Biochemical and Biophysical Research Communications 430 (2013) 999–1004

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Involvement of plasmin-mediated extracellular activation of progalanin in angiogenesis

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ARTICLE INFO

Article history:
Received 14 November 2012
Available online 19 December 2012

Keywords:
Progalanin
Galanin
Extracellular processing
Tranexamic acid
Angiogenesis
Matrix metalloproteinase

ABSTRACT

Progalanin is released from the small cell lung carcinoma line SBC-3A and converted to its active form by plasmin. To elucidate the role of progalanin activation in the extracellular compartment, matrix metalloproteinase (MMP) activity was studied in SBC-3A cells treated with progalanin siRNA, and angiogenesis was measured in tumor tissue originating from SBC-3A cell transplantation into mice. Progalanin siRNA caused downregulation of progalanin expression for approximately 8 days. MMP activity and angiogenesis were reduced in tumors induced by transplantation of progalanin siRNA-treated SBC-3A cells. In contrast, MMP-9 and MMP-2 activity and angiogenesis increased in tumors originating from progalanin siRNA-treated SBC-3A cells in the presence of galanin and progalanin. Furthermore, injection of tranexamic acid, a plasmin inhibitor, more markedly reduced MMP-9 and MMP-2 activity and angiogenesis in tumors originating from progalanin siRNA-treated SBC-3A cells and in tumor tissue originating from progalanin siRNA-treated SBC-3A cells in the presence of progalanin. The reduction of MMP-9 and MMP-2 activity with tranexamic acid was restored by galanin, but not by progalanin. Moreover, tranexamic acid reduced angiogenesis in control siRNA-treated SBC-3A cells. These results suggest that the activation of progalanin by plasmin in the extracellular compartment was involved in MMP-9 and MMP-2 activation and in angiogenesis in tumor tissue.

1. Introduction

Several neuropeptides have been shown to play a role in the survival of tumor cells, tumor growth, and metastasis. Such neuropeptides as bombesin/gastrin-releasing peptide (GRP), galanin, bradykinin, proopiomelanocortin (POMC) and arginine–vasopressin [1–4] are released from certain types of tumor cells, known as ectopic neuropeptide-producing cells. Although these peptides have been identified by radioimmunoassay and RT–PCR, the molecular forms of the peptides and their activities are poorly understood. Small cell lung carcinoma cells (SCLCs) release proGRP and POMC [5,6]. The release of these precursor forms is constitutive and associated with low levels of processing enzymes in tumor cells [5,6]. Therefore, the physiological significance of the release of precursor forms from tumor cells remains unclear. The function of precursors is also unknown. Neuropeptides act on specific G-protein coupled receptors in the cell membrane. The activation of G-protein coupled receptors induces cAMP production or cytosolic Ca^{2+} signaling. The somatostatin receptor is expressed in some tumor cell lines [7–9] while VIP receptors (VPAC1) are expressed in breast and lung tumors [10,11], and gonadotropin releasing hormone (GnRH) receptor is expressed in some breast tumors [12]. It is well known that many neuropeptides including VIP, GnRH, and galanin serve as tumor cell growth factors [12–16].

Our previous studies have demonstrated that the human SCLC cell line SBC-3A and the human breast cancer cell lines BT-549 and MDA-MB-436 produce progalanin and release it into culture media [17–19]. Furthermore, progalanin released from cancer cells is converted to the active form galanin(1–20) by extracellular plasmin, a protease [19], which is involved in angiogenic regulation in tumor tissue. The plasminogen precursor pro-plasmin is converted to active plasmin by enzymes such as tissue plasminogen activator (t-PA) and urokinase (uPA), which are abundantly expressed in tumor tissues [20]. Structure–activity relationship studies of galanin have shown that amino acid sequences 1–15 and 1–16 are essential for its biological activity [21,22], and it has been shown that SBC-3A cells express the galanin receptor 2 (GALR2) [18]. We therefore hypothesized that galanin(1–20) is produced from progalanin in tumor tissue and plays a role in tumor growth.

In the present study, the effect of the extracellular processing of progalanin by plasmin on matrix metalloproteinase (MMP) activity

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http://dx.doi.org/10.1016/j.bbrc.2012.11.124

and angiogenesis were evaluated in progalanin siRNA-treated SCLC cells. The effect of the extracellular processing of endogenous and exogenous progalanin was also evaluated in SCLC cells and progalanin siRNA-treated SCLC cells after the injection of tranexamic acid, a plasmin inhibitor.

2. Materials and methods

2.1. Cell lines

The human SCLC cell line SBC-3A [23] was kindly supplied by Dr. Atsuya Tsujimoto, Nippon Kayaku Inc., Tokyo. SBC-3A cells were cultured in RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (FBS, Moretate Biotech, Bulimba, Australia) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. When cells reached 80% confluency, they were dispersed with 0.05% (w/v) trypsin in phosphate-buffered saline (PBS) and harvested at a concentration of 10^4 cells/ml.

2.2. Animals

All experimental protocols were performed in accordance with the guidelines for the Care and Use of Laboratory Animals of the University of Shizuoka. Male KSN/slc mice were purchased from Nippon SLC Ltd. (Shizuoka, Japan) and housed under standard laboratory conditions (23 ± 1 °C, 55 ± 5% humidity) with access to tap water and food *ad libitum*. Lights were automatically turned on at 0800 and off at 2000.

2.3. Recombinant progalanin

Recombinant progalanin was prepared as described previously [18]. The cDNA encoding rat progalanin was obtained from rat hypothalamus by RT–PCR. The primers used were 5′-GGGG CAT ATG CCA ACA AAG GAG AAG AGA GG-3′ and 5′-GGGG CTC GAG GGA CTG CTC TAG GTC TTC TG-3′ to incorporate into the NdeI and XhoI sites, respectively. The PCR fragment was cloned in plasmid pET21-a(+) (Novagen), yielding pET-GAL. *Escherichia coli* ER2566 cells containing pET-GAL were grown to an OD_{600} of 0.6 in 1 L of LB-Amp medium at 37 °C, induced with 0.5 mM IPTG, harvested 4 h after culture and frozen. Frozen cells were resuspended in 50 mM Tris–HCl (pH 7.6), 5 mM EDTA, 10% glycerol and 1 M NaCl, and disrupted by sonification (Bioruptor, Tohsho Denki, Japan). After centrifugation at 30,000*g* for 30 min, the supernatants were loaded onto columns of Ni–NTA agarose (Qiagen) and recombinant progalanin was purified.

2.4. Preparation of culture media and tumor extraction for gel filtration chromatography

SBC-3A cell culture media were briefly centrifuged at 3000*g* for 10 min, and the supernatants were lyophilized and subjected to gel filtration chromatography. Tumor samples were obtained as described before [11]. Briefly, KSN/slc mice were implanted with SBC-3A cells (1×10^6 cells/100 μl PBS(−)) subcutaneously on the dorsal side. When tumors reached a diameter of 8–10 mm, tumor samples were excised. The tumors were heated in a boiling water bath for 10 min in 0.1 M acetic acid. After cooling, the acetic acid concentration was increased to 1 M and samples were homogenized in a Teflon pestle homogenizer. The homogenates were then centrifuged at 3000*g* for 30 min. Supernatants were lyophilized and used as tumor extracts.

2.5. Gel filtration chromatography

Culture media and tumor extracts were eluted on a Sephadex G-50 fine column (1.0 × 100 cm, GE Healthcare UK Ltd., England) using 1 M acetic acid as the eluent. The eluate was collected in 0.9 ml volumes and lyophilized. The lyophilized fractions were dissolved in the standard RIA diluent. The column was calibrated with BSA (molecular weight 69 kDa, void volume), lysozyme (14.4 kDa), human galanin (3 kDa), and dbcAMP (total volume).

2.6. Radioimmunoassay

Radioimmunoassay (RIA) was performed at 4 °C as described previously [24]. R0672 antibodies were raised in rabbits against synthetic human galanin(1–15), specific for the N-terminal region of galanin. Briefly, the standard diluent consisted of 0.01 M phosphate buffer (pH 7.4), 0.14 M NaCl, 0.025 M EDTA, and 0.5% (w/v) bovine serum albumin (BSA). In each assay, 0.1 ml of the standard or sample and 0.1 ml [^{125}I]-human galanin (approximately 3000 counts per minute (cpm)) were incubated with anti-human galanin serum R0672 (final dilution 1:21,000) for 24 h; normal rabbit serum (final dilution 1:50; 0.05 ml), goat anti-rabbit γ-globulin serum (final dilution 1:10; 0.05 ml) and 5% (w/v) polyethylene glycol 6000 (M.W. 7500; 0.5 ml) were then added to the mixture. After incubation for 2 h, samples were centrifuged at 2500*g* for 30 min. The supernatants were removed and radioactivity in the precipitate was counted with a gamma counter (ARC-100, Aloka, Japan). Human galanin was iodinated with iodine-125 by the chloramine-T method and separated by HPLC.

2.7. RNAi-induced progalanin knockdown assay

Progalanin siRNA and negative control (scrambled) siRNA were purchased from Sigma–Aldrich (St. Louis, MO, USA), and INTERFERin (Polyplus-transfection, NY, USA) was used to transfect the siRNA into SBC-3A cells. Progalanin expression in SBC-3A cells was measured by Western blot analysis.

2.8. Western blot analysis

SBC-3A cell extracts for SDS–PAGE were prepared as follows. SBC-3A cells were lysed on ice with 100 μl protein extraction buffer (500 mM Tris–HCl, pH 6.8 and 10% sodium dodecyl sulfate) and centrifuged at 12,000*g* at 4 °C for 10 min. The protein concentration of the cell lysates was measured by the Coomassie Brilliant Blue method using bovine serum albumin (BSA) as the standard. Samples (5 μg) of the cell lysates were separated on a 15% (w/v) polyacrylamide gel [25]. Proteins were blotted onto a nitrocellulose membrane (Protran BA85, GE Healthcare UK Ltd.) in a Mini trans-blot cell 3 (Bio-Rad Laboratories, Hercules, CA, USA) [26]. Nitrocellulose membranes were blocked with 1% (w/v) BSA. Blocked membranes were incubated with anti-galanin(1–15) rabbit antibody (R0672) and then with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG goat antibody (Biosource, Camarillo, CA, USA). The bands were subsequently visualized on a chemiluminescent detection system [27].

2.9. Matrigel plug assay

SBC-3A cells transfected with progalanin or negative control siRNA were dispersed with 0.05% (w/v) trypsin in PBS, and resuspended in Matrigel (BD Biosciences, New Jersey, USA) in the presence or absence of galanin (100 ng/0.5 ml in Matrigel, Peptide Institute, Inc., Osaka, Japan) or progalanin (100 ng/0.5 ml in Matrigel). The cells were implanted into the dorsal sides of KSN/slc mice subcutaneously with 0.5 ml Matrigel. Tranexamic acid (100 mg/kg,

Tokyo Kasei Kogyo, Tokyo, Japan) was administered intraperitoneally once a day for 7 days. Seven days after implantation, tumor tissue was excised and homogenized in a Polytron homogenizer (KINEMATICA AG, Lucerne, Switzerland). The homogenates were then centrifuged at 3000*g* at 4 °C for 30 min. Supernatants were used for hemoglobin measurement and for gelatin zymography analysis.

2.10. Gelatin zymography

MMP activity was measured using gelatin zymography [28]. The tumor extracts from the Matrigel plugs were denatured with SDS and separated with 10% polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, the SDS in the gels was removed with Tris–HCl buffer (pH 7.4) containing 2.5% Triton X-100, and then incubated in Tris–HCl buffer for 24 h, allowing digestion of the gelatin. The gels were visualized using Coomassie Brilliant Blue R250. The bands were densitometrically analyzed using Scion Image software (Scion corp., Frederick, MD, USA).

2.11. Hemoglobin content in tumor tissue

Angiogenesis was assessed by measuring the hemoglobin (Hb) content. Hb was measured by the cyanmethemoglobin method [29] with some modifications. The tumor extracts from the Matrigel plugs were diluted with 100 μl 0.5 mM sodium hydroxide, to which was added 20 μl 2% (w/v) potassium ferricyanide and 20 μl 0.5% (w/v) sodium cyanide. After 30 min incubation, Hb concentrations were determined by measuring the absorbance at 550 nm.

2.12. Data analysis and statistics

Data are represented as means ± SEM. Tukey–Kramer tests were used for statistical analysis.

3. Results

3.1. Characteristics of galanin-like immunoreactivity in culture media and tumor tissue

Galanin-like immunoreactivity (galanin-LI) was due to the presence of a 14 kDa protein in cultured SBC-3A cells (Fig. 1A), suggesting that the galanin-LI was caused by antibody binding to progalanin. In the extracts from tumor tissue, however, galanin-LI was due to the presence of a protein of around 2 kDa, in addition to the 14 kDa protein, in agreement with our previous papers [18,19]. In tumor tissue, the 2 kDa galanin-LI accounted for 58.7 ± 11% of total galanin-LI (n = 3).

3.2. Progalanin expression after progalanin siRNA transfection

We examined progalanin expression in SBC-3A cells transfected with either progalanin siRNA or control siRNA. Progalanin expression in SBC-3A cells was determined by Western blot analysis (Fig. 2). As expected, progalanin expression was inhibited by progalanin siRNA, and this inhibition was maintained for 8 days after transfection (see Fig. 3).

3.3. Involvement of progalanin activation in MMP activity

We examined the idea that progalanin, after activation by plasmin, is involved in regulating MMP activity in tumor tissue. MMP activity was determined by gelatin zymography. The activities of MMP-9 and MMP-2 were decreased in tumor tissue originating from progalanin siRNA-treated SBC-3A cells. MMP-9 activity was increased in tumor tissue originating from progalanin siRNA-treated SBC-3A cells in the presence of galanin and progalanin. In contrast, injection of tranexamic acid, a plasmin inhibitor, more markedly reduced the activities of MMP-9 and MMP-2 in tumor tissue originating from progalanin siRNA-treated SBC-3A cells and also in progalanin siRNA-treated SBC-3A cells in the presence of progalanin. The reduction of MMP-9 and MMP-2 activities with

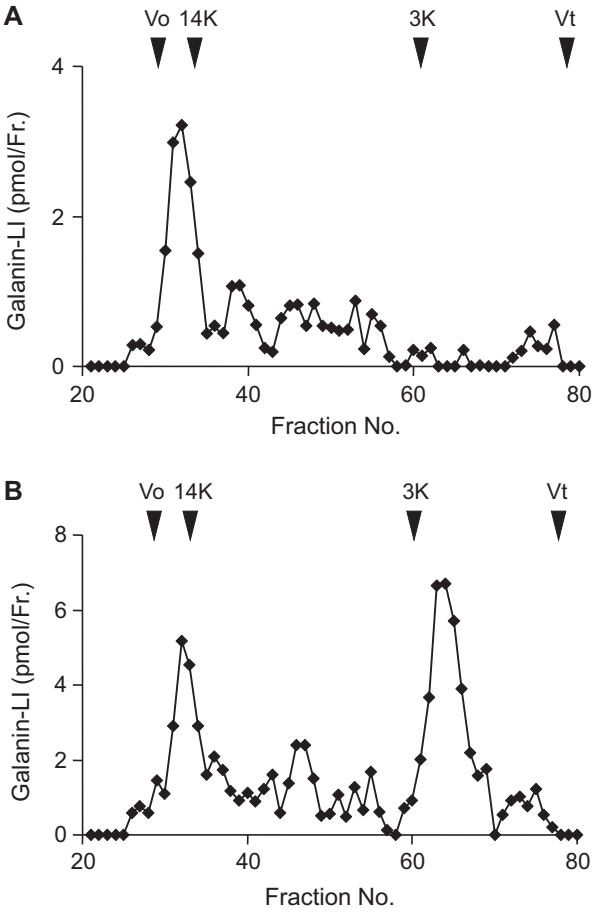

Fig. 1. Molecular forms of galanin-like immunoreactivity in culture media from SBC-3A cells (A) and tumor tissue extract (B) as revealed by gel filtration chromatography. Culture media from SBC-3A cells and tumor tissue extracts were loaded onto a Sephadex G-50 column with 1 M acetic acid as an eluent. Collected samples were assayed with a galanin-specific radioimmunoassay. Void volume (Vo) and bed volume (Vt) were calibrated using bovine serum albumin and dibutyryl cAMP, respectively. The eluted position of egg lysozyme and human galanin are indicated at 14 and 3 K, respectively.

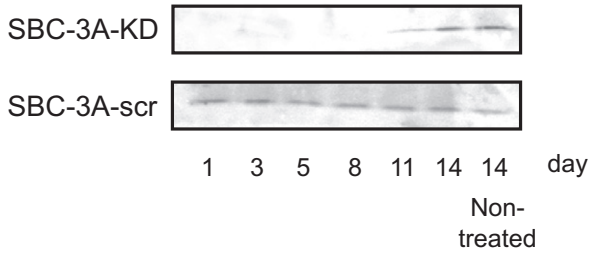

Fig. 2. Inhibition of progalanin expression by progalanin siRNA. SBC-3A cells were transfected with control (scrambled) or progalanin siRNA. Cell lysates were collected 1, 3, 5, 8, 11 and 14 days after transfection, and then separated by SDS–PAGE, followed by immunoblotting with anti-human galanin(1–15) antibody.

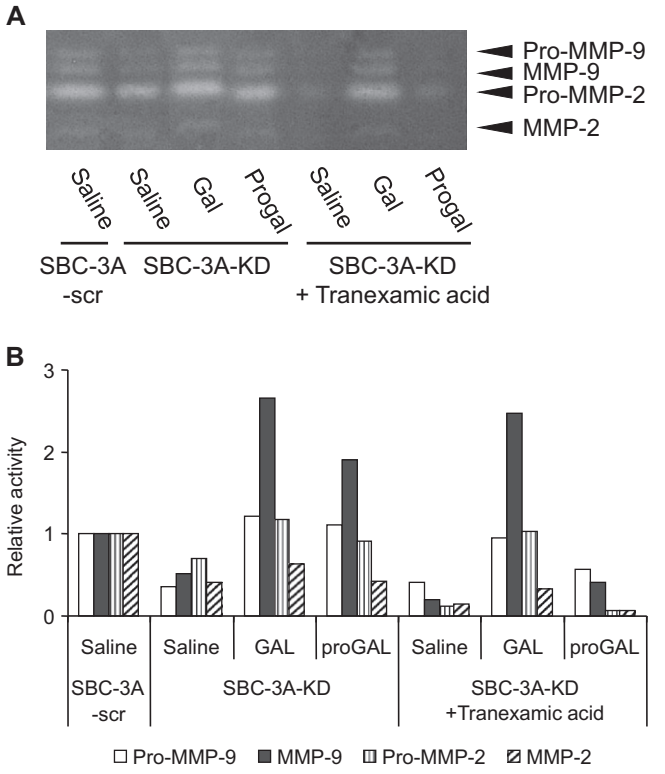

Fig. 3. The inhibition of pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 activities in tumor tissue by treatment with progalanin siRNA. The activities of pro-MMP-9, MMP-9, pro-MMP-2 and MMP-2 in Matrigel extracts were determined using gelatin zymography (A). SBC-3A cells transfected with progalanin siRNA (SBC-3A-KD) or control RNA (SBC-3A-scr) were suspended in Matrigel in the presence or absence of galanin (100 ng/0.5 ml Matrigel) or progalanin (100 ng/0.5 ml Matrigel), and the suspensions were subcutaneously implanted into the dorsal sides of KSN/slc nude mice. After implantation, tranexamic acid (100 mg/kg) was administered intraperitoneally once a day for 7 days. Seven days after implantation, Matrigel extracts were separated by SDS–PAGE using 10% acrylamide gel and 1 mg/ml gelatin. The bands digested were densitometrically analyzed (B). The rate of the digestive activity of tumor tissue treated with progalanin siRNA to that treated with control siRNA is represented as 1.

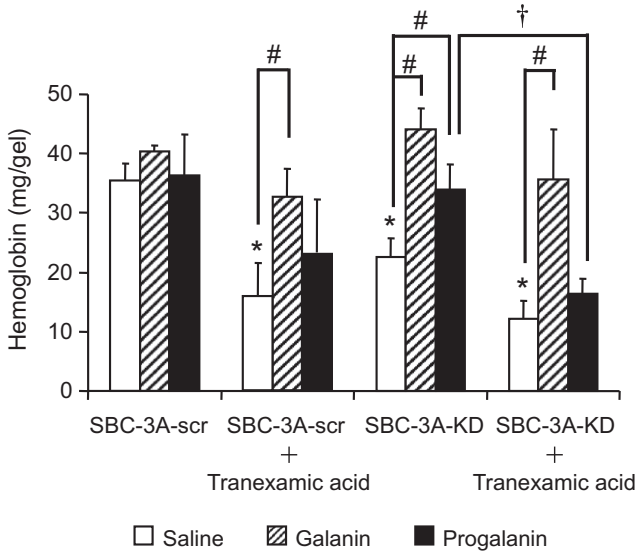

Fig. 4. Involvement of progalanin activation in angiogenesis. SBC-3A cells transfected with progalanin siRNA (SBC-3A-KD) or control siRNA (SBC-3A-scr) were suspended in Matrigel in the presence or absence of galanin (100 ng/0.5 ml Matrigel) or progalanin (100 ng/0.5 ml Matrigel), and the suspensions were subcutaneously implanted into the dorsal sides of KSN/slc nude mice. After implantation, tranexamic acid (100 mg/kg) was administered intraperitoneally once a day for 7 days. Seven days after implantation, tumor extracts were taken and their hemoglobin content measured by the cyanmethemoglobin method. ($n = 3$). $^{*}p < 0.05$, vs. SBC-3A-scr tumor treated with saline (control). $^{\#}p < 0.05$, vs. tumor treated with saline. $^{\dagger}p < 0.05$, vs. SBC-3A-KD tumor treated with saline.

tranexamic acid was restored in the presence of galanin, but not in the presence of progalanin.

3.4. Involvement of progalanin activation in angiogenesis

To assess whether progalanin activation is involved in angiogenesis, angiogenic activity was examined using the Matrigel plug assay (Fig. 4), in which Hb content was determined. The Hb content was 35.5 mg/gel in SBC-3A-scr tumors and was significantly decreased in tumor tissue originating from progalanin siRNA-treated SBC-3A cells (24.0 mg/gel). Furthermore, tranexamic acid, a plasmin inhibitor, decreased Hb content in tumor tissue originating from control siRNA-treated SBC-3A cells. Conversely, galanin increased the Hb content both in tumor tissue originating from control siRNA-transfected SBC-3A cells and in tranexamic acid-treated tumor tissue originating from progalanin siRNA-transfected SBC-3A cells. In contrast, progalanin increased the Hb content in tumor tissue originating from progalanin siRNA- transfected SBC-3A cells, but not in tranexamic acid-treated tumor tissue originating from progalanin siRNA- transfected SBC-3A cells.

4. Discussion

Galanin is widely distributed in the central and peripheral nervous systems. It produces biological effects such as neuroprotection [30], via the three galanin receptors (GALR1, 2 and 3), which belong to the G protein-coupled receptor superfamily [31]. In neuronal and endocrine cells, galanin is usually produced from the processing of progalanin by prohormone convertase. In several tumors such as neuroblastoma, colon carcinomas and squamous cell carcinomas that express galanin receptors, galanin regulates cell proliferation [32–37]. SCLC cells are well known to produce galanin ectopically. Although galanin is released in precursor form from some SCLC cells, little is known about the nature of precursor release. Because precursors cannot bind to receptors, they must first be activated in SCLC cells, which express galanin receptors. SBC-3A cells express galanin receptors, so it is possible that progalanin release from SBC-3A cells is associated with tumor growth. In the present study, progalanin release was evaluated with respect to angiogenesis, a requirement for tumor growth.

The major product released from SBC-3A cells that exhibited galanin-LI was a protein of approximately 14 kDa and was believed to be progalanin (Fig. 1A). In tumor tissues originating from SBC-3A cells, however, a protein of approximately 2 kDa exhibited galanin-LI and was produced in addition to the 14 kDa progalanin, as reported previously [18]. In structural analysis using MALDI-TOF mass spectrometry, the 2 kDa protein exhibiting galanin-LI was identified as galanin(1–20) [18]. Because the production of galanin(1–20) is inhibited in tumor tissues by administration of tranexamic acid, a plasmin inhibitor [19], plasmin seems to play a role in the conversion of progalanin into galanin(1–20). It is possible that this conversion is important in tumor growth.

Plasmin is an important protease in the mechanisms of angiogenesis in tumor tissues. A pro-plasmin called plasminogen is converted to active plasmin by enzymes such as tissue plasminogen activator (t-PA) and urokinase (uPA) in the extracellular compartment. The plasmin-mediated destruction of the extracellular matrix and basement membrane can enable the migration of endothelial cells and cancer cells [20]. Several studies have reported the effects of plasmin inhibitors like tranexamic acid on

endothelial cell invasion, angiogenesis, and tumor growth [38–41]. In the present study, in order to elucidate the involvement of the extracellular processing of progalanin in angiogenesis, MMP activity and angiogenesis were assessed with the Matrigel plug assay using SBC-3A cells treated with progalanin siRNA, causing progalanin expression to be downregulated for approximately 8 days. MMP activity and angiogenesis were reduced in tumor tissue originating from progalanin siRNA-treated SBC-3A cells. In contrast, MMP-9 and MMP-2 activity, as well as angiogenesis, was increased in tumor tissue originating from progalanin siRNA-treated SBC-3A cells in the presence of galanin and progalanin. Furthermore, injection of tranexamic acid, a plasmin inhibitor, markedly reduced MMP-9 and MMP-2 activity and angiogenesis in tumor tissue originating from progalanin siRNA-treated SBC-3A cells, and also in tumor tissue originating from progalanin siRNA-treated SBC-3A cells in the presence of progalanin. The reduction of MMP-9 and MMP-2 activity by tranexamic acid was restored in the presence of galanin, but not in the presence of progalanin. Interestingly, tranexamic acid also reduced angiogenesis in control siRNA-treated SBC-3A tumors. These results suggest that the activation of progalanin by plasmin in the extracellular compartment is involved in both MMP-9 and MMP-2 activity regulation and angiogenesis in tumor tissue originating from SBC-3A cells, a human SCLC cell line.

MMPs detach endothelial cells from adhesive proteins allowing the development of angiogenesis. In particular, MMP-2 overexpression is associated with the invasion of tumor cells and MMP-2 depletion is associated with the suppression of tumor growth [42,43]. Therefore, MMP-2 is a key enzyme in cell invasion, tumor growth and metastasis. In the present study, pro-MMP-2 was highly expressed in tumor tissue originating from SBC-3A cells. Galanin induces angiogenesis in a cotton thread-induced granulation assay in rats [44], in which it strongly induces pro-MMP-2 production.

In conclusion, the present study is the first to demonstrate that galanin is involved in angiogenesis via the production of MMPs in tumor tissues and that the extracellular processing of progalanin via plasmin is a novel mechanism in tumor growth.

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