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Fibrinogen facilitates the anti-tumor effect of nonnative endostatin

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Abstract

Endostatin is a potent inhibitor of tumor angiogenesis. Interestingly, nonnative endostatin also exhibits an anti-tumor effect, which remains a mystery so far. Here we show that intravenous injection of nonnative endostatin results in tumor inhibition effect. Soluble and active endostatin is isolated from human blood after the addition of nonnative endostatin *in vitro*. By fractionation of the whole blood, we surprisingly identify fibrinogen specifically binding to and inhibiting the aggregation of nonnative endostatin. Moreover, the anti-tumor activity of nonnative endostatin is substantially impaired in fibrinogen-deficient mice. Our studies demonstrate that fibrinogen facilitates the anti-tumor effect of nonnative endostatin, which also provides new insights into the novel physiological function of fibrinogen.

Keywords

endostatin; fibrinogen; nonnative; anti-tumor; aggregation

Endostatin, a 20 kDa C-terminal fragment of collagen XVIII, is originally identified as a potent tumor angiogenesis inhibitor [1]. Animal studies demonstrate that recombinant endostatin strongly inhibits the growth of a variety of murine and human tumors with no toxic side effects [1–3]. Notably, it is also reported that over expressed endostatin in the blood circulation by gene therapy had no anti-tumor effect [4–5]. One explanation is that the protein produced might be misfolded or aggregated [6]. On the other hand, repeated cycles of subcutaneous (s.c.) administration of nonnative endostatin of *E. coli* origin results in almost complete suppression of the tested primary tumors [1,7]. It is suspected that contamination by other factors produced in *E. coli*, such as endotoxin, is in fact responsible for the activity [8]. So far, the mechanism of these paradoxes is still unclear. The structure and function of endostatin has been

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systematically studied by our group [9–11]. It is expected that certain conformation or active site is necessary for endostatin to exhibit its biological functions.

Human fibrinogen is a circulating 340 kDa glycoprotein, playing a vital role in the “common pathway” of the coagulation process [12]. It has been established that fibrinogen induces proliferative signals by serving as a scaffold to support the binding of growth factors and to promote the cellular responses of adhesion, proliferation, and migration during wound healing, angiogenesis, and tumor growth [13]. The local deposition of fibrin(ogen) and fibrinogen degradation products (FDPs) is a universal and conspicuous feature of solid tumors, although neither fibrin(ogen) nor FDPs is essential for primary or secondary tumor growth [14–15]. However, the role of fibrinogen in the tumor growth and inhibition is still largely unknown.

In this paper, we intravenously (*i.v.*) inject nonnative endostatin into the tumor bearing mice and observe significant anti-tumor effect. Soluble and active endostatin is isolated from blood added with nonnative endostatin *in vitro*, which supports the hypothesis that nonnative endostatin into animals may result in conversion into an active form [1]. Fibrinogen is subsequently identified as a putative inhibiting factor for the aggregation of nonnative endostatin. Fibrinogen specifically binds to and forms complexes with nonnative endostatin, whose anti-tumor effect is substantially impaired in fibrinogen-deficient (fibrinogen^{-/-}) mice. Taken together, our findings suggest that nonnative endostatin may exhibit anti-tumor effect through conversion into an active form, which is facilitated by fibrinogen.

Materials and methods

Materials

E. coli-expressed recombinant human endostatin with MGGSHHHHH attached at the N-terminus was obtained from Protgen (CN). Commercial endostatin (>99% pure) was of clinical grade and was tested to be free of endotoxins and other bacterial products. Human plasma fibrinogen, fibronectin, IgG was purchased from Sigma (USA). Bovine serum albumin was obtained from Roche (CH). Polyclonal goat anti-rabbit immunoglobulin/HP was from DakoCytomation (DK). All other antibodies were from Protgen (CN).

Preparation of nonnative endostatin

Clinical grade endostatin was incubated in 8 M urea, 140 mM β -mercaptoethanol, 50 mM Tris-HCl, pH 8.5, at 37 °C, for 2 h, and then rapidly dialyzed into 20 mM acetate acid, pH 3.0 immediately before use. Under this condition, endostatin was in a soluble but nonnative state and its disulfide bonds were not formed [9,10].

Isolation of active endostatin from blood added with nonnative endostatin

Nonnative endostatin was added stepwise into 500 ml human blood to a final concentration of 0.2 mg/ml. Soluble and active endostatin was purified from supernatant after centrifugation, using Micro-Prep[®] High Q column (BioRad Laboratories) and Ni-NTA column (Qiagen, Inc.). See supplementary materials for details.

Endothelial cell migration inhibition assay

The migration assay with human microvascular endothelial cells (HMEC) cells was performed using 96-well Boyden chambers (Neuro Probe, Inc.) with a polycarbonate membrane (Poretics Corp.). The lower chamber was filled with Dulbecco's modified eagle's medium (DMEM) containing 10 ng/ml bFGF and 0.5% fetal bovine serum (FBS). The upper chamber was seeded with 10,000 cells/well, as well as either form of endostatin at concentrations of 1.75 and 17.5 μ g/ml or saline as a control. Cells were allowed to migrate for 6 h at 37 °C and 5% CO₂. At that time, the cells on the upper surface of the membrane were removed with a cell scraper,

and the migrated cells on the lower surface were fixed in 70% ethanol and washed in PBS before being photographed.

Interaction between nonnative endostatin and fibrinogen as well as other plasma proteins

Interaction between nonnative endostatin and fibrinogen as well as other plasma proteins *in vitro* was performed as follows: Fibrinogen solutions were prepared at concentrations of 1.5, 3, and 6 mg/ml, respectively. FN, BSA, IgG, transferrin and GroEL solutions were prepared at 3 mg/ml. Nonnative endostatin was added into equivalent volume of the above solutions containing fibrinogen, FN, BSA, IgG, transferrin and GroEL, respectively, with a final concentration of 0.2 mg/ml at 20 °C. After 2 h incubation, the solutions were centrifuged at $20,000 \times g$ for 15 min. Soluble endostatin in the supernatant was detected by Western blotting.

Dynamic light scattering

Nonnative endostatin was diluted into 3 mg/ml fibrinogen in PBS with a final concentration of 0.2 mg/ml. After incubation at room temperature for 2–3 h, the sample was clarified by centrifugation at $12,000 \times g$ for 10 min. Dynamic light scattering was performed with the supernatant using DynaPro-MSTC (Protein Solutions Ltd., High Wycombe, Bucks., UK). Data were analyzed by software DYNAMICS (version 5.26.60) supplied by the manufacturer.

Electron microscopy

Protein samples were prepared as for DLS measurement. Approximately 5 μ l sample was placed on the surface of glow-discharged carbon film grid. The liquid was removed after 30 seconds. The residual material remaining on the carbon films was negatively stained with 1% uranyl acetate. A Philips CM 120 transmission electron microscope, operated at an acceleration voltage of 100 kV, was employed for the detection.

Animal tests for anti-tumor effect of endostatin

The generation of fibrinogen^{-/-} mice has been described previously [16] and were backcrossed for >8 generations in Balb/c mice for this study. Human hepatocellular SMMC-7721 cells and LLC cells were cultured in 900-cm² roller bottles, and a suspension of 2×10^6 cells in 0.1 ml DMEM was injected *s.c.* to the dorsa of mice. SMMC-7721 cells were implanted in Balb/c nude mice and LLC in fibrinogen^{-/-} mice, respectively. When the tumor volume reached 90 to 110 mm³, mice were randomized into control and treatment groups (9 mice for the control group, and 7 mice for each treatment group). For treatment groups, standard endostatin or nonnative endostatin was administered *i.v.* daily. Control animals received *i.v.* daily injections of saline. The tumor volumes were determined using the formula $a^2 \times b \times 0.52$ (a is the shortest and b is the longest diameter).

Results

Nonnative endostatin can exhibit anti-tumor effect

To explore the ability of nonnative endostatin to carry out an anti-tumor effect, we performed anti-tumor experiments in Balb/c nude mice bearing SMMC-7721 tumors using *i.v.* injected native and nonnative endostatin (Nonnative endostatin is defined as fully reduced and denatured protein, see Methods. Native endostatin refers to *E. coli*-expressed recombinant human endostatin, which is correctly refolded to native conformation.). It was observed that, at a dose of 10 mg/kg, the nonnative endostatin inhibited the tumor growth by 49% comparable to that of native endostatin (46%) at a dose of 10mg/kg (Fig. 1A). The nonnative endostatin exhibited significant tumor inhibition effect even at a dose of as low as 1.5 mg/kg (data not shown). The anti-tumor experiment was performed on nude mice, which could exclude the possibility that immune response may be responsible for the anti-tumor effect of nonnative

endostatin. Endostatin was of clinical grade and was tested to be free of endotoxins and other bacterial products, so the effect could not be due to distorted physiological condition of mice either.

To demonstrate whether the injected nonnative endostatin can be converted into its active form, we added nonnative endostatin into human blood *in vitro*. Surprisingly, soluble and active endostatin was isolated from the supernatant after centrifugation (See supplementary materials for detailed isolation process). No soluble endostatin was obtained because of aggregation when nonnative endostatin was added into phosphate-buffered saline (PBS) (data not shown). The re-isolated endostatin showed the same characteristics to native endostatin in both SDS-PAGE and reversed-phase high performance liquid chromatography (RP-HPLC) (Fig. 1B and 1C). Anti-angiogenic activity assays demonstrated that the re-isolated endostatin inhibited the migration of human microvascular endothelial cells (HMECs) as effectively as native endostatin at the same concentration (Fig. 1D). The re-isolated endostatin we obtained was not from an endogenous origin because the exogenously added endostatin was His-tagged. These results suggest that nonnative endostatin can exhibit anti-tumor effect, which may be facilitated by its conversion from nonnative form to a soluble and active form. The aggregation of nonnative endostatin can be potentially inhibited by certain factor(s) in the blood.

Fibrinogen is isolated to potentially inhibit the aggregation of nonnative endostatin

To identify the potential factor(s) which inhibited the aggregation of nonnative endostatin, the blood was fractionated and fractions were analyzed by enzyme linked immunosorbent assay (ELISA). The activity of control fraction as indicated was assumed to be 100%. During the fractionation process, the corresponding protein content had been normalized to make sure that the observed effect was not due to dilution or concentration of plasma fractions. (See supplementary materials for detailed isolation process) The majority of the components that could effectively inhibit nonnative endostatin aggregation were found in the plasma (Fig. S1A), and could be precipitated in an active form at pH 4.0 (Fig. S1B). Fractionation by size exclusion chromatography (SEC) of the precipitate dissolved in PBS produced two major protein peaks (Fig. S1C), and peak 1 had the predominant activity (Fig. S1D). Analysis of peak 1 by SDS-PAGE revealed four major bands (Fig. 2A). Based on MALDI-TOF-MS peptide mass fingerprinting analysis, bands I, II, and III were identified as the α -, β -, and γ -chains of fibrinogen, respectively, while band IV was found to be fibronectin (FN). Other bands were not identified because of their low abundance or impurity. When commercial fibrinogen and fibronectin were tested at their average physiological concentrations in plasma [17, 18], only fibrinogen exhibited major activity, and no synergistic effect between fibrinogen and fibronectin was observed (Fig. 2B). To further exclude the possibility that the effect was due to non-specific effect of other high concentration plasma proteins, we compared fibrinogen, fibronectin, bovine serum albumin (BSA), IgG, and transferrin at the same concentration. Nonnative endostatin was added into the solutions of these proteins, but only fibrinogen prevented the aggregation of nonnative endostatin in a dose-dependent manner as revealed by Western blotting (Fig. 2C). Therefore, the result suggests that fibrinogen is the factor in blood which can specifically inhibit the aggregation of nonnative endostatin.

Fibrinogen can specifically interact with nonnative endostatin

To further confirm the specific interaction between fibrinogen and nonnative endostatin, we performed Co-immunoprecipitation (IP) experiment. Interestingly, nonnative endostatin was IP'd with fibrinogen, while native endostatin did not (Fig. 3A). The formation of the complexes between fibrinogen and nonnative endostatin was further verified. As detected by dynamic light scattering (DLS), fibrinogen alone in PBS buffer displayed hydrodynamic radii mainly between 7 nm and 20 nm with an average radius of 15 nm (Fig. 3B, upper panel). By contrast, when nonnative endostatin was also present, hydrodynamic radii ranged from 30 nm to 100

nm, with an average radius at 38.8 nm, were also detected (Fig. 3B, lower panel). These complexes were not aggregates of nonnative endostatin, because no soluble endostatin was detected in the supernatant if nonnative endostatin was added to PBS buffer in the absence of fibrinogen (Fig. 2C).

As detected by negative electron microscope (EM) (Fig. 3C), after nonnative endostatin was added into fibrinogen solution, polydisperse globule-like particles mainly about 40 nm in diameter were detected in the supernatant (Fig. 3C, right panel). The morphology of the particles was distinctly different from that of fibrin. Fibrinogen alone was thread-like molecule (Fig. 3C, left panel). Finally, endostatin was found to be in high molecular weight fractions with fibrinogen after the mixture of fibrinogen and nonnative endostatin was subjected to size exclusion chromatography (data not shown). These observations indicate that fibrinogen can specifically bind to nonnative endostatin and form large complexes.

Anti-tumor effect of nonnative endostatin is substantially impaired in fibrinogen^{-/-} mice

To further explore the effect of fibrinogen on the anti-tumor function of nonnative endostatin *in vivo*, the anti-tumor activity of native and nonnative endostatin was compared in fibrinogen^{-/-} mice (Fig. 4). Native and nonnative endostatin were administered intravenously by single bolus injection daily and the change of tumor volume was monitored every 2–3 days. Interestingly, in contrast to the result shown in Figure 1A, nonnative endostatin showed almost no tumor inhibition effect during the whole process of the study in contrast to native endostatin (Fig. 4A). There were no statistically significant differences ($P=0.3$) in the mean tumor sizes between mice treated with saline and with nonnative endostatin, while native endostatin inhibited tumor growth by 41% ($P=0.0013$) at the conclusion (Fig. 4B). There was statistically significant difference ($P=0.04$) in the tumor inhibition effect between nonnative endostatin and native endostatin (Fig. 4B). In addition, in the parallel study using wild type mice, both nonnative endostatin and native endostatin significantly inhibited tumor growth in the same pattern as Figure 1A (data not shown). This result suggests that fibrinogen facilitate the anti-tumor effect of nonnative endostatin *in vivo*.

Discussion

Our findings confirm the anti-tumor activity of nonnative endostatin and support the hypothesized conversion of nonnative endostatin into an active form (Fig. 1). The anti-tumor effect exhibited by nonnative endostatin has long been a mystery [1,6]. The findings add to our knowledge that fibrinogen may potentially be involved in and facilitate the anti-tumor function of nonnative endostatin (Fig. 2–4).

The potential mechanism by which fibrinogen facilitates the activity of nonnative endostatin may include the following aspects: 1) The binding of nonnative endostatin to fibrinogen may lead to the refolding of endostatin or recovery of certain active fragment of endostatin molecule. Importantly, this hypothesis is also in support of our previous report that fibrinogen has chaperone-like activity, which can bind to nonnative substrates and keep them in a refolding competent state [19]. Besides, there are also reports that certain fragments of endostatin may possess anti-tumor activity [20–21]. 2) Binding with fibrinogen may prolong or increase the half life of soluble endostatin in the circulation. It is understandable that the clearance and proteolytic degradation of free nonnative endostatin or its fragments would be very rapid in the blood by the extracellular quality control system [22]. 3) Fibrinogen interacts with endothelial cells through specific receptors such as $\alpha_5\beta_1$ and intercellular adhesion molecule-1 (ICAM-1) to support matrix or cell adhesion [23–24], these interactions might tether endostatin in an environment that is close to endothelial cells and facilitate the interaction of endostatin with its potential receptors. 4) When we isolated the potential factor(s) which inhibited the aggregation of nonnative endostatin, fibronectin was also identified. Although we did not

observe any synergistic anti-aggregation effect between fibronectin and fibrinogen (Fig. 2B), interestingly, fibronectin was reported to have protein disulphide isomerase activity [25]. It will be very interesting to further explore whether other co-factors such as fibronectin are also involved in coordination with fibrinogen *in vivo*.

Taken together, our findings not only provide new insight into the mechanism of anti-tumor effect of nonnative endostatin but also have indications on the novel physiological functions of fibrinogen.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1.

Nonnative endostatin can exhibit anti-tumor effect. (A) Growth inhibition of human hepatocellular SMMC-7721 tumor xenografts in Balb/cA nude mice induced by *i.v.* injected saline (○), nonnative endostatin 10 mg/ml (Δ), and native endostatin 10 mg/ml (X). The error bars depict standard deviation from the mean. (B) SDS-PAGE (15%) of re-isolated and standard endostatin. M, molecular weight marker. Lane 1, endostatin re-isolated from blood (non-reduced). Lane 2, endostatin re-isolated from blood (reduced). Lane 3, Native endostatin (non-reduced). Lane 4, Native endostatin (reduced). (C) High performance liquid chromatography (HPLC) analysis of re-isolated and native endostatin. (D) Cell migration assays for re-isolated and native endostatin.

Fig. 2.

Fibrinogen is isolated as a factor inhibiting aggregation of nonnative endostatin. (A) Reduced SDS-PAGE (12%) analysis of peak 1 from c. M, molecular weight marker. (B) Relative activities of unfractionated plasma, Fibrinogen (FG), and FN. The concentrations of fibrinogen and FN were 3 mg/ml and 0.16 mg/ml, respectively. (C) Fibrinogen can prevent the aggregation of nonnative endostatin. Nonnative endostatin was added to the solution as indicated, and endostatin in the supernatant was detected with antibody against endostatin. Lanes 1–3, fibrinogen at concentrations of 1.5, 3, and 6 mg/ml respectively. Lanes 4–8, proteins as indicated at concentration of 3 mg/ml. In panels A, B, and D, the values represent the means of three independent experiments, and error bars depict standard deviations from the mean.

Fig. 3.

Fibrinogen can specifically form complexes with nonnative endostatin. (A) Co-IP of fibrinogen and endostatin. Native endostatin was added into fibrinogen (lane 1) or PBS (lane 3) solutions, respectively. Nonnative endostatin was added into fibrinogen (lane 2) or PBS (lane 4) solutions, respectively. IP was performed with antibody against fibrinogen, and immunoblotting (IB) with antibody against endostatin. Detection of the complexes formed between fibrinogen and nonnative endostatin (B) Dynamic light scattering of fibrinogen alone (upper panel) and with nonnative endostatin (lower panel). (C) Negative-stained electron microscopic image of fibrinogen alone (left panel) and fibrinogen-endostatin complexes (right panel). The scale bars represent 100 nm.

Fig. 4.

The anti-tumor effect of nonnative endostatin is impaired in fibrinogen^{-/-} mice. (A) Tumor growth curve in the fibrinogen^{-/-} mice (n=7). (B) Tumor size in fibrinogen^{-/-} mice at the end of experiment. Mice were treated with saline (○), native endostatin (△), or nonnative endostatin (□). Error bars depict standard errors of the mean.