hyal-1 expression in normal cells is under investigation.

Hyaluronan is a major GAG in the matrix of fibroblasts (53, 56) as well as the ECM of the dermis. We have demonstrated here that fibroblasts derived from

normal human dermis contain hyaluronidase activity. This suggests that depolymerization of HA can occur locally within the dermis, as well as in the draining lymphatics and in the liver. This observation adds a potential new dimension to fibroblast biology and to the investigation of disorders of skin and connective tissues. This is particularly true of urticaria, the edema of dermis that often occurs as part of an allergic reaction, as well as sub-epithelial bullous lesions of skin, both of which involve excessive deposition of HA with its associated water-of-hydration. Such accumulations of dermal HA can arise from both increased synthesis as well as local catabolic failures. The present observations may provide new clinical targets for pharmacological intervention. And yes, Virginia, fibroblasts do have hyaluronidase.

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