Enhancement of Cathepsin B Activity in Irradiated Mouse Testes

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·X-irradiation/Testes/Lysosomes/Cathepsin B

The irradiation of mouse testes at 600 rads is accompanied by extensive tissue destruction which ultimately results in a 70% loss of organ weight but displays appreciable recovery which is usually complete by week 18. The attrition phase of testicular weight is concurrent with a conspicuous elevation in cathepsin B activity. This is interpreted to reflect the extensive proteolysis that must form the basis for such tissue weight loss. The recovery period, in contrast, is characterized by an attenuation in the activity of the enzyme. Further analyses reveal that the rise in hydrolytic activity is not due to lysosomal membrane breakage. Nor does it seem to be related to an increase in the number of lysosomes. Instead, our data are more consistent with the contention that the rise in cathepsin B activity may be associated with an increase in protein synthesis. As a result, some lysosomes may contain a larger number of enzyme molecules. This hypothesis is supported by our demonstration that radiation induces a distinct shift in the density of lysosomes toward the heavier components. The significance of such radiation-induced enhancement of protein synthesis in terms of a general response of tissues to radiation damage is discussed.

INTRODUCTION

Special interest in the function of lysosomal enzymes, including cathepsin B, has been spurred by their demonstrated involvement in a number of pathological conditions[1]. Invasive processes such as tumorigenesis and metastasis have long been correlated with the elevated activity of these proteolytic enzymes[2,3]. Thus the growth of a tumor has been shown to proceed through the release of enzymes whose function is thought to facilitate the destruction of healthy adjacent tissues[4-7].

The attrition of soft organs resulting from exposure to gamma radiation has also been reported to coincide with the autolytic activity of released lysosomal enzymes[8,9]. Based upon quantitative cytochemistry, the underlying mechanism has been purported to involve an increase in the levels of peroxides within lysosomal membranes, their breakage, and the subsequent escape of the enzymes into the cytoplasm[10,11].

As a special case of radiation-induced cellular attrition, we have focused our attention on the mouse testes[12-14]. The potential genetical consequences arising from such injury[15] either during accidental exposures or even in cases of cancer-related therapeutic methodologies[16] have been previously addressed but found wanting of an explanation. The lysosomal contribution to the underlying mechanism of such dysfunction is unknown and was, therefore, considered important to investigate as an initial step in the understanding of X-ray damage.

MATERIALS AND METHODS

All animals were obtained from Arizona State University and were derived from an SW stock. They were maintained on an *ad libitum* regimen of food and water. Irradiation was done with a Picker C4M/60 therapeutic unit under previously described conditions[14]. Control animals were also anesthetized but not exposed.

At designated intervals, pairs of testes from a pool of 8 mice were weighed to the nearest 0.1 mg, decapsulated and homogenized in cold TKMS buffer (0.05M Tris-HCl, 0.025M KCL, 0.005M MgC12, 0.25M sucrose, pH 7.5) to a final tissue concentration of 50 mg/ml. An aliquot, designated as the unfractionated homogenate, was set aside for analysis.

The fractionation procedure was carried out according to a modification of Hofer's method[17] which consisted in centrifuging the homogenate at 800 g for 5 minutes at 4°C in a Sorvall RC-2B centrifuge (SS-34 rotor). The resulting sediment, or the first pellet, was resuspended in TKMS buffer, tested for cathepsin activity (see below), and further sedimented at 16,000 g for 20 minutes. Both the resulting second pellet and its supernatant (cell-free extract) were similarly tested for enzyme activity.

The fluorometric method of cathepsin B assay was based on the original method of Greenberg[18] as modified by Barrett[19] using the synthetic substrate N-benzoyl-DL-arginine-2-naphthylamide (BANA) in dimethyl sulfoxide (2.2 mg/100 ml). Just before use, the substrate was diluted 100 fold with 0.1M phosphate buffer containing 0.2 mM EDTA and 0.2 mM dithiothreitol (pH 6.3).

To 2 ml of the diluted substrate 50 ul of the various fractions were added at 37°C and analyzed in a Turner spectrofluorometer (Model 430) at 345 nm (excitation) and 425 nm (emission). The reaction was continuously monitored for at leat 2 minutes with a Soltec (Model 3314) recorder. All pertinent data were expressed as arbitrary fluorescence units per unit time (F units/min.). Total activity of the samples was measured after the addition of 50 ul of Triton X-100 (1% stock in water). Following a short preincubation period of 1 minute at 37°C, the samples were analyzed as described above. In all instances, the spectroluorometer readings were compensated for sample scatter and calculations of activity of the samples and the detergent when the latter was also employed.

RESULTS

ENZYME ASSAY

Two separate experiments were performed each comprising 60 irradiated and 40 control animals. The following phases of cathepsin B activity were analyzed. The first, or the free activity, is presumed to represent the normal inward diffusion of the substrate (BANA) through intact lysosomal membranes with the consequent release of the fluorogenic naphthylamine. Its level of activity during assaying is representative of the undisturbed homogenate. The conspicuously large total activity, induced by the addition of Triton X-100, is the second function of the enzyme which reflects the added availability of the substrate to its enzyme through the dissolution of the lysosomal membranes. In both cases, it was found that, within

the time of monitoring, the reactions were linear and showed no lag period indicating that the substrate was not limiting and that the rate was proportional to the concentration of the enzyme. In our experiments the free activity of control homogenates averaged 11 F units/min. (N = 10) while the total activity rose to an average value of 27 F units/min. The third aspect of cathepsin B is its latency which is expressed as the difference between the two activities. In normal testicular tissues its mean value is 16 F units/min. or 59% of the total cathepsin activity. Latency is normally unexpressed under conditions where lysosomal membranes remain intact. Although cryptic in nature, latency reflects both the intrinsic character of the enzyme within intact lysosomes and the structural integrity of the lysosomal membranes. Hence temporal changes in latency associated with radiation damage may, therefore, be essential in assessing the integrity of the lysosomes as well as the nature of the enzyme within them.
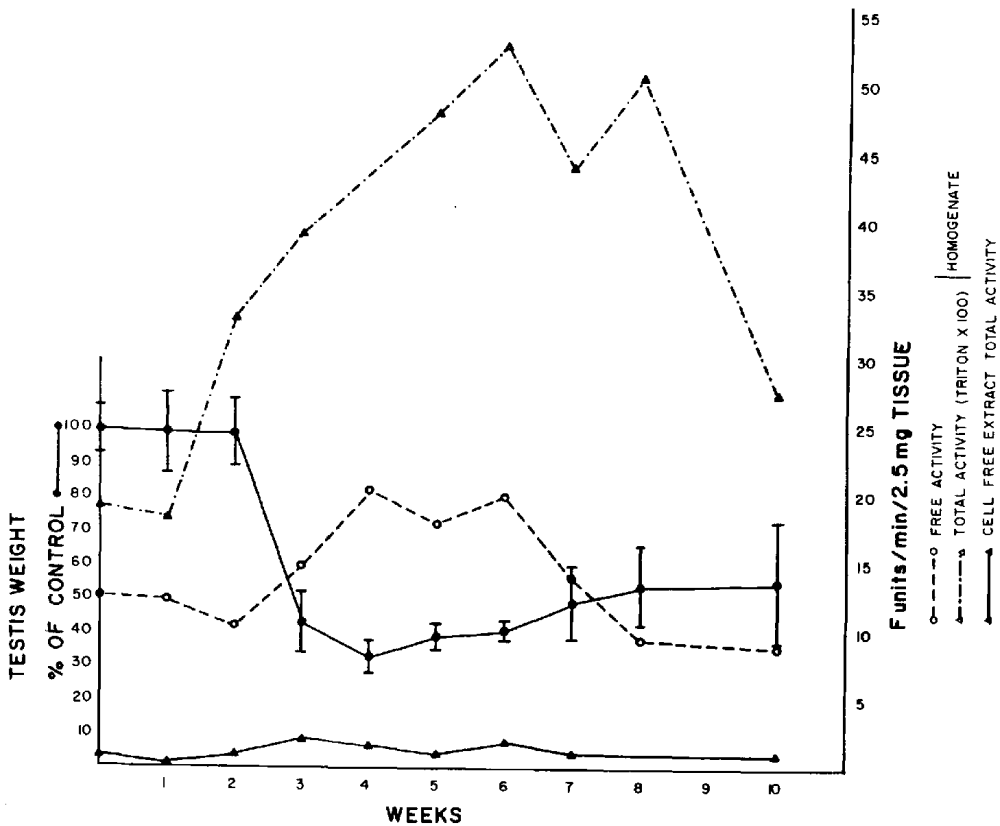

Fig. 1. The increase and subsequent decrease in the free, total and latent activity of cathepsin B in relation to testicular weight changes after an exposure of 600 rads. The total activity of the cell-free extract is also shown.

TESTICULAR WEIGHT AND CATHEPSIN B ACTIVITY

The damage that results from the irradiation of testicular tissues yields a condition of severe attrition. After a delay of two weeks, testicular weight abruptly declines to a point, 5 weeks after the initial exposure, where 70% of it is lost (Fig. 1). This is followed by a recovery period beginning at week 7 which is usually complete by week 18 (not shown). Characteristically, the initial attrition phase is accompanied by an increase in all phases of cathepsin B activity. Similarly, the recovery phase of testicular weight is punctuated by a corresponding attenuation in enzymatic activity.

Of the three phases of enzyme function, free activity shows the most modest increment from 13 F units/min. to 21 F units/min. This is in sharp contrast with latency which increases from 7 F units/min. on the day of radiation to 33 F units/min. 6 weeks after exposure thus representing a 5-fold elevation in activity. The coincidence between such enhancement and the pronounced testicular weight loss is very likely an indicator of the extensive proteolysis needed to induce the tissue losses observed. Changes in total activity are not aptly discussed since they merely reflect variations in latency already considered. The data make it clear also that the increase in both the free activity and latency is not due to the radiation-induced breakage of lysosomal membranes. Such rupture would have had the consequence of elevating the activity of the cell-free extract for which we have no evidence. In addition, it would have resulted in a measurable decrease in latency by correspondingly reducing the total activity of the samples. Hence considerations of events leading to the radiation-induced elevation in cathepsin B activity must reside within intact lysosomes themselves.

NUMBERS OF LYSOSOMES

The lack of experimental support for the radiation-induced breakage of lysosomal membranes led us to consider the possibility that an increase in the number of lysosomes per unit volume may be responsible for the enhanced activities observed. The need for more extensive proteolysis during the attrition phase could have served as a signal for the synthesis of additional lysosomes. An assay was, therefore, devised in which the total activity of irradiated homogenates was determined in the presence of increasing amounts of Triton X-100.

At a point in detergent concentration where no further increase in enzyme activity was detected, all lysosomal membranes were considered dissolved. It was further assumed that an effective increase in the number of lysosomes required a correspondingly larger concentration of the detergent for maximal release. With care being taken that the tissue concentration was always 50 mg/ml, maximal enzyme activity was always reached when 40 ul of Triton X-100 were added to the samples irrespective of the time of assaying after the exposure (Fig. 2; for reasons of clarity only the analysis of the attrition phase is shown). Excess amounts of the detergent led to a slight but measurable quenching of fluorescence. Albeit indirectly, the results do, therefore, suggest that a large increase in the number of lysosomes is not instrumental in causing the elevation in cathepsin B activity observed.

FRACTIONATION OF TESTICULAR HOMOGENATES

In the absence of any evidence for lysosomal membrane rupture or for an increase in the number of lysosomes, the elevation in cathepsin B activity, following exposure to gamma rays,
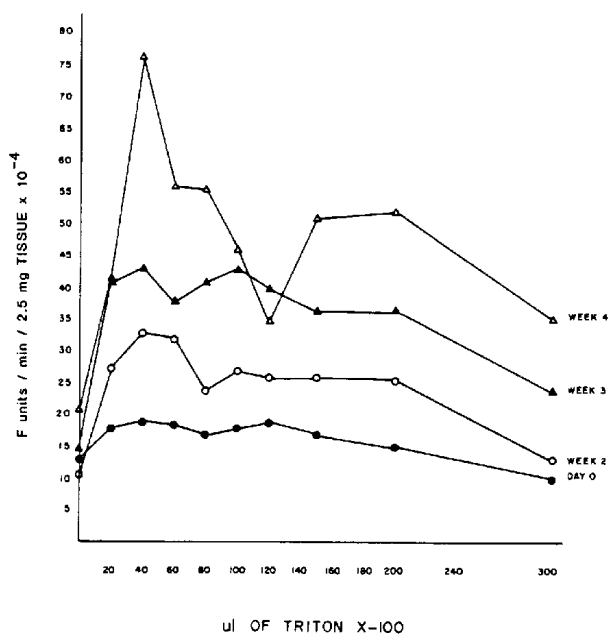
Fig. 2. Determination of cathepsin B activity following the addition of Triton X-100 in 10 ul aliquots to testicular homogenates. Portions of 0.5 ml were preincubated at 37°C for 1 minute after which 10, 20, 30 ul . . . etc. were added and mixed. To 2 ml of prewarmed substrate, 50 ul of the mixture were transferred, mixed, and immediately monitored. Each point on the curve represents the average of 4 separate determinations. All weeks were assayed as described but only the results of the first 4 weeks are shown to avoid the confusion of many overlapping curves. In all instances, maximal activity was reached when 40 ul of Triton X-100 were added.

was further investigated by characterizing the density distribution of the particles in control and irradiated homogenates. The approach chosen made use of the centrifugal profile of lysosomes as an inherent signature of their density in the various samples. Figure 3 shows the centrifugal profiles of the total activity of fractions (expressed as % of free activity) derived from control and week 7 samples. The total activity of cathepsin B was used as the indirect means of detecting the presence of lysosomes. Centrifugation of control homogenates at low force (800 g) yields two fractions: a pellet composed primarily of nuclei and cell debris and a supernatant thought to contain mitochondria, ribosomes and lysosomes. A significant increase in activity (250%) could be demonstrated in the pellet when it was subjected to membrane lysis with Triton X-100 thus indicating the presence of heavy lysosomes in addition to the nuclei and debris normally associated with it. As expected, the supernatant contained a large complement of lysosomes which were judged to be of lighter weight in view of their centrifugal
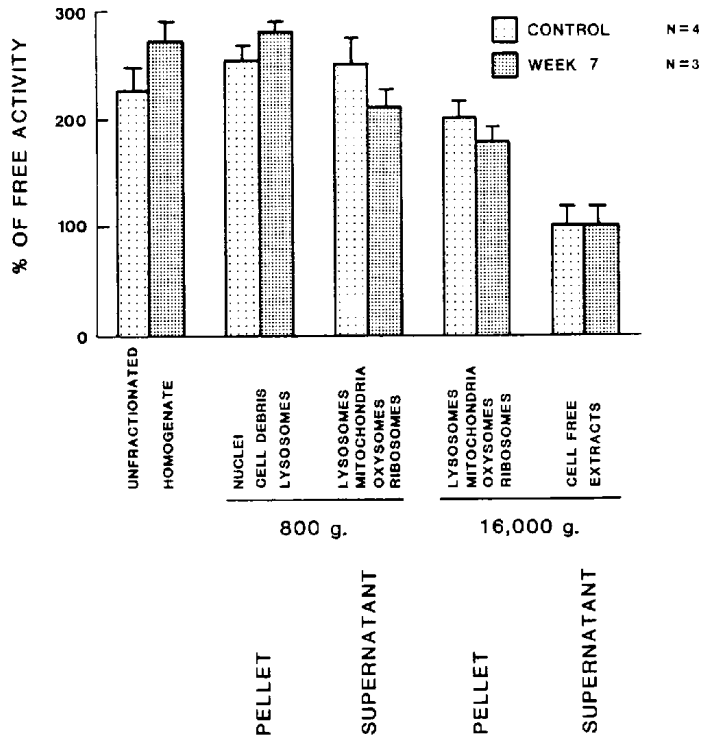
Fig. 3. Sedimentation profile of testicular samples obtained by centrifugation (see Materials and Methods). All fractions shown were assayed with Triton X-100 and the values expressed as % of free activity. The increase in the activity of the first pellet appears to result from a shift in the sedimentation profile of irradiated lysosomes since there is a corresponding decrease in the activity of the first supernatant and second pellet respectively. This is interpreted to represent an enrichment of the samples with heavier lysosomes following exposure to X-rays. The bars represent the standard deviation.

distribution. The total activity of the enzyme that could be generated in this sample with the addition of the detergent was of the same magnitude as that of the pellet. When this supernatant was further fractionated at higher centrifugal force (16,000 g), a second pellet and supernatant were also obtained. But in contrast to the previous fractions, only the pellet could be shown to contain lysosomes whose density was obviously lighter than those present in the first pellet. The corresponding supernatant, or the cell-free extract, was evidently devoid of lysosomes since no increment in activity could be ellicited following the addition of Triton X-100.

When week 7 samples were similarly examined, the unfractionated homogenates exhibited the increase in total activity expected and previously shown for irradiated samples (see Fig. 1). It could be demonstrated that this increment in activity was contributed, in large measure, by a corresponding increase in the total activity within the first pellet. But it became equally

clear that such an increase occurred at the expense of the total activity of the other fractions such as the first supernatant and the second pellet in which total activity declined.

This is interpreted to represent a shift in the density profile of irradiated lysosomes where the enrichment of the heavier particles appears to be enhanced. Under the conditions of this experiment, there are reasons to deduce, therefore, that the irradiation of testicular tissue leads to alterations in lysosomes which ultimate translate into the demonstrated shift in density during fractionation. As in the above results (see Fig. 1), the cell-free extracts showed no increase in activity when subjected to the effects of the detergent. This substantiates the contention that the elevation in cathepsin B activity is not due to membrane breakage but is inherent within altered lysosomes. It further attests to the gentleness of the technique and thus obviates the need to consider mechanical artifacts as a potential source of the enhanced enzyme activities observed.

DISCUSSION

It has been shown that the acute administration of 600 rads of gamma rays to the pelvic area of mice results in severe testicular weight attrition which, in turn, is accompanied by a drastic elevation in cathepsin B activity. The increase in enzymatic activity could not be related to a rupture of the lysosomal membrane. In fact, considerable resiliency to X-rays seems to characterize lysosomes at this dose as well as in ranges of exposures far higher that the one used in this study[9, 20, 21]. Suggestions that irradiation may affect the fragility of lysosomes have, of course, been advanced previously[22] but it is difficult to distinguish them from cases of reported radiation-induced increases in membrane permeability[9, 10]. Claims that exposure to radiation causes the activation of lysosomes[23, 24] have also been made but they are obscured by the lack of definition that attends the term "activation".

The elevation of cathepsin B activity could not be correlated with corresponding increases in the number of lysosomal particles. Small fluctuations in lysosomal numbers could have been masked by the increments of Triton X-100 used. However, had such variation in numbers been proportional to the observed augmentation in the total activity of the enzyme, a distinct shift in the concentration of the detergent would have been evident.

Considering the present evidence available, our data are more consistent with an increase in the density of lysosmes as a possible response to radiation damage. This reaction to injury may constitute a generalized phenomenon whose ubiquity may be more extensive than previously thought. For instance, the injection of a large dose of glucagon induces a shift in the median sedimentaiton characteristics of lysosomes toward the heavier components[25]. A similar shift in the size distribution has also been reported by Straus[26] in the case of induced autophagy in kidney cells stimulated with horseradish peroxidase.

Two possible causes for the increase in density can be considered. First, autophagy itself may generate a population of particles of a more complex and denser nature. Secondly, the change in cathepsin activity could also occur as a consequence of a net increase in the quantity of enzyme molecules per lysosomes accrued from enhanced protein synthesis. Admittedly, our procedures do not adequately discriminate between these two alternatives.

However, the little information that is available on the subject supports the latter contention as in the case of Sapsford's[27] demonstration that irradiated rat gonocytes accumulate additional nuclear proteins following exposure to X-rays. Other types of injuries, such as induced cryptorchidism with its attendant extensive testicular weight attrition, also bring about a significant rise in ^{3}H lysine incorporation[28]. It could also be shown that elevated lysosomal enzyme activity, in other experimental systems, depends upon an intact protein machinery since the administration of puromycin tends to depress it[29].

The enhancing effects of radiation injury on protein synthesis would significantly coincide with our results and contribute to the formulation of a comprehensive theory concerning a tissue's response to radiation damage. Accordingly, assuming the same rate of substrate diffusion into stimulated lysosomes, free activity would be enhanced in the presence of additional enzyme molecules. In a similar fashion, latency would also be augmented when revealed through the application of Triton X-100 to the experimental system. In neither of these assumptions, would it be necessary to envisage lysosome membrane rupture or permeability changes. Should it be shown that enhanced protein synthesis is indeed a fundamental response of testes to radiation damage, the inherent mechanism behind such activation after exposre will be important to determine. In addition, with the availability of techniques for obtaining enriched cellular populations from the germinal epithelium[30, 31], it should be possible to identify the cells involved in such activation. The important question of the permanence of X-ray damage on the reproductive system and its ultimate relation to aberrant inheritance patterns may also be assessed.

ACKNOWLEDGEMENT

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