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ENHANCEMENT OF CARTILAGE PROTEASE ACTIVITY DURING AGE AND GROWTH HORMONE-DEPENDENT GROWTH

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Summary

We wish to report an intriguing relationship between cartilage protease activity and rat growth rate. This was demonstrated by comparing protease activities of rats having different growth rates, i.e., normal rats of different ages, hypophysectomized and growth hormone-treated hypophysectomized rats. Protease activity, assessed by hydrolysis of a gelatin film by cartilage microtome slices, at pH 4.0, was time and temperature dependent. Preincubation of cartilage tissue at various temperatures resulted in an increase of protease activity from 4°C to 37°C and a decrease in activity from 37°C to 100°C. The activity of younger (4 week old) more rapidly growing rats, was greater than that of older, less rapidly growing animals. Hypophysectomy reduced protease activity to approximately one-third normal levels. However, injection of bovine growth hormone into hypophysectomized rats restored the activity. These results suggest that a positive correlation exists between cartilage protease activity and growth rate. Our results support the novel hypothesis that cartilage growth could be mediated, at least in part, via growth hormone-dependent proteolytic activity.

Introduction

The relationship of tissue growth to proteolytic activity is poorly understood. The most efficient mechanism to explain tissue growth ostensibly could involve the enhancement of anabolic processes and the reduction of catabolic processes of the tissue. Therefore, it is not surprising that some investigators have reported a decrease in protein degradation during growth in vivo [1] and in vitro [1,2]. However, in other studies, high protease activity was correlated

with transformed cell growth in vitro [3,4], tumor growth [5] and age-dependent muscle growth in vivo [6]. In addition, certain proteases can stimulate cell proliferation in vitro [7,8].

Little is known about the relationship of cartilage growth to endogenous protease activities. Cartilage proteolytic activities have primarily been studied in relation to degradative diseases, such as osteoarthritis. Interestingly, however, this disease is associated with both high levels of protease activity and enhanced cell proliferation [9—11]. Although this proliferation is obtusely interpreted as an attempt by the tissue to recover from the destructive disease, it could be suggestive of a direct causal relationship of protease activity to tissue growth. Thus, investigation of the relationship between protease activity and cartilage growth could yield important information regarding the mechanism of skeletal growth.

In this study, experiments were designed to study potential differences of cartilage acid protease activities during changes in growth rate. This was accomplished by comparing protease activities of rats having different growth rates, due to differences in age, and hormone treatment.

A preliminary account of this work was presented at the 61st meeting of the Endocrine Society, Anaheim, CA [30].

Materials and Methods

Laboratory animals and hormonal treatment

Normal and 21 day post-operative hypophysectomized male Sprague-Dawley rats (Zivic-Miller Laboratories, Allison Park, PA) were used in this study. A solution of bovine GH (NIH-GH-B-18, 1.5—500 μg total dose) in alkaline saline or solvent alone was injected subcutaneously, one dose per day for 4 days. Rats were killed on the 5th day along with normal rats comparable in age (normal age control group) or weight (normal weight control group) to hypophysectomized animals.

Detection of cartilage protease activity

Cartilage protease activity was assessed by a slightly modified method of Owers [12,13]. The assay was based on the hydrolysis of gelatin film membranes which have been fixed with glutaraldehyde onto glass microscope slides [13].

Although quantitation of this protease assay was arduous, it afforded the advantages of high sensitivity and low specificity. Therefore, the possibility of detecting potential perturbations in one or more acid protease(s) (even those heretofore unindentified) was maximized by using this assay system. A similar procedure has been used by others to study the parathyroid hormone-dependence of bone proteolytic activity [14]. In the standard assay, three costal cartilages (from ribs 3—5) were excised from each rat. Embedding medium (Tissue-Tek O.C.T. compound, Scientific Products) was applied to cartilage triplicates from 7—9 rats, representing all treatment groups, and solidified at —20°C. Sixteen micron thick cross-sectional slices (starting 1 cm from the sternum) were cut at —20°C using a cryostat. Each slice was placed on a gelatin membrane and overlaid with 0.03 M veronal-acetate buffer, pH 4.0. The sam-

ples were immediately incubated in a humid chamber at 37°C for the times indicated. After incubation the slides were washed with buffer and dried prior to quantitation.

To visualize gelatin digestion, the membranes were either mixed with India ink prior to fixation [13] or stained with Trypan blue after digestion. These methods gave essentially identical results, when expressed on a percentile basis. We have, therefore, combined data from both methods. Membranes to be stained with Trypan blue were cooled for 5 min at 4°C after the reaction with cartilage. They were then washed sequentially at about 4°C for 2—10 min in 0.03 M veronal-acetate, pH 8.0, 1 min in 95% ethanol, 1 min in 50% ethanol, 10 s in 0.03 M veronal-acetate, pH 5.5, 10 s in 1.0% Trypan blue in 0.03 M veronal-acetate, pH 5.6, 10 s in deionized water and 10 s in a second water wash. The membranes were dired at room temperature and washed twice with deionized water at room temperature for 5 min each. Membranes were dried before quantitation.

Quantitation of protease activity

Protease activity resulted in decreased stain under the cartilage slice. To quantitate this activity, a photometric method was developed. After incubation, the membranes were washed, stained, and photographed (Ultra-Phot II, C. Ziess). Hydrolyzed regions appeared as dark areas on the negative. Total activity correlated with the size and darkness of these spots. Hydrolyzed areas were scanned (from the negative) at 540 nm with a densitometer [15] and the absorbance was recorded. The area under each absorbance peak was determined with a planimeter and was defined as relative total activity. Specific activity was the quotient of total activity and relative surface area of each cartilage slice. The relative surface areas were obtained by planimetry of magnified cartilage photographs. DNA content of cartilage was determined in identical parallel experiments by the diphenylamine technique, using calf thymus DNA (Sigma) as standard [16]. Total protein was quantified using the Biuret procedure, using bovine serum albumin (Sigma) as standard [17]. Statistical analysis of results was performed according to the method of Dunnett [18].

Results and Discussion

Time and temperature dependence of protease activity

Optimal cartilage protease activity on gelatin membranes was previously shown to occur at pH 4.0, with little or no activity at neutral or alkaline pH [19]. Therefore, we used pH 4.0 in the standard assay and found substantial activity with 16 μ thick sections of rat costal cartilage tissue. Protease activity, at this pH, probably represents the effect of one or more of the lysosomal acid proteases called cathepsins. As shown in Table I, protease activity at pH 4.0 was time-dependent.

The effect of temperature on cartilage protease activity is shown in Table II. After preincubation at various temperatures, cartilage was frozen, sectioned, and incubated with gelatin substrate at 37°C, pH 4.0. Optimal activity was obtained at 37°C preincubation. Activity increased between 4 and 37°C, indicating a temperature-dependent process may be required for full expression of

TABLE I

TIME-DEPENDENCE OF CARTILAGE PROTEASE ACTIVITY

Cartilage sections were placed on India-ink stained gelatin membranes and incubated at 37°C, pH 4.0 for times indicated above. Values (mean ± S.E. (n) of two experiments) are expressed as relative protease activities (Hx, 5 h activity = 100%). Statistical differences between time points were determined by Dunnett's analysis. Hypophysectomized rats +45 μg bovine GH, hypophysectomized injected with bovine GH, 45 μg total dose; normal wt. normal rat matched in weight to hypophysectomized rat. Values in parentheses represent the number of animals. Little or no detectable activity was found prior to 1 h incubation using India ink-stained membranes.

Experimental groups	Relative protease activities at 37°C	
	2 h	5 h
Hypophysectomized rats	15 ± 3 (7)	100 ± 11 (5) *
Hypophysectomized +45 μg bovine GH	43 ± 16 (6)	226 ± 23 (7) *
Normal wt.	83 ± 15 (8)	443 ± 29 (7) *

* Different from 2 h activity of the same experimental group, $P < 0.01$.

activity. Temperatures greater than 55°C greatly reduced activity, presumably due to enzyme denaturation. These results cannot be attributed to temperature-dependent changes in gelatin substrate structure since only cartilage tissue, without gelatin substrate, was preincubated.

Growth hormone-dependence of protease activity

As shown in Fig. 1, hypophysectomy greatly reduced cartilage protease activity. This observation suggested that a pituitary hormone(s) may be required for stimulation or maintenance of this activity in cartilage. Since GH is a primary regulator of cartilage growth, a priori, it would seem to be a likely candidate to account for the pituitary-dependence of the activity. Injection of bovine GH for 4 days (1.5—500 μg total dose) into hypophysectomized rats enhanced protease activity approximately to levels found in normal rats (Fig. 1). This observation suggested that pituitary influence on cartilage activity was due primarily to the action of GH. Since most, if not all, of the biochemi-

TABLE II

EFFECT OF TEMPERATURE ON PROTEASE ACTIVITY

Acid protease activity of normal age control rat cartilage was determined after 1 or 3 h preincubation at various temperatures in 0.9% NaCl. Values (mean ± S.E. (n) of 1—2 experiments) are expressed as relative specific activities.

Temperature (°C)	Relative protease activity	
	1 h	3 h
4	60 ± 4 (3) *	60 ± 4 (6) **
23	91 ± 20 (2)	75 ± 5 (6)
37	100 ± 2 (3)	100 ± 10 (4)
55	—	58 ± 5 (4) **
75	—	29 ± 8 (4) **
100	53 ± 6 (3) *	46 ± 7 (7) **

* Different from activity at 37°C, 1 h preincubation, $P < 0.05$.
** Different from activity at 37°C, 3 h preincubation, $P < 0.01$.

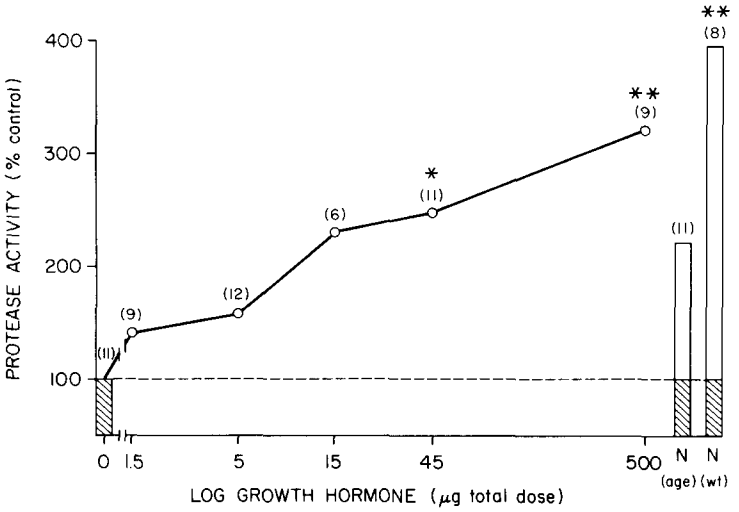

Fig. 1. Cartilage protease activity of normal, hypophysectomized and bovine GH-treated hypophysectomized rats. Hypophysectomized rats (21 days post operative) were injected for 4 days with a solution of bovine GH (NIH-GH-B-18, 1.5—500 μg total) in alkaline saline or with solvent alone. Costal cartilage was removed on day 5 and immediately frozen and assayed for proteolytic activity. Results are expressed as percentage of the hypophysectomized control average specific activity. Values are the average of four experiments. The S.E. averaged ≤13% of the mean. * Different from hypophysectomized-rat values, $P < 0.05$. ** Different from hypophysectomized-rat values, $P < 0.01$.

cal effects of GH on cartilage are believed to be mediated via GH-dependent somatomedins, it is probable that the GH-dependence of cartilage protease activity may actually be due, more directly, to the action of somatomedin than to GH itself.

Age-dependence of protease activity

Cartilage of the normal weight control group had greater specific activity than that of normal age control rats, indicating a probable age-dependence of the protease activity (Fig. 1). Further investigation of this phenomenon confirmed that the younger (4 week old), more rapidly growing animals, had higher protease activity than that of older, less rapidly growing animals (Table III). Specific activity in lieu of total activity, was used in the comparison because of substantial differences in cartilage slice cross-sectional areas. This age-dependency of cartilage protease activity was similar to canine hip joint cathepsin activity which decreased with age, but to a lesser extent per DNA than found in our experiments [20]. Our data are also compatible with Bezkorovainy's theory of aging [21], in which decreased protease activity with age causes increased levels of defective enzymes and thus less-efficient cellular function. Interestingly, the age-dependence of protease activity could be related to the hormone-dependence since secretion of, and tissue responsiveness to, GH diminishes with age [22,23].

It is possible that variation in protease activity could have been due to differences in cartilage cellularity. To investigate this possibility, cartilage DNA and protein content were determined in identical parallel experiments (Table

TABLE III

CORRELATION OF RAT AGE, BODY WEIGHT AND GROWTH VELOCITY WITH CARTILAGE PROTEASE ACTIVITY

Costal cartilage protease activity of normal rats differing in age and weight was determined. Protease values (mean ± S.E. (n) of 3—4 experiments) are expressed as percentage of the average activity of the 4-week old rats. Growth velocity values (mean of 16 or more rats per age group) were obtained from information supplied by Zivic-Miller Laboratories. Statistical differences were determined by Dunnett's analysis. The growth velocity is expressed as percentage increase of body weight per day.

Age (weeks)	Body weight range (g)	Growth velocity	Protease specific activity (%)
4 *	90—110	6.1	100 ± 7 (8)
5	115—130	4.7	76 ± 4 (8) ***
6	160—170	3.4	75 ± 4 (8) ***
8 **	270—290	2.9	56 ± 3 (13) ***

* Rats of equivalent weight to hypophysectomised animals.
** Rats of equivalent age to hypophysectomised animals.
*** Different from activity of 4 week old rats, $P < 0.01$.

IV). Both DNA and protein content of experimental groups varied no more than 50%. Since the observed protease activity often differed by more than 200% between groups, it appears that variation in cellularity had a minimal influence on differences in protease activity. In addition this enzyme activity was enhanced preferentially when compared to general changes in protein content.

Our observations indicate an enhancement of cartilage protease activity during age and GH-dependent cartilage growth. Skeletal growth involves the expression of several processes such as chondrocyte proliferation, extracellular matrix remodeling, macromolecular synthesis, and tissue calcification. Interestingly proteases have been implicated in many, if not all, of these processes [24—29]. For example, it is intriguing to note that trypsin [25], papain [26], and lysosomal lysates [27] can stimulate cartilage cell proliferation, in vivo. The

TABLE IV

CARTILAGE DNA AND PROTEIN CONTENT

Costal cartilage was weighed and homogenized (w/v) 1 : 20 in 0.005 M sodium phosphate buffer, pH 6.5. DNA and protein content of homogenates was determined after trichloroacetic acid precipitation. Values are mean ± S.E. (n) from 2—5 experiments.

Experimental group	DNA (mg/g tissue)	Protein (mg/g tissue)
Hypophysectomised	1.7 ± 0.1 (22)	60 ± 4 (22)
Hypophysectomised +500 μg bovine GH	1.9 ± 0.2 (18)	58 ± 2 (18)
Normal age	1.6 ± 0.2 (8)	70 ± 10 (8)
Normal wt.	1.8 ± 0.2 (13)	72 ± 6 (13)
Normal rats — age groups		
4 week	2.2 ± 0.2 (15)	64 ± 6 (15)
5 week	1.5 ± 0.2 (4)	70 ± 10 (4)
6 week	2.2 ± 0.3 (7)	64 ± 10 (7)
8 week	2.0 ± 0.2 (11)	96 ± 6 (11)

process of cartilage mineralization occurs primarily in regions where intercellular protein matrix has been partially degraded [28]. In addition, increased frequency of growth retardation and bone deformation apparently occurs in the off-spring of pregnant rats injected with leupeptin, a protease inhibitor [29]. Thus, it is enticing to speculate that the mechanism of GH-dependent skeletal growth may occur, at least in part, via a protease-mediated biochemical process(es). In addition, our observations may have import in the etiology and regulation of certain protease-related cartilage pathologies, such as osteoarthritis.

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