Metastatin: A Hyaluronan-binding Complex from Cartilage That Inhibits Tumor Growth

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ABSTRACT

In this study, a hyaluronan-binding complex, which we termed Metastatin, was isolated from bovine cartilage by affinity chromatography and found to have both antitumorigenic and antiangiogenic properties. Metastatin was able to block the formation of tumor nodules in the lungs of mice inoculated with B16BL6 melanoma or Lewis lung carcinoma cells. Single i.v. administration of Metastatin into chicken embryos inhibited the growth of both B16BL6 mouse melanoma and TSU human prostate cancer cells growing on the chorioallantoic membrane. The in vivo biological effect may be attributed to the antiangiogenic activity because Metastatin is able to inhibit the migration and proliferation of cultured endothelial cells as well as vascular endothelial growth factor-induced angiogenesis on the chorioallantoic membrane. In each case, the effect could be blocked by either heat denaturing the Metastatin or premixing it with hyaluronan, suggesting that its activity critically depends on its ability to bind hyaluronan on the target cells. Collectively, these results suggest that Metastatin is an effective antitumor agent that exhibits antiangiogenic activity.

INTRODUCTION

A potential therapeutic target on angiogenic endothelial cells is hyaluronan, a large negatively charged glycosaminoglycan that plays a role in the formation of new blood vessels (1). Particularly high concentrations of hyaluronan are associated with endothelial cells at the growing tips or sprouts of newly forming capillaries (2, 3). Similarly, when cultured endothelial cells are stimulated to proliferate by cytokines, their synthesis of hyaluronan is significantly increased (4). Interestingly, this stimulation is restricted to endothelial cells derived from the small blood vessels and is not seen in endothelial cells derived from larger ones (4). In the case of mature blood vessels, hyaluronan is present in perivascular regions and in the junctions between the endothelial cells (5, 6). Earlier studies have shown that exogenously applied hyaluronan has different effects on angiogenesis depending on its size, with macromolecular hyaluronan inhibiting vascularization in chicken embryos, and oligosaccharide fragments of hyaluronan stimulating vascularization in the chorioallantoic membrane (7–9). Thus, hyaluronan appears to be specifically associated with the endothelial cells of newly forming blood vessels and can influence their behavior.

In addition to hyaluronan, endothelial cells involved in neovascularization also express CD44 and other cell surface receptors for hyaluronan (10–12). Particularly, endothelial cells associated with neovascularization also express CD44 and other cell surface receptors for hyaluronan (10–12). In particular, endothelial cells involved in neovascularization express CD44 (11). In previous studies, we have shown that CD44 allows cells to bind hyaluronan so that it can be internalized into endosomal compartments, where the hyaluronan is degraded by the action of acid hydrolases (13, 14). Thus, the expression of CD44 by endothelial cells allows them to bind and internalize hyaluronan as well as any associated proteins. The fact that both hyaluronan and CD44 are up-regulated in endothelial cells involved in neovascularization suggests that the turnover of hyaluronan by these cells is much greater than that by cells lining mature blood vessels.

The increased turnover of hyaluronan in tumor-associated endothelial cells suggested a possible mechanism to specifically target these cells. Our initial idea was to use a hyaluronan-binding complex isolated from cartilage to deliver chemotherapeutic agents specifically to these endothelial cells. Purified by affinity chromatography, this hyaluronan-binding complex consists of tryptic fragments of the link protein and aggrecan core protein (5, 15, 16). We intended to couple the hyaluronan-binding complex to a chemotherapeutic agent such as methotrexate and use this derivative to attack endothelial cells. We hoped that this derivative would bind to the hyaluronan on the endothelial cells and then be internalized into lysosomes, where the methotrexate would be released by the action of acid hydrolases. Surprisingly, however, in the course of these experiments, we found that the hyaluronan-binding complex by itself (i.e., in the absence of a chemotherapeutic agent) inhibited angiogenic activity. Functionally, we termed the hyaluronan-binding complex, which inhibits tumor growth, Metastatin.

In the present study, we demonstrate that Metastatin has a number of intriguing biological activities, including inhibition of endothelial cell proliferation and migration, inhibition of angiogenesis, and suppression of tumor cell growth in chicken embryos and pulmonary metastasis in mice. These effects are blocked by preincubating Metastatin with hyaluronan, suggesting that the activity of Metastatin depends on its ability to bind hyaluronan on the target cells.

MATERIALS AND METHODS

Preparation of Metastatin. The hyaluronan-binding complex was prepared by a modified version of the method originally described by Tengblad (15, 16). Briefly, bovine nasal cartilage (Pel-Freez, Rogers, AR) was shredded with a Sure-Form blade (Stanley), extracted overnight with 4 M guanidine-HCl and 0.5 M sodium acetate (pH 5.8), and dialyzed against distilled water to which 10× PBS was added to a final concentration of 1× PBS (pH 7.4). The protein concentration was measured, and for each 375 mg of protein, 1 mg of trypsin (type III; Sigma, St. Louis, MO) was added. After digestion for 2 h at 37°C, the reaction was terminated by the addition of 2 mg of soybean trypsin inhibitor (Sigma) for each milligram of trypsin. The digest was dialyzed against 4 M guanidine-HCl and 0.5 M sodium acetate (pH 5.8), mixed with hyaluronan-coupled Sepharose, and then dialyzed against a 10-fold volume of distilled water. The hyaluronan-Sepharose beads were placed into a chromatography column and washed with 1.0 m NaCl followed by a gradient of 1.0–3.0 m NaCl. Metastatin was eluted from the hyaluronan affinity column with 4 M guanidine-HCl and 0.5 m sodium acetate (pH 5.8), dialyzed against saline, and sterilized by passage through a 0.2-μm pore filter. For SDS-PAGE analysis, the purified preparation was loaded onto a 10% BisTris nonreducing gel (Novex, Inc.) and subsequently stained with Comassie Blue. To identify the
Fig. 1. SDS-PAGE and NH$_2$-terminal analysis of Metastatin. Lane 1, molecular mass markers; Lane 2, Metastatin stained with Coomassie blue; Lane 3, Western blot of Metastatin immunostained with an antibody against the link protein. The fragment of aggrecan migrated as a diffuse band at ~85 kDa, whereas the truncated link protein was at 38 kDa. NH$_2$-terminal sequence analysis of the 38-kDa band indicated that the first 24 amino acids of the link protein have been cleaved and is indicated on the schematic diagram.

link protein by Western blotting, the proteins on the gel were transferred to a sheet of nitrocellulose and immunostained with the 9/30/8-A-4 monoclonal antibody. (The monoclonal antibody, developed by Dr. B. Caterson, was obtained from the Developmental Studies Hybridoma Bank under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.) This identity was further confirmed by NH$_2$-terminal sequencing (Fig. 1). In tests of the biological activity of Metastatin, controls consisted of Metastatin mixed with an excess mass of hyaluronan (LifeLore, Chaska, MN) or a heat-inactivated preparation made by placing it in a boiling water bath for 30 min.

**Endothelial and Tumor Cell Lines.** HUVECs were obtained from the Tumor Bank of the Lombardi Cancer Center (Georgetown University, Washington, DC). ABAEs were kindly provided by Dr. Luysan Li (Lombardi Cancer Center), and BREC were provided by Dr. Rosemary Higgins (Pediatrics, Georgetown University). These endothelial cell lines were cultured in 90% DMEM, 10% fetal bovine serum, and 2 mM L-glutamine. For the mouse metastasis assays, cells were generally used within each group, and the statistical significance was determined by Student’s $t$ test. Twelve or more eggs were used for each sample point.

**Mouse Metastasis Model System.** For the experimental melanoma model, mice were inoculated i.v. in the lateral tail vein with B16BL6 cells ($5 \times 10^4$ cells/animal) on day 0. Treatment was initiated on day 3 with 5 (0.2 mg/kg), 15 (0.6 mg/kg), and 49 (2 mg/kg) of Metastatin and continued daily until animals were sacrificed on day 14. After euthanasia, the lungs were removed, and surface metastatic lesions were enumerated under a dissecting microscope.

Mice were also inoculated with Lewis lung carcinoma cells, which were cultured in a conditioned medium. For the experimental melanoma model, mice were inoculated i.v. in the lateral tail vein with B16BL6 cells ($5 \times 10^4$ cells/animal) on day 0. Treatment was initiated on day 3 with 5 (0.2 mg/kg), 15 (0.6 mg/kg), and 49 (2 mg/kg) of Metastatin and continued daily until animals were sacrificed on day 14. After euthanasia, the lungs were removed, and surface metastatic lesions were enumerated under a dissecting microscope.

Chicken Chorioallantoic Membrane Assays. To measure angiogenesis, a chick chorioallantoic membrane assay was performed using a modification of the methods of Brooks et al. (17). For this, holes were drilled in the tops of 10-day-old chicken eggs to expose the chorioallantoic membranes, and filter discs (0.5 cm in diameter) containing 20 ng of human recombinant VEGF [20 μl (1 μg/ml); Pepro, Rocky Hill, NJ] were placed on the surface of each chorioallantoic membrane (day 0). The holes were covered with parafilm, and the eggs were incubated at 37°C in a humidified atmosphere. One day later, the eggs were given injections (via a blood vessel in the chorioallantoic membrane using a 30-gauge needle) of the various substances [Metastatin (80 μg/egg) or controls consisting of PBS or heat-inactivated Metastatin]. Three days later (day 4), the chorioallantoic membranes and associated discs were cut out and immediately immersed in 3.7% formaldehyde. For computer-assisted image analysis, the discs were divided into quarters with fine wires, and the blood vessels in each quarter were digitally photographed and analyzed by an Optimas 5 program to calculate the vessel area and length normalized to the total area measured. The means and the SEs were calculated from all quadrants within each group, and the statistical significance was determined by Student’s $t$ test. Twelve or more eggs were used for each sample point.

**Cell Growth Assays.** To determine the effects of Metastatin on cell growth, the cell lines were subcultured into 24-well dishes at a density of approximately $5 \times 10^3$ cells/well for the endothelial cell lines (HUVEC, ABAE, and BREC) and $5 \times 10^3$ cells/well for tumor cell lines (B16BL6, TSU, and Lewis lung carcinoma). For the dose-response experiments, the medium was changed every other day, and at the end of 6 days, the cells were released with 0.5 mM EDTA in PBS, and the cell number was determined with a Coulter counter (Hialeah, FL).

**ELISA Assay for Hyaluronan.** Cells were grown to confluence in 24-well dishes, and the conditioned medium was collected, incubated with a biotinylated version of the Metastatin (16), and then transferred to plates precoated with hyaluronan (umbilical cord; Sigma). The hyaluronan present in the conditioned medium interacts with the biotinylated Metastatin so that less of it will be left to bind to hyaluronan attached to the plate. At the end of the incubation, the plates were washed, and the amount of biotinylated Metastatin remaining attached was determined by incubating the plates with streptavidin coupled to peroxidase (Kirkegard & Perry, Gaithersburg, MD) followed by a soluble substrate for peroxidase. The amount of hyaluronan in the conditioned medium was calculated by comparison with a standard curve with known amounts of hyaluronan (16).

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The abbreviations used are: HUVEC, human umbilical vein endothelial cell; ABAE, adult bovine aorta endothelial cell; BREC, bovine retinal endothelial cell; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor.
Wound Migration Assay. A suspension of HUVECs (5 × 10⁶ cells in 5 ml of 98% M199 and 2% fetal bovine serum) was added to 60-mm tissue culture plates that had been precoated with gelatin (2 ml of 1.5% gelatin in PBS, 37°C, overnight) and allowed to grow for 3 days to confluence. An artificial “L”-shaped wound was generated in the confluent monolayer with a sterile razor blade by moving the blade down and across the plate. Plates were then washed with PBS, and 2 ml of PBS were added to each plate along with 2 ml of sample in M199 and 2% fetal bovine serum in the presence and absence of 5 ng/ml bFGF. After an overnight incubation, the plates were treated with Diff-Quik for 2 min to fix and stain the cells. The number of cells that migrated were counted under ×200 magnification using a 10-mm micrometer over a 1 cm distance along the wound edge. Ten fields for each plate were counted, and an average for the duplicate was calculated.

RESULTS

Characterization of Metastatin. Metastatin was isolated from bovine nasal cartilage by affinity chromatography on hyaluronan-Sepharose. As shown in Fig. 1, Metastatin consisted of two molecular factions as determined by SDS-PAGE, a sharp band at 38 kDa that corresponds to the link protein, and a diffuse band at approximately 85 kDa that represents a tryptic fragment of the aggrecan core protein (5, 15, 16). The diffuse nature of this latter fraction is probably due to variations in the degree of glycosylation and glycosaminoglycan content. The identity of the link protein was verified by immunoblotting with a specific monoclonal antibody against this protein (Fig. 1). In addition, NH₂-terminal sequence analysis of the 38-kDa band revealed that the purified protein was missing the first 24 amino acids. Previous studies have shown that this complex binds to hyaluronan with high affinity and specificity (5, 16). Indeed, a biotinylated version of the preparation has been widely used as a histochemical stain to localize hyaluronan in tissue sections (5, 16).

Because cartilage is known to contain various protease inhibitors, which may contribute to its antimetum properties (18), we wanted to determine whether Metastatin possessed such attributes. For this reason, we used a chromogenic assay (Diapharma Group, Inc., West Chester, OH) to assess the effect of Metastatin on the following enzymes: (a) trypsin; (b) chymotrypsin; (c) plasmin; and (d) elastase. At concentrations as high as 100 µg/ml, Metastatin did not inhibit the activity of any of the enzymes tested (data not shown).

Effect of Metastatin on Metastatic Tumors. In initial experiments, we found that Metastatin was effective at inhibiting pulmonary metastases of B16BL6 cells. When mice were given daily i.p. injections of Metastatin 3 days after tumor inoculation, lung metastases were strikingly reduced (Fig. 2A). Fig. 2B shows that the number of surface lung metastases (>0.5 mm) in the mice treated with 15 and 49 µg Metastatin/day were reduced by more than 80%. The dose-response curve shown in Fig. 2C was constructed from two independent experiments and shows that Metastatin decreased the number of metastatic colonies in a dose-dependent manner with an EC₅₀ of approximately 10 µg (0.4 mg/kg). Significantly, when Metastatin preparations were premixed with macromolecular hyaluronan, the antimitastatic activity was blocked, and the mean number of surface pulmonary metastases was comparable to that seen in control mice (Fig. 2B). This suggests that the ability of Metastatin to bind hyaluronan is required for its anti-tumor activity.

Similar results were obtained with the Lewis Lung carcinoma cell line, which is a more aggressive mouse tumor model. As shown in Fig. 3, A and B, Metastatin inhibited pulmonary metastasis of Lewis lung carcinoma cells in a dose-related fashion, as reflected in the weight gain of the lungs. Furthermore, Metastatin was effective when given by two different routes, i.p. and i.v. (Fig. 3, B and C).

Effect of Metastatin on in Vitro Cell Proliferation and Migration. In the next series of experiments, we wanted to determine whether Metastatin has any effect on the growth of either endothelial or tumor cells in tissue culture. For these experiments, the cells were grown in the presence of varying concentrations of Metastatin for 6 days, and then the final cell numbers were determined. Metastatin inhibited the proliferation of the endothelial cell lines HUVEC, ABAE, and BREC (Fig. 4A) and two of the tumor cell lines (B16BL6 and Lewis lung carcinoma cells) but had no effect on the TSU cells (Fig. 4B). Similar results were obtained when proliferation was monitored by incorporation of bromodeoxyuridine (data not shown). It is important to note that the growth inhibition of B16BL6 cells was partially blocked when the preparation of Metastatin was premixed with an excess of hyaluronan (Fig. 4B).

One possible explanation for the lack of TSU cell sensitivity to
Metastatin could be the amount of hyaluronan that they secrete because it has an inhibitory effect. To test this possibility, conditioned media from confluent cultures of the different cell lines were collected and analyzed for hyaluronan by a modified ELISA. TSU cells were found to secrete significantly larger amounts of hyaluronan into the medium than the other cell lines (7 mg/ml versus 0.5 mg/ml, respectively). Indeed, this level of hyaluronan would be sufficient to block the effects of added Metastatin.

We also examined the effects of Metastatin on the migration of endothelial cells, another important factor in the process of angiogenesis (19). In this assay, we examined the effect of Metastatin on the migration of HUVECs using the wound migration assay. Fig. 5 shows that at a concentration of 10 μg/ml, Metastatin inhibited the migration of HUVECs by 50% as compared with controls treated with bFGF alone. Again, similar results were obtained when migration was monitored using Nucleopore filters (data not shown).

**Effect of Metastatin on VEGF-induced Angiogenesis.** The fact that Metastatin could inhibit both the growth and migration of endothelial cells *in vitro* suggested that it might also be able to block angiogenesis *in vivo*. To test this possibility, we examined the effect...
of tumor cells under the following conditions: (a) a single i.v. injection of Metastatin into the chorioallantoic membrane of chicken embryos inhibited the growth of B16BL6 mouse melanoma cells and TSU human prostate cancer cells; (b) multiple i.p. injections of Metastatin prevented the experimental metastasis of B16BL6 and Lewis lung carcinoma cells to the lungs of mice; and (c) three i.v. injections of Metastatin were sufficient to inhibit the formation of Lewis lung carcinoma metastasis. In each case, Metastatin did not have an obvious detrimental effect on the host and was neutralized by complexing with soluble hyaluronan.

Metastatin is a member of a family of hyaluronan-binding proteins that also includes CD44, tumor necrosis factor-stimulated gene 6 (TSG-6), versican, neurocan, and brevican (20). Interestingly, Metastatin is similar to other factors that influence angiogenesis in that it is a fragment of a larger complex. For example, Angiostatin is a fragment of plasminogen, Endostatin represents a fragment of collagen XVIII, and serpin consists of a fragment of antithrombin (21–24). It is possible that the production of the peptide fragments is part of a feedback loop important in the down-regulation of angiogenesis.

In addition to Metastatin, a number of other antiangiogenic factors have been isolated from cartilage. Indeed, cartilage has been extensively studied as a source of molecules that could account for its avascular nature. Langer et al. (25) first reported a bovine cartilage fraction isolated by guanidine extraction and purified by trypsin affinity chromatography that inhibited tumor-induced vascular proliferation. In addition, Moses et al. (26) have recently isolated Troponin I from veal scapulae, which was shown to have antitumor and antiangiogenic properties. Lee and Langer (27) have described a guanidine-extracted factor from shark cartilage that inhibited angiogenesis and suppressed tumor vascularization. Similarly, Moses et al. (18) isolated a factor from cultures of scapular chondrocytes that inhibited angiogenesis in the chicken chorioallantoic membrane and appeared to be a protease inhibitor. However, it is likely that our preparation of Metastatin acts through a distinct mechanism because it has no detectable antiprotease activity and is inhibited by the addition of hyaluronan. It is tempting to speculate that Metastatin may contribute to the avascular nature of cartilage. Along these lines, we have previously found that hypertrophic chondrocytes produce large amounts of free hyaluronan, which may neutralize the effects of Metastatin in this region and thereby allow blood vessels to invade (28).

The results of this study suggest that Metastatin has antiangiogenic properties as demonstrated by its ability to block VEGF-induced formation of blood vessels in the chicken chorioallantoic membrane. The antiangiogenic effect of Metastatin was also consistent with our finding that it blocked both the proliferation and migration of cultured endothelial cells. Whereas Metastatin can directly attach tumor cells, we believe that most of its antitumor activity is due to its inhibition of angiogenesis because after its injection, the first cells that it would encounter are the endothelial cells, which would be exposed to the highest concentration. In addition, this antiangiogenic mechanism is suggested by the fact that Metastatin blocked the growth of TSU cells in vivo (i.e., on the chicken chorioallantoic membrane) but had little or no effect on their proliferation in vitro. In this particular case, it seems likely that Metastatin was acting indirectly on the TSU tumor cells by blocking angiogenesis.

In other cases, the antitumor activity of Metastatin may be due to the combined action of direct killing of the tumor cells and the inhibition of angiogenesis. Indeed, Metastatin does appear to partially inhibit the growth of B16BL6 in tissue culture, and it could presumably have a similar effect in vivo. Because many blood vessels that are associated with tumors are leaky (29), Metastatin may be able to escape the circulation to interact directly with the tumor cells and block their proliferation. Along these lines, a recent study by Maniotis et al. (30) has indicated that some tumors have the ability to form

**DISCUSSION**

In this study we report that Metastatin, a cartilage-derived hyaluronan-binding complex consisting of proteolytic fragments of bovine link protein and aggrecan, is able to block the growth and metastasis of tumor cells under the following conditions: (a) a single i.v. injection of Metastatin into the chorioallantoic membrane of chicken
vasculature independent of endothelial cells. The tumor cells themselves appear to take on the characteristics of endothelial cells and are responsible for the formation of blood vessels. It is possible that such dual-acting tumor cells could also respond to Metastatin.

The biological effects of Metastatin appear to be closely linked to its ability to bind hyaluronan. If the preparation of Metastatin was premixed with hyaluronan, then this reversed its inhibitory effects on tumor growth in vivo and in vitro and its effects on the growth and migration of cultured endothelial cells. This indirectly suggests that Metastatin is binding to hyaluronan associated with the target cell. In the case of endothelial cells, particularly high levels of hyaluronan are localized to the tips of newly forming capillaries in the chicken chorioallantoic membrane and rabbit cornea (2, 3). A variety of other cell types show a similar relationship between proliferation and the production of hyaluronan (31–34). Whereas the hyaluronan present on proliferating tumor and endothelial cells could interact directly with Metastatin in the blood, the hyaluronan in other locations would not be exposed to high concentrations of the complex. Most normal cells would be protected by the fact that high concentrations of hyaluronan are present in connective tissues such as the dermis, lamina propria, and capsules (5, 35, 36), which would help to neutralize the Metastatin that diffused into these regions. It is important to note that under normal physiological conditions, hyaluronan in the blood is maintained at low levels by the liver and lymphatic system (37, 38). Thus, the circulating Metastatin should retain its hyaluronan binding activity.

Cell surface hyaluronan may serve as a target for other inhibitors of angiogenesis and tumor growth. For example, Endostatin, a ~20-kDa fragment of the COOH-terminal of collagen XVIII that inhibits angiogenesis (21, 22), may also be able to bind hyaluronan, as suggested by the presence of specific structural motifs (39). Secondly, a soluble, recombinant version of immunoglobulin fused with CD44 that binds to hyaluronan can inhibit the growth of human lymphoma cells that express CD44 in nude mice (40, 41). TSG-6, which is secreted by a variety of cells after stimulation with inflammatory cytokines, is able to both bind hyaluronan and block tumor cell growth (42). In each of these cases, these factors may be interacting with hyaluronan on the surfaces of target cells to exert their effects on angiogenesis and tumor growth.

In preliminary studies, we have found that Metastatin induces apoptosis in the target cells. However, at present, the mechanism by which Metastatin is able to do this is unclear. One possibility is that after Metastatin has bound to hyaluronan on the cell surface, it is taken up by the cells into lysosomes, where it is broken down into smaller fragments that enter the cytoplasm and induce apoptosis, perhaps by interacting with the mitochondrial membrane. Alternatively, Metasta-
tint could be interacting directly with the plasma membrane of the target cells, causing damage that in turn induces the apoptotic cascade. Clearly, future experiments will be directed toward elucidating the mechanism by which Metastatin induces apoptosis in the target cells.

In conclusion, we have found that Metastatin is able to block tumor growth in two model systems, and this effect depends on its ability to bind hyaluronan. Metastatin appears to target both tumor cells and endothelial cells that are involved in neovascularization. We postulate that during angiogenesis, the endothelial cells up-regulate their synthesis of hyaluronan, which then serves as a target for the injected Metastatin. Thus, Metastatin may represent a new type of antitumor agent, which targets cell surface hyaluronan.

ACKNOWLEDGMENTS

We are grateful to Dr. Theresa LaVallee and Wendy Hembrow for their assistance with the migration assay.

REFERENCES


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