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Kidney Cortex Lysosomal Acid Proteinase Activity during Induction of a Protein-Losing Nephropathy with the Aminonucleoside of Puromycin.

Paul D. Bartlett, PhD, and Laura Katona, MS*

Effects have been studied of induction of aminonucleoside-nephrosis in the rat on kidney cortex lysosomal acid proteinase activity (pH 3.2) and on such activity in the cytosol from which the lysosomes were isolated. In parallel with the onset of the massive proteinuria of the disease process but not preceding this effect, activity in the cytosol fraction was significantly increased. Significantly increased activity in the lysosomal fraction, on the other hand, was not observed until the proteinuria was well-established. Comparative study of the release of acid proteinase activity, from intact lysosomes isolated from normal and from aminonucleoside-nephrotic rat kidneys, suggest—in the latter case—either increased permeability or increased lysis of lysosomal membranes, permitting release of the proteolytic activity. Absence of any in vivo or direct effect of aminonucleoside on the release of the acid proteinase from normal intact kidney lysosomes, however, cautions against this interpretation of the findings.

During nephrotoxic serum nephritis in the rat, proteinuria develops in two stages: (a) initial albuminuria, probably arising as a result of changes in glomerular basement membrane (GBM) structure at the intra- or intermolecular level (the nature of which still remains to be determined) and (b) massive and unselective proteinuria due to polymorphonuclear (PMN)-mediated enzymic damage to the GBM.

Proteinuria in this disease thus involves an initial PMN-independent step followed by a PMN-dependent step. While considerable insight has been provided into the nature and cause of the proteinuria of the PMN-dependent step, the mechanism of induction of PMN-independent proteinuria is still unknown. Aminonucleoside-induced nephrosis in the rat provides a particularly convenient experimental model since induction of this protein-losing nephropathy occurs independently of PMN accumulation and avoids the problem of separating primary (ie, PMN-independent) from secondary (ie, PMN-dependent) effects characteristic of the immunologically induced disease. We proceeded on the assumption that proteinuria of the aminonucleoside-induced disease results from an enlargement of the interstices (ie, the intra- and

* Department of Biochemistry and Molecular Biology, Edsel B. Ford Institute for Medical Research

Address reprint requests to Dr. Bartlett at E.B.F.I., Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202
intermolecular spaces) of the GBM collagen-glycoprotein matrix. We sought experimental evidence supporting the hypothesis that pathogenesis of such proteinuria involves alterations in specific enzyme activities associated with the formation and/or cleavage of the collagen and non-collagen components of the collagen-glycoprotein matrix.

Renal cell proteases (e.g., the cathepsins), sialidase, glycosidases, glucosyl- and galactosyl-transferases, and lysyl oxidase—either singularly or in combination—might thus induce subtle changes in the tertiary and/or quaternary structure of the collagen-glycoprotein components of the GBM and, in so doing, alter the semi-permeability characteristics of the GBM. We have focused our attention on kidney lysosomal acid proteinases. There is, for example, a fairly broad spectrum of proteolytic enzymes; the lysosomal cathepsins which through synergistic and concerted action may affect almost complete degradation of a wide variety of proteins. In this context, the capacity of lysosomal enzymes to degrade completely the collagenous component of connective tissue and glycoproteins and the function of a collagenolytic cathepsin in degrading collagen would seem of special interest. Evidence also has been presented for a possible key role of lysosomal enzymes as mediators of the inflammatory process. The reported in vitro stabilization of lysosomal membranes by certain steroidal and non-steroidal anti-inflammatory drugs, as evidenced by a reduction in the release of lysosomal enzymes, is also an interesting finding since certain of these drugs are also efficacious in the treatment of protein-losing nephropathies.

We report here the results of studies of in vivo and in vitro effects of aminonucleoside, 6-dimethylamino-9-(3'-amino-3'-deoxy-β-D-ribofuranosyl)-purine, on pH 3.2 acid proteinase activity in kidney cortex lysosomes and in the cytosol from which they were isolated.

**Methods**

Sprague-Dawley virgin female rats weighing 175-200 gm were used in all studies. For isolation of lysosomes and the cytosol fraction, rats were sacrificed in parallel in groups of three (i.e., a group of three normal untreated animals and a group of three rats treated with aminonucleoside). Treated rats were injected intraperitoneally with 1 ml of 1.5% aminonucleoside solubilized in isotonic saline, and sacrificed at various post-injection time intervals.

Kidney cortex lysosomes and cytosol fraction were isolated as described by Shibko and Tappel. Enzyme activities were measured in the lysosome fraction after disruption by freezing and thawing ten times. The cytosol fraction was assayed without further treatment. Acid proteinase assays were conducted at pH 3.2 using bovine hemoglobin as a substrate. To avoid interference from aminonucleoside which also absorbs at 280 nm, the products of the assay reaction were determined by Miller's modification of the Lowry et al procedure. Urinary excretion of protein by both normal and treated rats was determined in terminal 24-hour period collections prior to sacrifice. All rats were fed ad libitum but food was withdrawn during the final 24 hours of the experimental period.

**Results**

From the results of the work of Shibko and Tappel on several subcellular fractions of rat kidney homogenate, it was clearly shown that total catheptic activity measured at pH 5 with hemoglobin substrate was largely localized in the lysosomal fraction. Initial studies conducted in our laboratory on the distribution of activity assayed with the same substrate and at pH 3.2 confirmed this finding.

Results reported herein are thus primarily concerned with acid proteinase activities in the kidney cortex lysosomal fractions but
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also include cytosol fractions from which the lysosomes were isolated. Information concerning such activity in the cytosol fraction is of interest, since increases in acid proteinase activity in the latter could reflect an aminonucleoside-induced leakiness in the lysosomal membrane, and provide a mechanism by which cellular and GBM damage is initiated in the disease process.

Table I summarizes the results of two independently conducted experiments in which lysosomal and cytosol acid proteinase activity were assayed during the course of induction of aminonucleoside-nephrosis in the rat. To simplify expression of results, the activities are expressed in terms of mg protein released from hemoglobin substrate per 100 mg cytosol or lysosomal fraction protein per hour. Although the acid proteinase activity in the cytosol fraction prepared from normal rat kidney cortex varied considerably from experiment to experiment, the day to day variations in any one experiment were relatively small, compared with those for the lysosomal fraction. This occurred in spite of meticulous attention to washing of lysosomal fractions and centrifugal forces for pelleting. To minimize day to day variations which might be due to these and other unknown parameters, results are also expressed in terms of ratios of the acid proteinase activity in the cytosol or lysosomal fraction prepared from the treated rat kidney and normal rat kidney. From this data it can readily be seen that, with the onset of massive proteinuria (corresponding to the 6th post-injection day), the ratio of such activities is considerably increased in the cytosol fraction.

Changes in the lysosomal activity ratios were not observed until the final day of the experiment at which time the disease process was fully developed. The absence of any alteration in the activity ratio preceding the onset of proteinuria (eg, as early as the 4th post-injection day) preclude a direct effect of aminonucleoside on the acid proteinase activity and any causal relationship to the development of the proteinuria.

Data for an in vitro experiment summarized in Table II substantiate this conclusion. No in vitro or direct effect of aminonucleoside on the acid proteinase activity of normal lysosomes is discernible when intact lysosomes are incubated with aminonucleoside in a concentration of 3.5 x 10⁻⁴ molar for periods of 15′ and 60′ at 37°C. Expressed in terms of total lysosomal acid proteinase activity released by the usual lysis procedure, we found that normal kidney cortex lysosomes incubated in the absence and presence of aminonucleoside released 34.9 and 34.5% of the total activity in 15′ and 57.3 and 52.5% in 60 minutes.

Since the increase in lysosomal membrane permeability to acid proteinase activity might occur during the period of in vivo exposure to aminonucleoside, we conducted a comparative study of the leakiness of lysosomes isolated from the kidney cortex of normal untreated control rats and from a similar group of rats treated with the aminonucleoside of puromycin.

Table III summarizes the results of such an experiment in which normal and aminonucleoside-nephrotic kidney cortex lysosomes were incubated in 0.34 M EDTA in 0.6 M sucrose, pH 7.1 at 37°C for one- and two-hour periods. Expressed in terms of total acid proteinase activity released by the usual lysing procedure of freezing and thawing, approximately 20% more activity is released from the aminonucleoside nephrotic rat kidney cortex lysosomes during the first hour of incubation than is released from the normal lysosomes.

During the second hour of incubation an additional 32.6% of the total activity was released from lysosomes isolated from the normal rat kidney cortex but that from aminonucleoside-nephrotic rats decreased 10.8%. This is an interesting finding since it occurred even though the release of lysosomal protein measured during the second hour of incubation was actually increased. Thus 16.5% of the total protein was released from the lysosomes isolated from
### Table I

**TIME-COURSE STUDIES OF RAT KIDNEY CORTEX LYSOSOME AND CYTOSOL ACID-PROTEINASE IN AMINONUCLEOSIDE-NEPHROSIS**

<table>
<thead>
<tr>
<th>Experiment Number</th>
<th>Days Post-Injection*</th>
<th>Urine Protein† (mg excreted/24 hr)</th>
<th>Acid-Proteinase Activity</th>
<th>Lysosomes</th>
<th>Cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
<td>Treated**</td>
<td>Ratio</td>
</tr>
<tr>
<td>I.</td>
<td>2</td>
<td>2.4</td>
<td>80</td>
<td>78</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.7</td>
<td>100</td>
<td>85</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>62.6</td>
<td>92</td>
<td>125</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>227.</td>
<td>91</td>
<td>153</td>
<td>1.68</td>
</tr>
<tr>
<td>II.</td>
<td>2</td>
<td>3.4</td>
<td>61</td>
<td>63</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>6.9</td>
<td>69</td>
<td>68</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>93.1</td>
<td>62</td>
<td>72</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>89.9</td>
<td>63</td>
<td>91</td>
<td>1.44</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>145.2</td>
<td>64</td>
<td>113</td>
<td>1.77</td>
</tr>
</tbody>
</table>

* Days (24 hour period) after a single intraperitoneal injection of 15 mg aminonucleoside dissolved in isotonic saline (1 ml).
** AN = aminonucleoside-treated; N = Normal; AN/N = Ratio of Activities
† Average excretion of 15 normal rats = 2.4 mg/24 hr.
### Table II

**IN VITRO EFFECTS OF AMINONUCLEOSIDE ON NORMAL KIDNEY CORTEX LYPOSOMAL ACID PROTEINASE ACTIVITY**

<table>
<thead>
<tr>
<th>Source of Lysosomes</th>
<th>Lysed Lysosomes</th>
<th>Intact Lysosomes Incubated*</th>
<th>Percentage of Total Lysed Activity Released In 15 min.</th>
<th>Percentage of Total Lysed Activity Released In 60 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>(mg protein released/mg lysosomal protein/hr)</td>
<td></td>
</tr>
<tr>
<td>Normal Rats</td>
<td>6.7</td>
<td>2.3</td>
<td>3.5</td>
<td>34.5</td>
</tr>
<tr>
<td>Normal Rats**</td>
<td>6.1</td>
<td>2.1</td>
<td>3.5</td>
<td>34.9</td>
</tr>
</tbody>
</table>

* Lysed by freezing and thawing 10x.
** Added aminonucleoside to give a final concentration of 3.5 x 10^{-4} molar.
*** Incubated at 37°C.

### Table III

**STUDY OF THE RELEASE OF ACID PROTEINASE ACTIVITY AND OF PROTEIN FROM NORMAL AND AMINONUCLEOSIDE NEPHROTIC RAT KIDNEY CORTEX LYPOSOMES DURING INCUBATION 37°C.**

<table>
<thead>
<tr>
<th>Source and Treatment of Lysosomes</th>
<th>Normal Rat</th>
<th>Aminonucleoside-Nephrotic Rat*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lysed**</td>
<td>Incubated***</td>
</tr>
<tr>
<td></td>
<td>1 hr.</td>
<td>2 hr.</td>
</tr>
<tr>
<td>Acid proteinase activity released:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) mg protein released/mg lysosomal protein/hr. from substrate</td>
<td>6.25</td>
<td>2.91</td>
</tr>
<tr>
<td>(b) % of total activity released</td>
<td>46.6</td>
<td>79.2</td>
</tr>
<tr>
<td>Lysosomal Protein released:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) % total of protein†</td>
<td>32.2</td>
<td>41.5</td>
</tr>
</tbody>
</table>

* Rats injected I.P. with 15 mg. aminonucleoside in isotonic saline and sacrificed 10 days later.
** Lysosomes lysed by freezing and thawing 10x.
*** Incubated at 37°C for indicated time before assay.
† % Protein released = 100 (mg protein released from lysosome by 37°C incubation/mg protein (total) released from lysosomes by lysis procedure)
aminonucleoside-nephrotic rat kidney cortex while only 6.5% additional was released during the same period by the normal kidney cortex lysosomes.

In lysosomes isolated from the kidneys of the aminonucleoside-treated rat, loss of protease activity during the second hour of incubation might be attributed to formation and release of some inhibitor molecule in the incubation medium during the prolonged period of acid protease activity or even perhaps to the accumulation of overwhelming concentrations of hydrolytic products which interfere with the formation and/or breakdown of an enzyme-substrate complex (ie, hemoglobin-acid protease complex).

Discussion

Evidence from the laboratories of several investigators now seems to point to phenomena of considerable interest in respect to the mechanism of induction of a protein-losing nephropathy in the rat with the aminonucleoside of puromycin. In this context demonstration by Gang and Mautner of the appearance of protease activities in the serum of aminonucleoside-nephrotic rats prior to the onset of proteinuria and of morphologic alterations coinciding in time with the onset of excretion of GBM-like proteins and increased excretion of acid and alkaline proteases in the urine of such animals focuses attention not only on the release of proteolytic enzymes but also their possible implication in damaging the GBM and altering its semi-permeability characteristics. Pertinent also in this respect are membranal alterations induced in kidney by trypsin injections and the in vivo effects of aminonucleoside on the labilization of pancreatic zymogen granules resulting in the release of proteases into the circulation. Mayer et al were also able to demonstrate a direct concentration dependent in vitro effect of aminonucleoside on the release of hydrolytic enzymes from isolated pancreatic zymogen granules. While the demonstration in our studies of a relatively greater release of acid protease activity and of protein from lysosomes isolated from kidney cortex of aminonucleoside-nephrotic rats than from those isolated from normal rat kidney cortex can be considered as in accord with the view that aminonucleoside or some one of its metabolites induces alterations in the tertiary and/or quaternary structure of lysosomal membranes, this effect does not appear to be causally related to the onset of the proteinuria in such animals. This is in contrast to the implication that labilization of membranes of pancreatic zymogen granules and of neutrophils (extrarenal alterations) by aminonucleoside, resulting in the release of proteolytic enzymes into the circulation and subsequent damage to the GBM, is causally related to the initiation and onset of the proteinuria characteristic of the disease process.

In the absence of aminonucleoside-induced alterations in kidney cortex lysosomal and cytosol acid protease activity prior to the onset of proteinuria and in the absence of a direct in vitro effect of aminonucleoside on normal kidney cortex lysosomal activity, the marked elevation in acid protease activity, occurring in a later time sequence, might be interpreted as reflecting an effort on the part of the renal cell to rid itself of the exceptionally high concentrations of protein arising from tubular re-absorption of plasma proteins entering the urinary space. The possibility of enhanced damage to GBM arising from the proteolytic action of increased lysosomal and cytosol acid protease activity may, however, contribute significantly to erosion and loss of GBM structure as the disease progresses.
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REFERENCES


