Anti-angiogenic Effect of Argatroban and Angiopoietin 1/2 Balance in Experimental Cancerous Angiogenesis

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Abstract

Close correlations between coagulation, fibrinolysis cascade, and physiological angiogenesis have been reported. Angiogenesis in cancer tissue is also correlated with this cascade, although no particular mechanism has yet been analyzed. Clinical articles have revealed that anticoagulants may affect tumor angiogenesis, however, few reports have utilized experimental cancerous angiogenesis models. Furthermore, new anticoagulants, such as selective thrombin inhibitors, have not been examined. We focused on a new anticoagulant, argatroban (1.2 mg/kg/day), and investigated its antiangiogenic effects in the mouse dorsal air sac model and Matrigel assay. Macroscopic vascular structure, vascular area count, CD34 micro vessel density, and angiogenic factors, angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2) and vascular endothelial growth factor (VEGF) were measured in the dorsal air sac model using S-180 sarcoma cells and COLO320 DM colon cancer cells. Tube formation in HUVEC cells was examined by Matrigel. With administration of argatroban, tumor angiogenesis was significantly inhibited. Because argatroban did not inhibit tube formation on HUVEC in Matrigel assay, this effect may not be a direct action on endothelial cells. In this experimental model, the Ang-2 to Ang-1 ratio was always elevated during tumor angiogenesis, and this phenomenon may have caused abnormal tumor vascular architecture. These results suggest that argatroban may exert an antitumor effect through inhibition of tumor angiogenesis. In addition, changes in the Ang-2 /Ang-1 ratio may be related to antiangiogenic effects in cancer tissues.

Key words
Angiogenesis, Argatroban, S-180, COLO320DM, CD34

Introduction

Administration of anticoagulant heparin in cancer therapy has been reported since the 1960s. Recent meta-analysis has shown that low-molecular weight heparin improves the prognosis of cancer patients that exhibit venous thromboembolism. However, few reports using experimental cancerous angiogenesis models exist. In addition, the new selective thrombin inhibitor anticoagulants have not been examined.

Argatroban is a potent selective thrombin inhibitor that has a strong inhibitory activity when compared with heparin. Although a direct effect on the binding site of thrombin has been reported, this inhibitor also inhibits serine protease and exerts effects on endothelial cells as well as inflammatory and coagulation cytokines. The pharmacokinetics of argatroban in the coagulation cascade are not fully understood. In contrast to heparin, argatroban is not affected by tissue factor pathway inhibitor (TFPI) or mobilization of platelet factor IV. Because its anticoagulant action is not altered by hemococoncentration or hemodilution, it may be useful in cancer-associated thrombosis. For these reasons, we hypothesize that argatroban may have antitumor effects by exerting antiangiogenic activ-

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On the other hand, it has been reported that angiogenesis plays an important role in cancer growth, and thus various studies and therapies have focused on tumor angiogenesis. If the specific characteristics of tumor vessels can be elucidated, more effective antiangiogenic therapies may be put into practice. Clinically, angiography shows that tumor vessels are both large and small, and that they are twisting, winding and leaky when compared with normal vessels. Microscopically, there are numerous immature vessels, mosaic formations, and intussusceptive growth in cancer tissues. These alterations may be caused by substantial proliferative activity or imbalance of angiogenic factors in cancer tissues, although this is not clear. Angiopoietin is one of the new angiogenic factors, possibly involved in vasculogenesis and angiogenesis. Angiopoietin-1 (Ang-1) is thought to be a natural antagonist of angiopoietin-2 (Ang-2). Ang-2/Ang-1 balance may be important to control vascular elongation and mature under VEGF existence. Here, we focus on Ang-2/Ang-1 balance and examine changes in this balance together with the antiangiogenic effects of argatroban in experimental cancerous angiogenesis.

Materials and Methods

1. Mice sarcoma cell culture of peritoneal passage

Sarcoma180 (S-180) cells originating from mouse sarcoma were kindly provided by the Cell Resource Center, Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. Cells were passaged intraperitoneally in ICR mice and were used at concentrations of 1×108/ml.

2. Human colon cancer cell culture

Human colon cancer COLO320DM cells were purchased from Dainippon Pharmaceutical Corporation (Osaka, Japan). Cells were cultured in RPMI 1640 and were used at concentrations of 5×10^4/ml.

3. Preparation of anticoagulant

Powdered argatroban was kindly provided by Mitsubishi Pharma Corporation (Osaka, Japan) and was dissolved at the recommended concentration. (10 mg/ml, recommended by Mitsubishi Pharma Co.) The final concentration was 0.02 mg/ml. We gave mice argatroban which concentration was 1.2 mg/kg/day.

4. Dorsal air sac method for angiogenesis in mice

ICR male mice (age: 4~5 weeks) were purchased from Nihon SLC Corporation (Sizuoka, Japan) and were bred at the St. Marianna University School of Medicine, Post-Graduate School, Institute of Experimental Animals (Kawasaki, Japan). Five mice in each group were used in the experiments. S180 and COLO320DM were suspended 0.2 ml of culture medium, and each medium or PBS (control group) was injected into Millipore chambers (filter pore size: 0.45 μm, Millipore Co., MA, USA). These chambers were subcutaneously implanted into dorsal sites of each mouse. (Fig. 1) Experimental groups were injected with anticoagulant (Argatroban: 1.2 mg/kg/day) intraperitoneally once daily. Four days after implantation, the skin from the dorsal sites was gently removed under pentobarbital anesthesia. A silicon gasket (Millipore) of the same shape as the chamber was placed on the chamber-attached skin and photographs were taken using a digital camera (EOS kiss digital, Canon, Tokyo, Japan). All procedures in this investigation were approved by the Animal Welfare Committee of our institution.

5. Image analyzer

Vascular areas of the dorsal skin were evaluated using a Leica QWin Image analyzer (Leica, Tokyo, Japan) and were compared between each group.

6. Immunohistochemistry for CD34

a) Histological sections

Chamber-attached skin was removed and fixed with 4% paraformaldehyde (pH 7.4) at 4°C for 24 h. The skin was then immersed in 20% sucrose (pH 7.4) and embedded in OCT compound using liquid nitrogen. Longitudinal sections (7 μm) of subcutaneous tissue were made and attached to silane-coated sides.

b) Immunohistochemistry

Air-dried sections were washed with phosphate-buffered saline (PBS; pH 7.2) and blocked with endogenous peroxidase using 0.3% H2O2 for 30 min. A 10-fold dilution of anti-mouse CD34 rat monoclonal antibody (MEC14.7 HM1015, HyCult biotechnology b.v. Uden, Netherlands) and a Histofinesimple stain mouse MAX-PO (Rat) kit (414311, Nichirei, Tokyo, Japan) were employed in immunohistochemistry.

c) Determination of micro vessel density (MVD)

Vessel staining on CD34 immunohistochemistry was defined as MVD. According to Weidner et al., hot spots for MVD were identified at 100× magnification, and thus MVD in a 0.72-mm² area were counted at 200× magnification; counts were
performed 3 times in each area and the mean values were calculated.

7. Western blotting for angiopoietin-1 and-2, VEGF

a) Tissue extract

Subcutaneous tissues were carefully removed from skin tissues and were homogenated in 2 ml of 10 mM Tris-HCl (pH 7.5) using an Ultrasonic Disruptor (UP-201, Tomy, Tokyo, Japan) 3 times for 15 min each. After centrifugation at 14000 rpm at 4°C for 15 min, the supernatant was collected and used.

b) Measurement of Ang-1, Ang-2, and VEGF

A total of 50 μg of sample protein was treated with 5X SDS and electrophoresis was performed using 10% polyacrylamide gels. After membrane transfer (Immunobilin-P Transfer Membrane, Millipore, USA), membranes were blocked with buffer A for 1 h, and were washed with Tris buffer containing 0.05% Tween20. Membranes were incubated with anti-mouse Ang-1 goat polyclonal antibody (N-18 sc-6319, Santa Cruz Biotechnology, CA, USA) at 300× dilution, anti-mouse Ang-2 rabbit polyclonal antibody (ANG21-A 25001A, Alpha Diagnostic International, SA, USA) at 200X dilution, or anti-mouse VEGF rabbit polyclonal antibody (RB-222, Lab Vision, CA, USA) at 200X as primary antibodies, or were incubated with anti-mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH) rabbit polyclonal antibody 2275-PC-1, Trevigen, MD, USA at 1000X dilution as a positive control. Membranes were stained with nitroblue tetrazolium solution (NBT), and bromochloroindolyl phosphate solution (BCIP) for HR staining. Relative amounts of protein for each angiogenic factor were calculated using Scion Image Ver. 4.0.2, Scion Co. Maryland, USA based on band intensity.

8. DNA content of cultured cancer cells in implanted chambers

Four days after chamber implantation, cultured cells in the chamber were immersed in DNA digestion buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 0.5%SDS, 0.1 mg/ml proteinase) at 55°C overnight. Phenol was then added to the cells and centrifugation was performed at 12,000 rpm for 5 min. Chloroform was added to the supernatant, which was centrifuged, followed by isopropanol addition, and further centrifugation. The pellet was mixed with 70% ethanol, centrifuged, and dried. TE buffer was added to the pellet and was incubated at 55°C overnight. DNA content was calculated based on optical density using a spectrograph (DU 530, Beckman, CA, USA).

9. Tube formation of human umbilical vein endothelial cell (HUVEC) in Matrigel

HUVEC were seeded onto Biocoat Matrigel Micro Test Plates. A range of concentrations of Argatroban was added to the plates. After 18 h of incubation, Matrigel was fixed and analyzed for tube formation by phase-contrast microscopy.

10. Comparison of data

Vascular area and CD34 immunohistochemistry were examined in the PBS group, the S-180 group, the COLO320DM group, the S-180+Argatroban group, and the COLO320DM+Argatroban group. In addition, Ang-1, Ang-2, VEGF, and
DNA content were measured in the PBS group, the S-180 group and the S-180+Argatroban group. Matrigel assay was performed with various concentrations of Argatroban.

11. Statistical analysis

Statistical analysis of the mean value in 2 groups was performed using the Mann Whitytoney-U test. Statistical significance was defined as P<0.05. Prism for Windows (Ver. 2.01, GraphPad Software Inc., CA, USA) was used for statistical analysis.

Results

1. Image analysis of dorsal subcutaneous vessels

Vessels in the subcutaneous chamber in the PBS group were of the same pattern as the surrounding subcutaneous vessels. In contrast, numerous microvessels were generated and vascular dilation was seen in the S-180 and COLO320DM groups. Aberrant vascular architecture was observed. Vascular area was 24.6 mm² in the PBS group, compared with 53.3 mm² in the S-180 group (p=0.0001). In the S-180+Argatroban group, vascular area was 36.5 mm² and argatroban significantly inhibited experimental tumor angiogenesis (p=0.0001). Vascular area was 49.0 mm² in the COLO320DM group and this was significantly higher that in the PBS group. However, administration of argatroban significantly decreased this area in the COLO320DM+Argatroban group (26.4 mm²). (Fig. 2, 3)

2. CD34 Microvessel Density

MVD was 36.3 in the PBS group but was significantly higher at 146.9 in the S-180 group (p=0.0001). In contrast, MVD decreased significantly to 91.5 in the S-180+Argatroban group (p=0.0022). In longitudinal sections, the vessel morphology changed dramatically by stimulating cancer cells. Small and large vessels as well as stromal cells were markedly increased in the S-180 and COLO320DM group when compared with the PBS group. This alteration was significantly inhibited by argatroban without resulting in stromal cell increase. (Figs. 4, 5)

3. Western blotting of Ang-1, Ang-2, and VEGF expression

Although there were no statistical differences, Ang-1 expression (P=0.093) was decreased while Ang-2 (P=0.086) and VEGF (P=0.06) expression was increased in the S-180 group when compared with the PBS group. Ang-1 expression was higher than Ang-2 expression in the PBS group, while Ang-2 expression was higher than Ang-1 expression in the S-180 group. The ratio of Ang-2 to Ang-1 was significantly higher in the S-180 group than in the PBS group (p=0.035). In the S-180+Argatroban group, of the expression levels of these angiogenic factors tended to return to those in the PBS group. (Ang-1; P=0.1, Ang-2; P=0.097, VEGF; P=0.08) The ratio of Ang-2 to Ang-1 was significantly lower in the S-180+Argatroban group than in the S-180 group (P=0.043). (Fig. 6)

4. DNA content of culture cells in the chamber

In order to measure changes in cell number in the chamber, DNA content was measured. DNA content was lower in the S-180+Argatroban group than in the S-180 group. However, this difference was not statistically significant (p=0.25). (Fig. 7)

5. Tube formation of HUVEC in Matrigel

Several concentrations of argatroban (1, 10, 50, 100 μg/ml) were added to HUVEC on Matrigel. Although HUVEC branched and formed capillary-like tubes in the control group, there was no obvious effect of Argatroban on tube formation of HUVEC.

Discussion

There is a close correlation between venous thromboembolism (VTE) and cancer, and thus the coagulation system appears to be activated in such patients. The cause of this phenomenon is understood as part of the general pathology of cancer. Folkman et al. reported that angiogenesis plays an important role in cancer growth. They focused on the fact that vessel growth is essential for nutrition and oxidation of the tumor, and the theory of tumor dormancy was published, which lead to therapies that aim to inhibit tumor growth through angiogenesis inhibition. This favorable theory inspired numerous clinical or basic studies of angiogenesis to be performed. We previously showed that high levels of plasma VEGF in patients with advanced colon cancer are closely correlated with activation of platelet aggregation. It is thus obvious that the coagulation and fibrinolysis systems are correlated with physiological or cancerous angiogenesis. The current potent angiogenic inhibitors angiotatin (180 KDa Plasminogen) and endostatin (182 KDa CollagenXVIII) are also coagulation-system related drugs. In a recent review, Rickles et al. reported correlations between thrombin, cancer growth, and angiogenesis. They also cited several
Figure 2. Comparison of initial and binary images of dorsal sides of mice with PBS, S180 and S180 + Argatroban.

a, Initial image of subcutaneous dorsal blood vessels of an ICR mouse implanted with a Millipore chamber containing PBS; a’, binary image of subcutaneous blood vessels.

b, Initial image of subcutaneous blood vessels of an ICR mouse implanted with a chamber containing S180 cells; b’, binary image of subcutaneous blood vessels.

c, Initial image of subcutaneous blood vessels an ICR mouse which was given 1.2 mg/kg/day of Argatroban (intraperitoneally) from day1 to day3 after transplantation, implanted with a chamber containing S180 cell; c’, binary image of subcutaneous blood vessels.
articles suggesting the possibility of therapeutic applications of antithrombotic drugs (low-molecular weight heparin, unfractionated heparin) against cancer. However, the effects of these anticoagulants against cancer growth and angiogenesis remain uncertain. Direct thrombin inhibitors (lepirudin, desirudin, bivalirudin, and argatroban), a newly developed category of antithrombotic drugs, have been recently developed. The action of these agents differs from that of heparin or warfarin-type drugs. Argatroban was synthesized by Okamoto et al. in 1980 and is a low-molecular weight anticoagulant that inhibits production of thrombin directly. It is an synthetic arginine-based drug and is characterized by stronger inhibition of thrombin than heparin, no production of antibodies and reversible anticoagulation effects.

Coagulation systems operate through inhibition of factor XIIa, antagonism of protein C activation, activation of thrombin-dependent fibrinolytic inhibitor, inhibition thrombin related platelet hemostasis and vasomodality, or modulation of inflammatory cytokines.

For these reasons, we hypothesized that argatroban may have inhibitory effects on cancerous angiogenesis. The results of this study clearly show that argatroban possesses antiangiogenic activity against angiogenesis experimental cancer models in both mouse and human cell line in vivo.

Dorsal air sac model originated by Folkman is one of the angiogenesis measurement methods. This method is capable of measuring angiogenic activity of various cultured cancer cells. In the present study, S180 mouse sarcoma cell and Colo320 DM human colon cancer cell were used in order to
compare various kinds of cells. Argatroban inhibited subcutaneous angiogenesis in two kinds of cells. Not only the macroscopic vascular area but also the microscopic microvessel density using CD34 were inhibited by argatroban. Inhibition of dilatation of established vessel tended to be greater than proliferation of microvessels. In order to investigate whether this action is a direct action to endothelial cells or not, in vitro Matrigel assey was done using HUVEC.

The results of Matrigel assay suggest that this effect may not be due to a direct action on endothelial cells. Because the angiogenic factors Ang-1, Ang-2, and VEGF tended to be inhibitory by argatroban, the antiangiogenic effect may be caused by the inhibition of thrombin-related activity in angiogenesis mediated by platelets or other angiogenic factor-releasing cells. In the histology of chamber-attached subcutaneous tissues, the number of stromal cells was markedly increased in the S-180 group when compared with the PBS group. This phenomenon was not affected by administration of argatroban. Therefore, the antiangiogenic effects may be associated with circulating cells, particularly platelets. Several articles have described correlations between platelets, angiogenesis, and cancer.16-19

Thrombin is thought to possess an angiogenic action of clotting dependent and independent mechanism. (Fig. 9) Clotting dependent action is correlated with platelet and fibrin, fibrin clot secrete VEGF and basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF) and stimulate angiogenesis.

Clotting independent action is that thrombin binds to protease-activated receptors which promote release of VEGF, VEGF receptor, TF, bFGF, and MMP-2. It was supposed that the effect of argatroban inhibits thrombin, in consequence, inhibits angiogenesis via clotting-dependent and independent mechanisms.

In order to observe the influence of argatroban administration to cancer cell proliferation, DNA content of cancer cell in chamber was measured. There was a tendency of decrease of DNA content in chamber cells after argatroban administration. Argatroban may possess an antiproliferative effect for cancer cells. Antiangiogenic drugs for clinical cancer therapy recently have been developed. Thrombosis is one of the problem side effects of these drugs.20-21 Other anti-cancer agents also activate thrombin. Combined use of these drugs and an anticoagulant may be a reasonable approach to minimize adverse effects. Based on the results of this experiment, the use of argatroban during chemotherapy may be useful for not only inhibiting tumor angiogenesis but also in decreasing the side effects.

Antiangiogenic therapy specifically targeting tumor vessels has not yet been reported. Physiologically, various angiogenic factors play roles in vasculogenesis and angiogenesis. Angiopoietin is one such angiogenic factor related to vessel maturation, elongation, etc. It has been reported that Ang-1 affects pericyte coverage and vessel maturation, while Ang-2 affects elongation of vessels releasing VEGF. Ang-1 and Ang-2 bind competitively to the same receptor, Tie-2 receptor. Therefore, Ang-1/Ang-2 balance is important in physiological angiogenesis.

Ang-1/Ang-2 balance in cancer tissues has been reported in several articles.22-26 However, no reports have investigated the dorsal air sac model of angiogenesis. The results of our experiment showed that Ang2 expression was higher than Ang1 expression in the S-180 group. In contrast, Ang1 expression was higher than Ang2 expression in the PBS group after 4 days of implantation. Expression of Ang-2 may always be higher than that of Ang-1 in cancer-surrounding tissues. These results suggest that immature vessels or aberrant vessels are more common in cancer tissues because vascular elongation is always occurring at higher levels than vascular maturation in cancer tissues. Our results revealed
Figure 6. Expression of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), vascular endothelial growth factor (VEGF), angiopoietin-2,1 balance for western blotting analysis. The mean±S.D. of 5 samples and 3 groups is depicted.

Figure 7. Effect of Argatroban with a chamber containing S180 cells. The mean±S.D. of 5 samples.

Figure 8. Chemical structure of argatroban.
that cancerous angiogenesis may be characterized by consistently higher levels of Ang-2 when compared with Ang-1. Therefore, although Ang-1 promotes angiogenesis in normal tissue, it may inhibit angiogenesis in cancer tissue. This suggests that Ang-1 may possess antitumor effects in cancer tissues in vivo.

It has been reported that angiopoietin is secreted from endothelial cells, fibroblast, macrophage, platelets, and tumor cell. In this experiment, the alteration of VEGF or angiopoietin may originate from total secretion of these cells. Tumor cell under hypoxic environment accelerate transcription of hypoxia inducible factor (HIF). HIF directly up-regulates Ang-2 expression or indirectly control via VEGF. These pathways may also be correlated with this experiment. In recent study, it was suggested that vascular stabilizer action of Ang-1 is related to PDGF expression. Inhibition of angiogenesis of argatroban may be correlated with these thrombin-platelet aggregation-PDGF pathway. In this experiment, VEGF and Ang-2 from tumor cells were inhibited by administration of argatroban. It is unclear that this action is a direct action to tumor cells or is an indirect action from platelets or stromal cell. No experiments exist that evaluate the correlation between anticoagulant and angiopoietin in vivo. Future experiments needed to analyze these mechanisms.

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Reference

抗凝固薬による培養癌細胞の血管新生抑制の効果
およびその検討

抄 録

凝固線溶系は生理的血管新生と密接に関係しており、癌の血管新生についても同様である。過去の報告から凝固線溶に関わる薬剤は癌血管新生に影響を及ぼすとされるが、実験モデルでの報告は少なく、最近開発された新規抗凝固薬についてはその詳細は明らかにされていない。

我々は新規抗凝固薬である Argatroban に着目し、その癌血管新生抑制効果をマウス実験モデルとマトリゲルを用いて検討した。Argatroban 投与後 (1.2 mg/kg/day) の癌血管新生に対する影響を肉眼的血管構築の変化と血管面積、CD34 微少血管密度 (MVD), 皮下組織の血管新生因子 (Ang-1, Ang-2, VEGF) を測定し、比較検討した。

Argatroban 投与群の新生血管は非投与群と比べ、有意に抑制され、免疫組織学的にもその MVD も有意な低下がみられた。血管新生因子も抑制傾向がみられたが、マトリゲルによるHUVEC の毛細管形成能は抑制されず、血管内皮細胞への直接作用は少ないと考えられた。また血管新生因子の検討から Ang1/2 balance は常に正に傾いて血管伸張が有意に上昇する状態にあり、特徴的な癌血管構築を形成する可能性が示唆された。

以上より Argatroban は癌血管新生を抑制する可能性が示唆され、抗腫瘍効果が期待できると考えられた。また癌組織の Ang1/2 balance から癌血管新生の抑制戦略には Ang1 による競合的 Ang2 抑制などによる Ang シグナルの抑制が効果的である可能性が示唆された。