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Murine Myeloid Leukemia: *in Vivo* Suppression by Sericystatin A, a Proteinase Inhibitor from Leukocytes

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Abstract—*Parenteral administration of sericystatin A, an intracellular leukocyte inhibitor of the proinsulin-splitting enzyme cathepsin B, prolongs the mean survival time of mice with insulin-dependent myeloid leukemia. Sericystatin A reduces the supranormal blood levels of substances detectable by insulin-specific radioimmunoassay in these animals; this effect is abolished by concomitant insulin administration. It is concluded that sericystatin A reduces tumor proliferation indirectly, i.e. by reducing circulating insulin levels.*

INTRODUCTION

WE RECENTLY described the *in vivo* suppression of growth of an insulin-dependent murine myeloid leukemia by somatostatin [1]. This hormone decreases the supranormal levels of immunoreactive insulin (IRI) in such tumorous animals. It was proposed that this effect, possibly together with the diminution of growth hormone levels, is the mechanism of the modulation of the growth rate of this insulin-dependent tumor [1].

The recent discovery that the lysozomal protease cathepsin B converts proinsulin to insulin *in situ* [2] and the availability of purified sericystatin A, a natural specific intracellular inhibitor of cathepsin B, and related cysteine proteinases [3, 4] enable a test of this hypothesis.

Here we report that sericystatin A, administered intraperitoneally to mice bearing myeloid leukemia, diminishes both IRI in blood and prolongs their mean survival time; this effect is abolished by the simultaneous application of minute doses of insulin. However, this inhibitor does not exert any effect on tumorous cells *in vitro*, indicating that their growth *in vivo* is retarded mainly by modulation of blood IRI levels.

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MATERIALS AND METHODS

The myeloid leukemia was transplanted by intravenous inoculation of one thousand or one million splenocytes into male RF/O mice [5]. The maximum (exponential) proliferation rate of leukemic cells in the spleen was observed between the seventh and ninth days after inoculation. Cell suspensions were obtained by passing spleens through sterile nylon gauze. The number of viable cells was determined by trypan blue exclusion.

Mice were kept in plastic cages, not more than five in each. They were fed with standard pelleted food and had water *ad libitum*.

Cells were grown in Parker's medium in Bellco (Vineland, NJ, U.S.A.) microplates. Each well contained one million tumor cells in 200 μl medium plus 50 μl of the appropriate concentration of sericystatin A and 25 μl of [^{3}H]-thymidine (Radiochemical Centre, Amersham, U.K.). The efficiency of thymidine incorporation was assessed according to ref. [6].

The proteinase inhibitor sericystatin A, with the approximate molecular mass of 13,000, used in these experiments was isolated from porcine peripheral leukocytes as described previously [3, 7]. This protein inhibits cysteine proteinases (cathepsins B, H, L and S, and papain) and intracellular proteinases of the chymotrypsin type ([4] and references therein).

Crystalline bovine insulin (Pliva, Zagreb, Yugoslavia) was administered subcutaneously.

The immunoreactive insulin in the animals' sera was determined by the double antibody method [8].

The results were statistically evaluated by the Mann-Whitney U test.

RESULTS

Because the progression of the insulin-dependent murine myeloid leukemia is paralleled by the increase of IRI in blood [1], we measured the effects of sericystatin A on blood levels of IRI. We injected either 0.1 ml Hanks' solution or a single dose of sericystatin A in the same volume of Hanks' solution (0.5, 2.5 or 5.0 mg sericystatin A per kg body mass) into tumorous, otherwise untreated mice on the eleventh day following tumor inoculation (terminal phase of disease). Ninety minutes after the injection of Hanks' solution or sericystatin A, blood was drawn for IRI determination.

The supranormal levels of IRI in the untreated tumorous animals are diminished significantly by the sericystatin A dose of 2.5 mg/kg ($P<0.05$; Fig. 1). However, further increase of the sericystatin A dose did not result in more efficient IRI suppression and, therefore, the dose of 2.5 mg per kg body mass (50 μg per animal) was used in most experiments.

Figure 2 displays the effects of sericystatin A on the mean survival time of mice suffering from myeloid leukemia. Control mice were treated

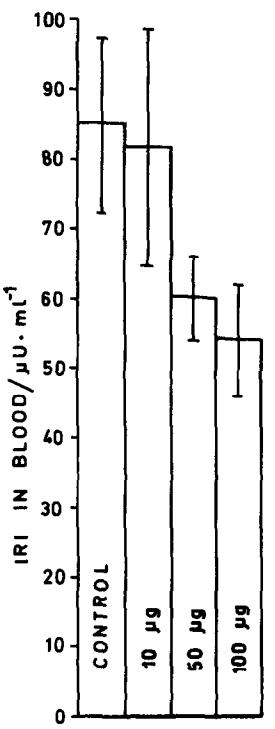

Fig. 1. The levels of immunoreactive insulin (IRI) as a function of sericystatin A dose on the 11th day after tumor inoculation in blood of non-treated animals 90 min after they received either a single i.p. injection of 0.1 ml Hanks' solution ('control') or 10, 50 or 100 μg sericystatin A in 0.1 ml Hanks' solution per animal. Each group consisted of five animals. IRI in blood of animals treated with 50 and 100 μg sericystatin A per animal differs significantly (P <0.05) from the control group. The bars denote standard deviation.

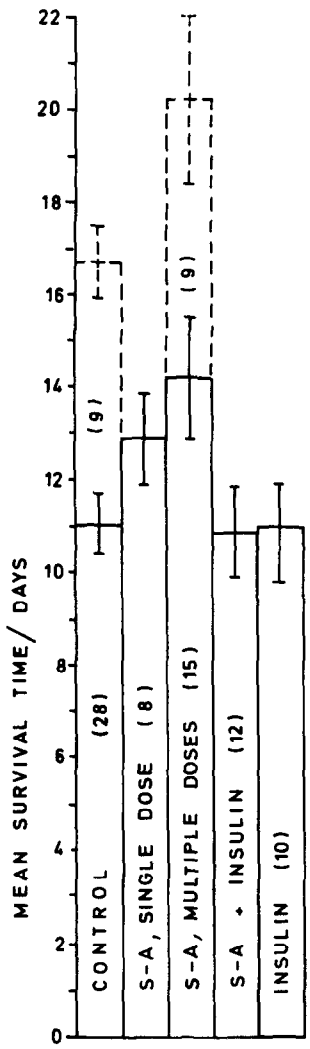

Fig. 2. The mean survival time ± standard deviation of mice suffering from myeloid leukemia which received either a single i.p. injection of 0.1 ml Hanks' solution the day following tumor inoculation of one million tumor cells or every second day thereafter ('control'). The 'S-A, single dose' group received a single i.p. injection of 100 μg sericystatin A per animal on the day following tumor inoculation. The 'S-A, multiple doses' group received 50 μg sericystatin A every second day. The 'S-A + insulin' group received the same treatment of multiple sericystatin A injections which were followed by s.c. injections of 25 mU crystalline insulin per animal. The 'insulin' group received multiple s.c. injections of 25 mU insulin per animal only. The numbers in parentheses denote the total number of animals in the group. The mean survival times of animals treated with sericystatin A differ significantly (P <0.001) from the control group. Also, the animals treated with multiple doses differ significantly (P <0.05) from animals treated by a single inhibitor injection. The dashed columns represent results obtained with animals inoculated with one thousand splenocytes only.

either with a single i.p. injection of Hanks' solution on the first day following inoculation of one million tumor cells or every second day thereafter. No difference was noted between these two groups and, therefore, they are represented together as the single control group in Fig. 2. A single 0.1-ml i.p. injection of 5.0 mg sericystatin A per kilogram body mass (100 μg per animal) prolonged the mean survival time of leukemic animals significantly ($P<0.001$). This effect is probably due to the inhibitor-induced delay of the exponential phase of tumor growth. Even more significant was the treatment with a 0.1-ml i.p. injection of 2.5 mg sericystatin A per kg body mass (50 μg per animal) every second day ($P<0.05$ with respect to the group treated with the single dose of sericystatin A). A similar life prolongation was observed in animals inoculated with one thousand splenocytes. These effects were completely abolished if each of the sericystatin A injections administered every second day was followed by an s.c. injection of 1.25 IU of bovine crystalline insulin per kg body mass (25 mU per animal) in 0.1 ml Hanks' solution per animal (Fig. 2). The corresponding treatment with insulin alone had no effect on animals' life duration (Fig. 2).

To assess whether the suppressing effect of sericystatin A is exerted on the tumorous cells directly, we measured the spontaneous incorporation of [^{3}H]-thymidine into splenocytes from tumorous animals in the terminal phase of disease (11th day) incubated in sericystatin A. The control group received 0.1 ml Hanks' solution i.p. every second day, while other animals were administered either sericystatin A (2.5 mg/kg in 0.1 ml Hanks' solution per animal) alone or together with insulin (1.25 IU/kg). In Fig. 3a it can be seen that in Parker's medium, splenocytes from tumorous animals pretreated with sericystatin A *in vivo* incorporate significantly less [^{3}H]-thymidine than those from untreated animals ($P<0.01$) or from those which received insulin ($P<0.01$). However, the addition of sericystatin A to cell cultures did not affect the [^{3}H]-thymidine incorporation by splenocytes from animals treated with Hanks' solution or sericystatin A (except in the single case of maximum sericystatin A concentration in cultures of cells from animals pretreated with sericystatin A; Fig. 3b). Moreover, we assessed the toxicity of 32–8192 ng sericystatin A for one million splenocytes in 1 ml of medium. Differentiating the dead cells by the trypan blue method, we found 85% of cells alive at the beginning of incubation, and this percentage did not change significantly in any sericystatin A concentration up to the termination of incubation in the 120th minute.

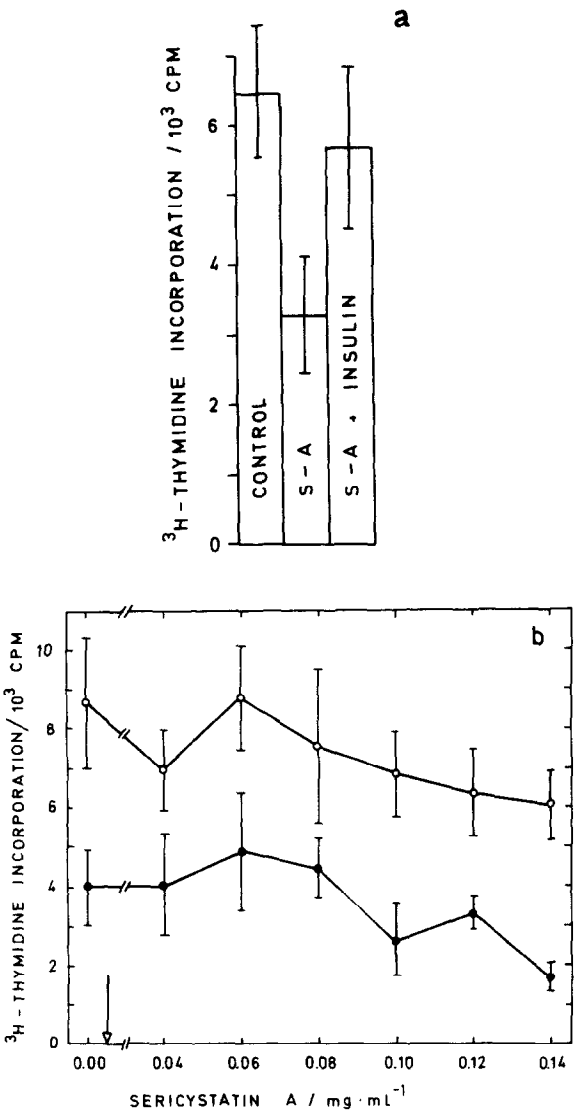

Fig. 3. (a) Incorporation of [^{3}H]*-thymidine into cultured myeloid leukemia cells from animals pretreated with injections either of* 0.1 ml *Hanks' solution ('control'),* 50 μg *sericystatin A ('S-A') or* 50 μg *sericystatin A together with* 0.25 mU *crystalline insulin ('S-A + insulin'). The bars denote standard deviation. (b) The same for the 'control' (open circles) and 'S-A' (full circles) groups as a function of sericystatin A concentration in the medium. The arrow indicates the concentration of sericystatin A* in vivo *(neglecting the* in vivo–in vitro *differences of catabolic rates and assuming the same densities of the tissue and culture medium).*

DISCUSSION

We chose the myeloid leukemia of mice as a model to study the effects of modulators of insulin synthesis and release (and, possibly, of insulin-related substances) because it is an insulin-dependent tumor [1] and because the distribution of animal survival times is narrow. Sericystatin A is recognized not only as an inhibitor of cathepsin B, the proinsulin-splitting enzyme, but also as possessing antimetastatic properties. These properties are believed to be due to the inhibition of

tumor-related proteinases involved in metastasis formation [9, 10].

The 25% prolongation of survival by sericystatin A is important because the myeloid leukemia is one of the most malignant murine tumors. In animals inoculated with one thousand splenocytes only, the relative life prolongation by sericystatin A is the same as in those inoculated with one thousand times more cells.

As the tumor-retarding effects of sericystatin A are completely abolished by the concomitant insulin injections, it is plausible that sericystatin A modulates growth of this insulin-dependent tumor by reducing IRI levels in blood rather than by any other mechanism. While minute doses of insulin (25 mU per animal) shorten animals' lives, 20–50 times larger doses *prolong* this parameter in various murine tumors [11]. The results of [^{3}H]-thymidine incorporation *in vitro* clearly demonstrate, together with the absence of cytotoxicity, that the effects of sericystatin A are indirect. Namely, at inhibitor concentrations comparable to those *in vivo*, the efficiency of *in vitro* [^{3}H]-thymidine incorporation by the tumor cells depends only on their previous treatment with sericystatin A *in vivo* (the maximum inhibitor concentrations with some direct inhibitory effect *in vitro* should be compared with the dose of 5 μg sericystatin A/g *in vivo*, which resulted in significant tumor inhibition).

Aside from the observation of the tumor retarding effect, it is of importance to note that the present experiments demonstrate unequivocally that sericystatin A can modulate secretion of immunoreactive insulin (and possibly of insulin-related substances). This can mean that sericystatin A and similar inhibitors of cathepsin B might be used to control proinsulin splitting in malignancies where the excessive insulin induces hypoglycemia. In this respect studies of sericystatin A uptake and effects in insulinoma cells might be enlightening.

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