Serum T-kininogen Levels Increase Two to Four Months before Death*

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We have reported an accumulation of T-kininogen mRNA in the liver of aging Sprague-Dawley rats. T-kininogen is a cysteine proteinase inhibitor. Since a disruption of the intracellular protein degradation machinery is known to occur during senescence, we wished to further define the role of this protein in the aging process. As a first step, we have measured T-kininogen levels both in serum and within the liver. We have found that serum protein levels are indeed augmented during senescence, although not as dramatically as the mRNA (2.5-fold versus 8.3-fold). Immunochemistry, as well as Western blot analysis suggests that this is due to the presence of T-kininogen within hepatic cells in aged rats. Life-long dietary restriction, a known age-prolonging treatment, decreases the overexpression of the protein in 24-month-old rats. Later, diet-restricted animals still show an increased expression from the gene, the effect being delayed but not abolished by dietary manipulation. Interestingly, a longitudinal study indicated the existence of a positive correlation between the time of increase of serum T-kininogen and the time of death of the animal. Serum T-kininogen was found to increase 2.5-4 months before death.

By differential screening of a cDNA library constructed from old Sprague-Dawley rat liver mRNA, we have recently found that T-kininogen gene expression is induced during aging (Sierra et al., 1989). In a previous report, we have demonstrated that the increase in T-kininogen mRNA appears to be independent of acute inflammatory processes, and it operates at the gene transcription level (Sierra et al., 1989). While the original screening was done with the sole purpose of defining a marker of aging, it is obvious that an examination of the possible role of this protein in the senescence process is important for our understanding of the full range of biological changes than accompany aging. For this reason, we have now undertaken a study aimed at determining the relative levels of this protein both in the serum and in the liver from aging animals.

T-kininogen is a member of a family of proteins whose serum levels increase during experimental as well as physiological inflammatory conditions in rodents (the so-called Acute Phase Reactants) (Anderson et al., 1984; Birch and Schreiber, 1986; Furuto-Kato et al., 1985; Kushner, 1982). It has been shown that aged animals often have a decreased response to inflammatory stimuli, as exemplified by a reduced responsiveness of lymphocytes to cytokines (Martin et al., 1989; Thoman and Weigle, 1981), a reduced febrile response (Gleckman and Hibert, 1982), and an increased susceptibility to sepsis and endotoxin shock (Knook and Brouwer, 1989). The role of many of the acute phase proteins is related to the defense of the organism against further damage (usually by inhibition of released proteases) or the salvage of debris (such as heme groups, for example) for recycling in the liver (Pey and Gauldie, 1990). T-kininogen is a potent cysteine proteinase inhibitor (Anderson and Heath, 1985) and, at least in vitro, it can give rise to the vasoactive peptide T-kinin through the action of high concentrations of trypsin (Okamoto and Greenbaum, 1983). It is not presently clear whether this conversion can occur in vivo since no protease with the correct specificity has thus far been described in serum. For this reason, we are concentrating our efforts on the possible role of T-kininogen as a cysteine proteinase inhibitor. Most cysteine proteinases described so far have an intracellular location and are thought to be directly involved in intracellular protein turnover (Bond and Butler, 1987; Kushner, 1982). The apparent breakdown of this machinery during senescence might be responsible for the observed accumulation of anomalous as well as partially ubiquitinilated proteins in cells derived from aging organisms (Ivy et al., 1984, 1989, 1990; Lavie et al., 1981; Rothstein, 1985). Direct infusion of a known cysteine proteinase inhibitor (leupeptin) into the brain, as well as other internal organs, similarly leads to the accumulation of both age pigment (lipofuscin) and ubiquitinilated proteins (Ivy et al., 1988, 1990). These observations have led some investigators to propose a theory of aging based on the inhibition of intracellular cysteine proteinases (Ivy, 1987).

In this paper, we report an increase in serum as well as intrahepatic T-kininogen levels in senescent Sprague-Dawley rats. This effect is found to tightly correlate with the time of death of the animal, the induction occurring between 2.5 and 4 months before death. Furthermore, dietary restriction, a well-known age-prolonging treatment, effectively diminishes the overexpression of T-kininogen in 24-month-old rats. This effect is, however, transient since food-restricted animals do show an induction of T-kininogen later in life. As in control animals, induction occurs several months before death.

MATERIALS AND METHODS

Animals and Diets—Sprague-Dawley male rats were used in all experiments. They were housed individually in our specific pathogen-free facility and, unless otherwise noted, were fed ad libitum a laboratory chow Nafag 857 (Nafag, A.G., St. Gallen, Switzerland). These rats have a median lifespan of 23.85 ± 0.66 months. Dietary treatments were started at 8 months of age. One restricted group
received 70% of the mean dietary intake of the ad libitum group (median lifespan 26.25 ± 0.48 months), and another group was fasted 2 nonconsecutive days a week for a period of 24 h (median lifespan 25.51 ± 0.58 months). The semipurified diet used contained 24% protein (lactalbumin) and 6% fat. The restricted group received a vitamin supplement (to the level of the ad libitum intake) so as to avoid malnutrition.

Whenever necessary, acute inflammation was induced by a single subcutaneous injection of turpentine (400 μl/100 g of body weight), and RNA or protein samples were collected after 24 h.

Protein Analysis—For most experiments, blood was collected by aortic puncture at the time of sacrifice. For the time course experiment, however, blood was collected every 6 weeks by retro-orbital puncture. Serum T-kininogen and α1-acid glycoprotein levels were then determined by rocket immunoelectrophoresis as previously described (Baumann et al., 1987; Laurell and McKay, 1981) or Western immunoblotting after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Burnette, 1981). For immunofluorescence studies, small pieces of liver were fixed in Bouins for 1 day, they dehydrated, and embedded in paraffin. The presence of T-kininogen was determined by fluorescence microscopy using rabbit polyclonal anti-rat T-kininogen-specific antibodies (Baumann et al., 1987).

RNA Analysis—Total liver RNA was extracted by a modification of the acid guanidinium-phenol-chloroform method of Chomczynski and Sacchi (1987), as modified by Puissant and Houbeline (1990). Total RNA was then electrophoretically fractionated on glyoxal gels (McMaster and Carmichael, 1977), transferred to GeneScreen membranes (Du Pont New England Nuclear), fixed by exposure to UV light, and hybridized to random primed probes in 50% formamide at 42 °C overnight. The cloning of cDNA sequences used as probes has been previously described (Sierra et al., 1989).

RESULTS

Serum T-kininogen Levels Are Induced during Aging—In a previous study, we established that T-kininogen mRNA is more abundant in the liver of old animals (24 months), as compared to younger counterparts (Sierra et al., 1989). As a natural follow-up to these findings, we wanted to determine whether the level of the protein product was also increased during senescence. Since under inflammatory conditions T-kininogen is secreted into the bloodstream, we first measured the protein levels in the serum of young and old animals. The results are presented in Fig. 1. Both Western blot analysis and rocket immunoelectrophoresis measurements indicate that aging male Sprague-Dawley rats have a serum level of T-kininogen significantly higher than younger counterparts. As a control, we have measured the levels of albumin, α1-acid glycoprotein, and α2-macroglobulin. In our hands, aging does not affect the serum levels of α1-acid glycoprotein or albumin. The level of α2-macroglobulin is slightly elevated. This increase is, however, not controlled at the transcriptional level (data not shown) and is therefore unlikely to reflect an inflammatory process.

Dietary Restriction Eliminates the Overexpression of T-kininogen mRNA in 24-Month-old Rats—As a way of further clarifying the relationship between aging and the induction of T-kininogen, we tested the effect of dietary restriction on the expression of the gene. Dietary restriction has long been known to effect a prolongation of the median life span in laboratory animals (Fernandes et al., 1976; Guigoz and Munro, 1985; Masoro, 1988; Weindruch et al., 1979; Yu et al., 1982; Yu, 1987). Furthermore, the age-related decline in a variety of biochemical parameters has been shown to be either abolished or at least greatly reduced or delayed by dietary restriction (Chatterjee et al., 1989; Masoro et al., 1982; Richardson et al., 1987). Therefore, it was of considerable interest to determine whether or not dietary restriction has any effect on the age-related expression of T-kininogen. We first measured the effect of diet manipulation on T-kininogen mRNA levels in rats of 24 months of age.

Fig. 2A indicates that the T-kininogen mRNA induction observed in 24-month-old animals fed ad libitum (lane 3) is substantially reduced in dietary restricted 24-month-old animals (lanes 4 and 5). While a small decrease in the level of α1-acid glycoprotein in the dietary restricted samples is also apparent, results obtained with individually tested animals (data not shown), as well as normalization relative to ribosomal RNA precludes the possibilities of either inflammatory processes or unequal RNA loading affecting our results. Dietary restriction also has an effect on the expression of the gene at 15 months of age (lanes 1 and 2). We do not at present have an explanation for this effect since our previous observations have shown that the overexpression of the gene is not yet observable in 15-month-old fed ad libitum. Analysis of mRNA levels in individual animals confirmed that the relatively high expression observed in the 15-month-old group fed ad libitum (lane 1) is not due to the presence of a few inflamed animals within the group, as determined by the lack of overexpression of α1-acid glycoprotein (data not shown).

Dietary Restriction Diminishes Serum T-kininogen in 24-Month-old Rats—The results shown in Fig. 2A indicate that dietary restriction can at least partially reverse the age-related
overexpression from the T-kininogen genes in 24-month-old animals. To further confirm these results, we analyzed serum T-kininogen levels from these same rats. Fig. 2B shows an immunoblot prepared from a sodium dodecyl sulfate-polyacrylamide gel containing pooled serum samples and probed with antibodies that specifically recognize rat T-kininogen and α1-acid glycoprotein. This experiment clearly shows that, at the protein level, the effect of dietary restriction is qualitatively similar to the effect previously observed at the mRNA level, namely, that dietary restriction is capable of diminishing the overexpression of the protein in 24-month-old animals (lanes 4 and 5, compared to lane 3). Interestingly, at the protein level there is no difference due to diet between the two samples from 15-month-old animals, in spite of the fact that the serum utilized for this experiment was obtained from the same animals used for the RNA analysis.

**T-kininogen Induction Occurs Shortly Before Death**—Fig. 2 indicates that dietary restriction can effectively abolish overexpression from the T-kininogen genes in 24-month-old rats. Two possible explanations for this phenomenon can be offered: dietary restriction can either abolish or delay the overexpression from the genes. In order to test which of these hypotheses is correct, we collected blood every 6 weeks from animals under different diet conditions (n = 18 for each group). Levels of serum T-kininogen (and α1-acid glycoprotein, not shown) were determined by Western blot analysis. The results are presented as a level relative to that obtained in young animals (17-months-old) against the fraction of lifetime accomplished at the moment the sample was taken (see text). 0 = ad libitum fed. ∆ = restricted to 70% of food intake. ⊀ = fasted 2 days/week. Statistical analysis of the data indicates that the last three points of each group (starting at 85–89% of lifespan) are statistically significant (p ≤ 0.01), when compared to the first sample point (60–64% of lifespan).

in T-kininogen (p ≤ 0.01) occurs at between 85 and 89% of lifespan. This represents between 2.5 and 4 months before death. Again, for this experiment, the lack of increase in α1-acid glycoprotein was taken to indicate the absence of inflammatory processes (not shown). We can therefore conclude that dietary restriction delays but does not abolish overexpression from the T-kininogen genes in old rats.

**Intrahepatic T-kininogen Levels Increase during Senescence**—Fig. 1 indicates that the level of T-kininogen protein in the serum is statistically elevated during senescence. However, this difference is not quite as dramatic as the difference we have observed at the mRNA level. Indeed, we have determined that liver T-kininogen mRNA content is increased 8.3-fold in aging rats (Sierra et al., 1989), and the increase in serum protein is only 2.5-fold (see Fig. 1B). One possible explanation for this apparent discrepancy could be that the protein is not efficiently secreted by the liver of old animals, therefore leading to its accumulation within the hepatocytes. The high levels of albumin secretion in old animals (Chen et al., 1973; Van Bezooijen, 1984, and data not shown) argue against an overall breakdown of the hepatocyte secretory pathway during senescence. We nevertheless decided to test this possibility by directly measuring the level of T-kininogen within the hepatocytes. Fig. 4 shows the results of immunofluorescence staining of liver samples derived from animals of different ages. It is clear that, with age, increasing amounts of T-kininogen immunoreactive material are present within the tissue. The staining observed in the tissue derived from senescent animals (panel D, 24-month-old) is distinct from that observed during experimental inflammation (panel A) in that no granules are apparent, suggesting a different intracellular localization of the protein. The fact that high levels of this material also exist during experimental inflammation, even in young animals, prevents us from making any conclusions about the effect of aging on the secretory rate of this particular protein. While the immunofluorescence studies clearly show an intrahepatic increase of T-kininogen with age, it is difficult to directly quantitate these results. For this reason, we decided to repeat the experiment, this time using
T-kininogen and Aging

In a previous report, we have shown that T-kininogen mRNA levels are elevated during aging of male Sprague-Dawley rats. This increase is rather dramatic (about 8-fold) and statistically reproducible (Sierra et al., 1989). We have now undertaken to measure whether this increase in mRNA is reflected in a concomitant increase at the protein level. Immunoblot assays of total serum proteins allowed us to conclude that, in aging rats, T-kininogen levels are indeed elevated as compared to younger counterparts. The induction of serum levels has practical significance since it allows the determination of this senescence marker without the need to sacrifice the animal, thus allowing longitudinal studies such as those presented in Fig. 3.

The induction of serum T-kininogen, while statistically significant, is however less remarkable than the corresponding increase in mRNA level. Many potential explanations can be offered, such as increased intrahepatic degradation or diminished secretion. Outside the liver, the potential formation of enzyme-inhibitor complexes, followed by increased kidney clearance could also explain these results. By using in situ immunocytochemistry staining, we have shown the presence of the protein within a subset of hepatocytes in the liver of aging animals (Fig. 4, panel D). Similarly, semiquantitative immunoblotting on samples obtained after liver perfusion have shown that the protein is found intracellularly in higher amounts in old as compared to young animals (Fig. 5). Intrahepatic T-kininogen is approximately 7-fold higher in old as compared to young animals, while in this particular set of experiments the average serum increase was found to be 4.5-fold (Fig. 5). These observations together can probably account for the difference observed between mRNA and serum protein levels.

We can therefore also rule out the possibility that the mRNA which accumulates with age is not functional. T-kininogen is a potent cysteine proteinase inhibitor (Anderson and Heath, 1985), and most cysteine proteinases described so far have an intracellular location (Bond and Butler, 1987; Katunuma and Kominami, 1983). It has been observed that bloodborne T-kininogen can penetrate into cells other than hepatocytes (Chao et al., 1988), thus providing at least a theoretical way for it to encounter its substrate, the intracellular proteases. Independently of this observation, it is quite important to consider that a significant amount of T-kininogen is indeed found intracellularly in aged animals, at least at its site of production, the hepatocyte. Therefore, it is possible, if not likely, that during senescence T-kininogen can inhibit the intrahepatic protein degradation machinery. In the present work, we have not yet addressed the issue of intracellular compartmentalization. Indeed, cathepsins, the most abundant intracellular cysteine proteinases, are located in lysosomes, while T-kininogen is expected to follow a secretory pathway. The nongranular appearance of T-kininogen in immunocytochemistry staining (Fig. 4, panel D) suggests the possibility that the protein is either deviated from this pathway or re-internalized via receptor-mediated "shuttling."
Inhibition of the intracellular protein degradation machinery has long been advocated as a plausible explanation for the accumulation of aberrantly modified peptides often observed in aging cells (Ivy et al., 1984, 1989; Lavie et al., 1981; Rothstein, 1985). The presence of T-kininogen in an intracellular location could therefore provide a biochemical basis for this phenomenon. Interestingly, injection of Leupeptin or E-64C (two synthetic cysteine proteinase inhibitors), but not serine proteinase inhibitors, into a variety of organs of several species, results in the accumulation of so-called “age-pigment,” characteristic of aging cells, as well as partially degraded, ubiquitinated protein products (Ivy et al., 1984, 1989; Ivy, 1987). Until we determine the intracellular localization of T-kininogen in cells from senescent animals, it is not possible to predict which, if any, of these protease systems is affected by the elevation in T-kininogen levels.

If the presence of T-kininogen were to have a direct effect on the mechanisms of cellular aging, it would be expected that this protein, or a functional equivalent, would accumulate in a variety of different aging organisms. So far, we have limited our studies to male Sprague-Dawley rats, and it is therefore too early to conclude anything about the effect of T-kininogen on the process of aging in general. We have, however, studied the expression of the gene under conditions in which the lifespan of the animal is changed. In fact, when male Sprague-Dawley rats are kept in our facilities, and fed ad libitum, their medium lifespan is 23.5 months. If the food intake of these same animals is reduced by 30%, their lifespan is then increased to 25–26 months. Under these conditions, we have observed that the overexpression of T-kininogen is delayed, as expected (Fig. 2), while the degree of overexpression (observed later in life in diet-restricted animals) is not considerably changed (Fig. 3).

Among many other metabolic effects, dietary restriction is known to delay death by lowering the incidence of disease states, such as nephritis (Masoro and Yu, 1989). It could be argued that the induction of T-kininogen seen in ad libitum fed animals is due to a particular type of inflammation that transcriptionally induces only one acute phase gene (T-kininogen) in the absence of overexpression from the other members of the family. The delay in the appearance of such a pathology in dietary-restricted animals would explain the observed delay in induction of the gene in these animals. However, no such pathology has so far been described in the literature, as all pathological states that induce an acute phase response induce concomitant expression from all relevant genes (Fey and Gaukro, 1990; Kushner, 1982).

The mechanisms by which T-kininogen gene expression is induced in aging liver are not known, and therefore, the effect of diet restriction cannot be properly evaluated. Nevertheless, it has been reported that food restriction enhances the proteolytic capacity of the aging liver (Ward, 1988). This phenomenon could be explained by the lower degree of T-kininogen induction observed under these conditions.

Our finding that there exists a positive correlation between the time of induction of T-kininogen and the time of death of the animal is intriguing. One possible explanation is that, as the time of death approaches, massive cell death leads to a quasi-inflammatory situation that induces the expression of this particular acute phase gene. We do not favor this hypothesis for two reasons: first, acute inflammation leads to overexpression of the whole family of acute phase genes, not just one of them. Second, it would seem unlikely for animals to be able to survive 2.5–4 months after such a massive cell death has occurred. Many other possible explanations for these findings can be offered. In view of existing theories about aging, we presently favor that the overexpression of the gene, for whatever reason it might be triggered, leads to a breakdown of the hepatocyte protein degradation machinery. This in turn could lead to the accumulation of toxic by-products, leading to renal failure as well as ultimately causing cell death. At present we lack any direct evidence to support this hypothesis, but it could be easily tested by overexpressing the gene either in culture cells or in transgenic animals.

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