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Research paper

Inhibition of metastasis development by daily administration of ultralow doses of RNase A and DNase I

Olga Patutina [a], Nadezda Mironova [a], Elena Ryabchikova [a], Nelly Popova [b], Valery Nikolin [b], Vasily Kaledin [b], Valentin Vlassov [a], Marina Zenkova [a,*]

[a] Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, Russian Academy of Sciences, Lavrentiev av. 8, Novosibirsk 630090, Russian Federation
[b] Institute of Cytology and Genetics, Siberian Branch, Russian Academy of Sciences, Lavrentiev av. 10, Novosibirsk 630090, Russian Federation

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ABSTRACT

Recent data on the involvement of miRNA and circulating tumor-derived DNA in regulation of tumorigenesis showed a great prospect for these molecules as a novel class of therapeutic targets and gave a new start for the study of enzymes cleaving nucleic acids as potential antitumor and antimetastatic agents. In the present paper using two murine tumor models with pulmonary or liver metastases we studied the antimetastatic potential of RNase A and DNase I and performed a search for possible molecular targets of the enzymes. Herein, we show for the first time that daily administration of ultralow doses of RNase A (0.5–50 μg/kg) and DNase I (0.02–2.3 mg/kg) inhibits the development of metastasis to 60–90% and RNase A exerts 30% retardation of tumor growth. Remarkably, the increase in RNase A dose from 50 μg/kg to 10 mg/kg leads to a disappearance of antitumor and antimetastatic effects. Simultaneous treatment of tumor-bearing animals with RNase A and DNase I leads to an additive effect and results in almost total absence of metastases. The use of RNase A as an adjuvant in conjunction with conventional cytostatic cyclophosphamide results in a reliable enhancement of antitumor and antimetastatic effect of the therapy compared with the use of these agents individually. The search for possible molecular mechanism of antimetastatic effect of nucleases showed that daily administration of the enzymes reduced the pathologically increased level of extracellular nucleic acids and increased nuclease activity of the blood plasma of tumor-bearing mice back to the level of healthy animals. Thus, we unequivocally show that the proposed protocol of treatment of tumor-bearing animals with RNase A and DNase I has a general systemic and immunomodulatory effect, leads to a drastic suppression of metastasis development, and in perspective may become an effective component of intensive complex therapy of cancer.

1. Introduction

Development of metastasis is an enormously complex process that is of great importance to the clinical management of cancer since the majority of cancer mortality is associated with disseminated disease rather than the primary tumor. The main way to fight metastatic spread is still chemotherapy. This method of treatment is at the top of its capacity; therefore, there is an urgent need to devise new basic as well as adjuvant approaches toward treating highly malignant tumors.

The actual complexity of the metastatic cascade encompasses not only the biology of tumor cell itself but generally the rest of the organism in which it resides. Recent studies have revealed that tumor dissemination is accompanied by an imbalance in nucleic acid metabolism manifesting as the increased levels of circulating nucleic acids and decreased nuclease activity of blood plasma of cancer patients [1–3]. Abnormal elevation of nucleic acid concentration in plasma is related to the upregulated expression and active secretion of specific tumor-derived miRNA [4–6] and DNA [7,8], which researchers associate with intensive process of tumor progression and poor prognosis. These findings suggest that circulating oncogenic miRNAs and DNAs are not only promising cancer biomarkers but also novel starting point for the drug development.

The discovery of oncogenic miRNAs and DNAs as novel targets for cancer therapy encouraged us to assess the antitumor potential of enzymes of nucleic acid metabolism RNase A and DNase I. The concept of investigation of RNase A and DNase I as antitumor agents has been emerged as far back as 1950s, but obtained results were fragmentary and contradictory [9–12], whereas cytotoxic effect of other homologs of RNase A superfamily (onconase, BS-RNase) was unambiguous [13,14]. Nevertheless, guaranteed safety of RNase A

* Corresponding author. Tel.: +7 383 363 51 60; fax: +7 383 363 51 53.
E-mail address: marzen@niboch.nsc.ru (M. Zenkova).

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for mammalians emboldened us to reevaluate the antitumor activity of RNase A. We previously demonstrated that RNase A as well as DNase I significantly inhibited metastasis by inducing metastasis pathomorphosis (apoptosis, necrosis and destruction of oncocytes) and RNase A additionally retarded the primary tumor growth by 30–40% [15]. One of the crucial and distinguishing discoveries of this study is that the effective dosages are ultralow and located to the microgram range.

In the present study we performed the quantitative evaluation of antimetastatic effect of multiple low dosages of RNase A and DNase I and search for possible molecular targets of the enzymes.

2. Materials and methods

The oligodeoxyribonucleotide DNA_{60} was synthesized by phosphoramidite method and purified by HPLC. RNase A (13,700 g/M) and DNase I (2155 AU/mg) were purchased from Sigma. Fragment of HIV-1 RNA (96 nt) was prepared by *in vitro* transcription [16]. HIV-1 RNA and DNA_{60} were labeled at the 5′-end using γ-^{32}P ATP and T4-polynucleotide kinase [16].

2.1. Tumor transplantation and design of animal experiments

All animal procedures were carried out in compliance with the approved protocols and recommendations for proper use and care of laboratory animals [ECC Directive 86/609/EEC]. Ten- to 11-week old female C57Bl/6 mice bearing Lewis lung carcinoma (LLC) and 12- to 14-week old female A/Sn mice bearing Hepatoma A-1 (HA-1) were used. Solid tumors LLC or HA-1 were induced by intramuscular injection of LLC or HA-1 cells (10^6) suspended in 100 μl of saline buffer into the right thighs of mice. To generate a model of LLC metastasis tumor cells (1.25×10^5) suspended in 400 μl of saline buffer were inoculated into the lateral tail vein of mice.

On day 4 after tumor transplantation, each mouse bearing LLC was assigned to one of the nine groups ($n = 20$): 1, control, received saline buffer; 2–9, received RNase A at doses of 0.1 μg/kg–10 mg per kg. In the next experiment on day 8 after tumor transplantation, each mouse bearing HA-1 or LLC was assigned to one of the nine groups ($n = 7$): 1, control, received saline buffer; 2–4, received RNase A at doses of 0.35–7 μg/kg; 5–9, received DNase I at doses of 0.02–2.3 mg/kg. Effects of combined therapy with RNase A and DNase I were studied in LLC-bearing mice: animals were injected with mixture 1 (0.02 mg/kg of DNase I, 0.7 μg/kg of RNase A) or mixture 2 (0.02 mg/kg of DNase, 0.35 μg/kg of RNase A). In the experiment with the model of LLC metastasis on day 4 after intravenous inoculation of LLC cells each mouse was assigned to one of the three groups ($n = 10$): 1, control, received saline buffer; 2, received RNase A at a dose of 0.7 μg/kg; and 3, received DNase I at a dose of 0.12 mg/kg. In all experiments nucleases were administered intramuscularly daily except weekends (10–12 injections per experiment).

The effect of combined therapy with RNase A and cyclophosphamide was studied in the experiment with LLC-bearing mice. On day 4 after tumor transplantation, each mouse was assigned to one of the four groups ($n = 8$–10): 1, control, received saline buffer; 2, received RNase A; 3, received cyclophosphamide; and 4, received RNase A and cyclophosphamide in combination. RNase A at a dose of 0.7 μg/kg was administered daily intramuscularly; cyclophosphamide was injected intraperitoneally twice on day 7 and 11 after tumor transplantation at doses of 150 and 100 mg/kg.

Tumor size was determined every other day using caliper measurements in three perpendicular dimensions. Tumor volumes were calculated as $V = (\pi/6 \times \text{length} \times \text{width} \times \text{height})$. Tumor doubling time (DT) was calculated as $DT = (t - t_0) \times \ln 2/(\ln V - \ln V_0)$, where $(-t_0)$ indicates the length of time between two measurements of tumor size and V_0 and V denote the tumor volume at two points of the measurement [17].

2.2. Measurement of cytokine levels in the blood serum

Blood samples were collected from four groups of LLC-bearing mice on day 20 after tumor transplantation: 1, control, received saline buffer; 2, mice treated with RNase A at a dose of 0.7 μg/kg; 3, mice treated with cyclophosphamide at doses of 150 and 100 mg/kg; and 4, mice, received both RNase A and cyclophosphamide at the same doses. Blood serum was prepared by clot formation at 37 °C for 30 min and at 4 °C overnight followed by clot discard and serum centrifugation (4000 rpm, 4 °C, 20 min). Serum samples were stored at −70 °C until analysis.

The levels of TNF-α, IL-6, IFN-α and IFN-γ in blood serum of mice were measured using Mouse IFN gamma, Mouse IL-6 and Mouse TNF alpha Colorimetric ELISA Kits (ThermoScientific, USA) according to the manufacture protocols. The level of IFN-α was measured using Mouse Interferon Alpha (Mu-IFN-) ELISA Kit (ThermoScientific, USA).

2.3. Measurement of concentrations of circulating nucleic acids

To perform the analysis EDTA plasma samples was prepared as follows: blood of experimental animals (0.8 ml) was collected into the tubes containing 0.2 ml of sterile phosphate-buffered saline (PBS) supplemented with 50 mM EDTA and separated into plasma and blood cell fraction by centrifugation at 1200 rpm for 20 min. The blood plasma was collected, centrifuged at 2400 rpm for 20 min, and kept at −20 °C. EDTA plasma samples were collected from four groups of mice on day 15 after tumor transplantation 60 min after the last injection: 1, healthy C57Bl/6 mice; 2, the mice bearing LLC received saline buffer; 3, the mice bearing LLC treated with DNase I at a dose of 0.12 mg/kg; and 4, the mice bearing LLC treated with RNase A at a dose of 0.7 μg/kg. EDTA plasma samples were also collected from similar groups of the A/Sn mice bearing HA-1. DNA and RNA were extracted from 0.1 ml of plasma sample according to [18]. DNA concentration was measured using a Hoechst 33258 assay [19]. RNA samples were treated with RNase free DNase I and quantified with SYBR Green II according to manufacturer's recommendations.

2.4. Measurement of total DNase and RNase activities in plasma samples

The reaction mixture containing [^{32}P]-DNA_{60} (50 000 cpm, 10^{-6} M), 45 μl of EDTA plasma sample and 30 mM $CaCl_2$ was incubated at 30 °C for 10–75 min. The reaction mixture containing HIV-1 [^{32}P]-RNA (50 000 cpm), 45 μl of plasma sample, and 100 μg/ml *Escherichia coli* tRNA was incubated at 30–33 °C for 10–80 min. DNA or RNA cleavage products from reaction mixture were isolated by phenol–chloroform extraction followed by ethanol precipitation. DNA and RNA cleavage products were analyzed in 12% polyacrylamide/8 M urea gel.

Effective rate constants for total DNase and RNase activities of plasma samples at 30 °C (k_{eff}) were determined by fitting the experimental data to a single exponential equation $P_t = P_\infty \cdot (1 - \exp^{-k\text{eff}\ t})$ [16], where P_t and P_∞ are the fractions of the substrate cleaved at the time t and the end point, respectively.

2.5. Analysis of the number and area of metastases

Surface metastases in the lungs were enumerated using binocular microscope. For morphometric analysis the lungs and liver were fixed in 10% neutral-buffered formalin, routinely processed, and embedded in paraffin. Paraffin sections (5 μm) were stained with