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ARTICLES

Agkistin-s, a Disintegrin Domain, Inhibits Angiogenesis and Induces BAECs Apoptosis

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Abstract Previous work in our laboratory has shown agkistin, a snake venom metalloproteases (SVMPs) from the venom of *Agkistrodon halys*, possesses antiplatelet aggregation activity. In this study, we further examined the antiangiogenic activity of agkistin-s, the disintegrin domain of agkistin. Recombinant agkistin-s was produced in *Escherichia coli* by subcloning its cDNA into pET28a vector, and the effect of purified agkistin-s was evaluated. At the concentration of 0.5–1.5 μM, the recombinant agkistin-s exhibited inhibitory activities on the bovine aortic endothelial cells (BAECs) migration and proliferation in a dose-dependent manner. In addition, it exhibited an effective antiangiogenic effect when assayed by using the 10-day-old embryo chick CAM model and effectively inhibits the tube-like structure formation. Furthermore, it potently induced BAECs apoptosis as examined by flow cytometric assays. J. Cell. Biochem. 99: 1517–1523, 2006.

Key words: agkistin-s; disintegrin; apoptosis; BAECs; angiogenesis

Angiogenesis, the process of new capillaries sprouting from existing vessels, is essential for the growth of solid tumors and their metastases [Folkman, 1971; Folkman and Shing, 1992]. Like most embryonic tissues, tumors have the ability to build up their own blood vessel network, including endothelial cells proliferation, migration, invasion, and differentiation [Hanahan and Folkman, 1996]. In mice tumor models, several disintegrins, a family of cysteine-rich polypeptides isolated from snake venoms, effectively inhibit tumor angiogenesis specifically without obvious effects on the normal vasculature and produced potent antitumor effects [Kang et al., 1999; Huang et al., 2001; Markland et al., 2001; Yeh et al., 2001; Golubkov et al., 2003; Trochon-Joseph et al., 2004]. The molecular mechanisms were advocated that tumor angiogenesis was disturbed by disintegrin blocking endothelial cells adhesion to the ECM to induce endothelial cell apoptosis and suppress integrin signaling pathways [Hynes, 1992; Brooks et al., 1994; Sastry and Horwitz, 1996; Hantgan et al., 2004]. It appears that disintegrin inhibitors specifically target the newly formed vasculature in tumors by directly inhibiting endothelial cell proliferation, migration, or differentiation, but not quiescent blood vessels. Antiangiogenesis has become one of the most exciting approaches in the development of cancer drugs. Disintegrins

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may be used to develop the potential therapeutic agents for angiogenesis-related diseases, including cancer [Cao, 2004].

In the former reports, we have cloned a new snake venom metalloproteinase (agkistin) from the venom of *Agkistrodon halys* [Wang et al., 2003]. SVMPs are classified into four groups by their domain structures [Hite et al., 1994]. The sequencing data revealed that agkistin is a new member of P-II class SVMPs, containing a metalloproteinase and a disintegrin domain. Previous study showed agkistin was able to inhibit human platelet aggregation and induce apoptosis of HMECs [Wang et al., 2003]. Here, we investigated the function of agkistin-s, the disintegrin domain of agkistin. It was shown that agkistin-s induce BAECs apoptosis and possess antiangiogenic activity.

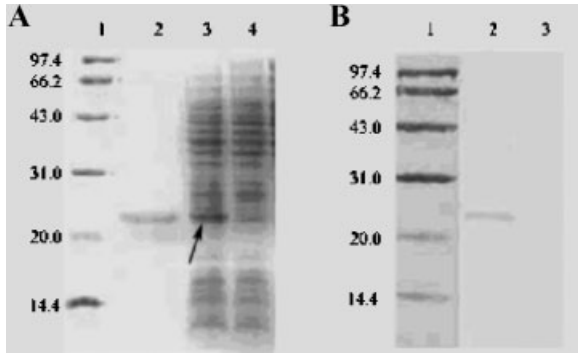

Fig. 1. SDS–PAGE and Western blotting analysis of recombinant agkistin-s. **A**: SDS–PAGE analysis: 1, molecular weight marker; 2, sample of purified recombinant agkistin-s; 3, sample induced with IPTG, 4, sample without induced with IPTG. **B**: Western analysis: 1, molecular weight marker; 2, sample of purified recombinant agkistin-s; 3, sample without induced with IPTG.

MATERIALS AND METHODS

Materials

BAECs were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% FBS, antibiotics (1 μM penicillin, 1 μM streptomycin) at 37°C in 5% CO_2 incubator. Fetal bovine serum was purchased from GIBCO. Prof. Renbao Gan generously provided BAECs.

Expression and Purification of Agkistin-s

The cDNA of Agkistin-s was cloned into pET28a vector and transformed into *Escherichia coli* BL21 (DE3). The cloning of agkistin-s has been described previously [Wang et al., 2003]. The transformant was grown in Luria–Bertani medium at 30°C and harvested 6 h after induced by Isopropyl-β-D-thiogalactopyranosid (IPTG). The harvested cells were broken by ultrasonic and centrifuged at 10,000*g* for 20 min. The supernatant were added to the Ni-iminodiatic acid column, the column was then washed and target protein was eluted according to the protocol provided by Novagen. The collected target protein was dialyzed under PBS (concentrations 1×, 0.5×, 0.1× in turn) and lyophilized, finally analyzed by SDS gel electrophoresis and Western blotting with antipolyhistine monoclonal antibody (Sigma).

BAECs Proliferation Assays

The proliferation assay was performed as described previously [O'Reilly et al., 1994]. BAECs were seeded at 24-well culture plates. When cells formed a confluent monolayer, target protein was added into the well with different concentrations. After 24 h incubation, cells were dispersed in trypsin and counted with a Coulter counter.

Scratch Assay

BAECs were harvested and plated onto gelatin-covered cell culture plates. The scratch was made by a pipette tip, and was approximately 1 mm in width. Detached cells were rinsed away with PBS, and then added medium containing 20% FBS. Agkistin-s was added at different concentrations (0.5, 1, and 1.5 μM)

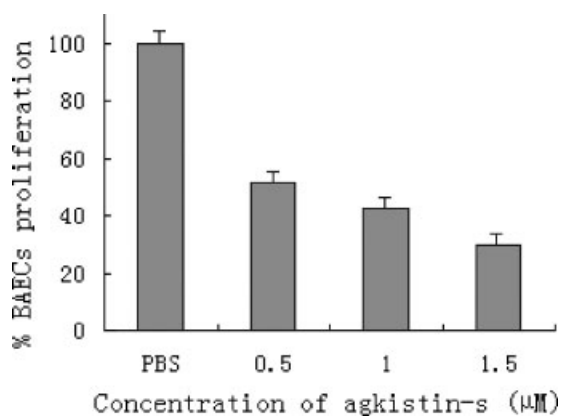

Fig. 2. Inhibition of BAECs proliferation. BAECs were cultured on 24-well plates and agkistin-s was added into the well with different concentrations. After incubation for 24 h, cells were dispersed in trypsin and counted.

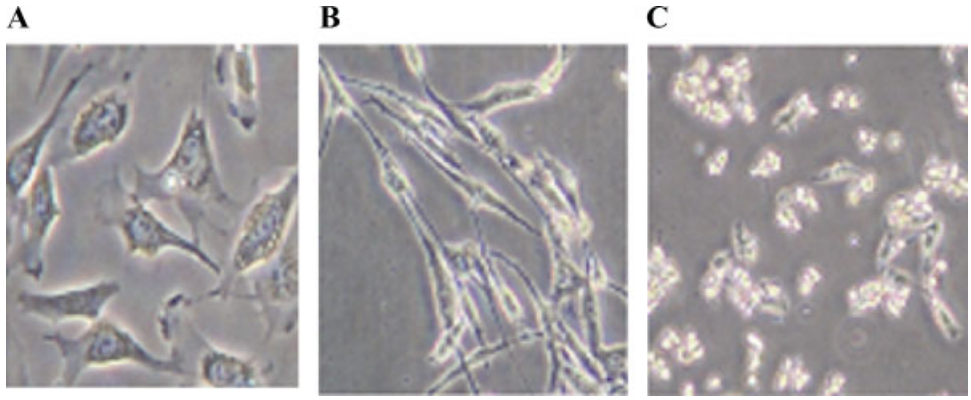

Fig. 3. Agkistin-s effect on BAECs morphology. Morphology of BAECs treated with PBS for 12 h (**A**), BAECs treated with 1 μM for 12 h (**B**), and BAECs treated with 1 μM for 24 h (**C**). (magnification, 200-fold).

immediately after the scratch was made. PBS was added into control plate. Digital image of cells was taken after 24 h of incubation.

Chicken CAM Assay

According to the previously reported method [Brooks et al., 1999], 10-day-old fertilized eggs were carefully cracked, 1 cm^2 filter paper disc containing agkistin-s were implanted on the chorioallantoic membrane (CAM) of individual chicken embryo and then sealed with sterile cellophane type. After 48 h incubation at 37°C with 60% humidity, the CAM was fixed by methanol/acetone (1/1), then observed and photographed under a stereomicroscope.

Tube Formation Assay

BAECs tube formation assay was performed on 24-well plates coated with 350 μl collagen gel per well according to method previously described by Wang [Wang et al., 2005]. Collagen 1 (Sigma) was solubilized in 10 mM acetic acid and stored at 4°C. Collagen gel mixture was prepared by adding 8 volumes of collagen 1 solution, 1 volume of 0.2 N NaOH, 200 mmol/l HEPES, and 1 volume of 10 × DMEM on ice. The mixture gel was added into 24-well plates and polymerized at 37°C for 30 min. Then cells were seeded on gel at a density of 5×10^4 cells/well and incubated at 37°C for 1 h. After adhesion, the medium was removed by aspiration and cells were covered by adding the mixture gel (150 μl/well). Once solid, 0.5 ml fresh medium containing 1% FBS was added to each well. Recombinant protein was added into the medium. Then the gel layer was visualized with a microscope.

Apoptosis Assay

To assess the effect of agkistin-s on apoptosis, BAECs were analyzed by flow cytometric detection of fluorescein isothiocyanate (FITC)-labeled annexin V. Briefly, BAECs were treated with increasing concentrations of agkistin-s during two time periods (24 and 48 h). Then cells were harvested, washed in PBS and

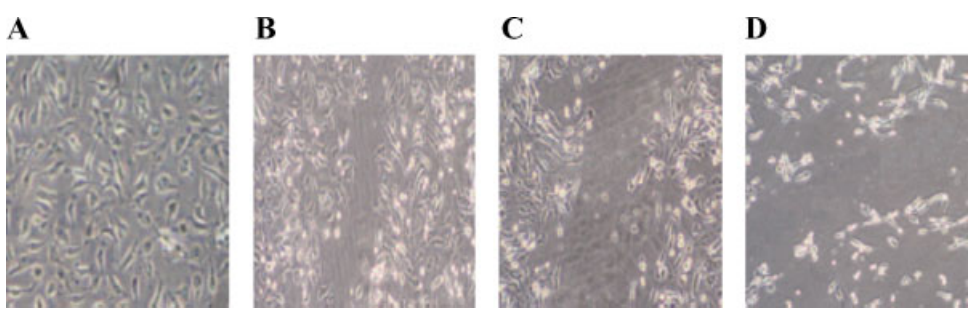

Fig. 4. Agkistin-s effect on BAECs migration. The picture (magnification 100-fold) was taken 24 h later for cells treated with PBS (**A**), cell pretreated with 0.1 μM agkistin-s (**B**), cell pretreated with 0.5 μM agkistin-s (**C**), cell pretreated with 1 μM agkistin-s (**D**).

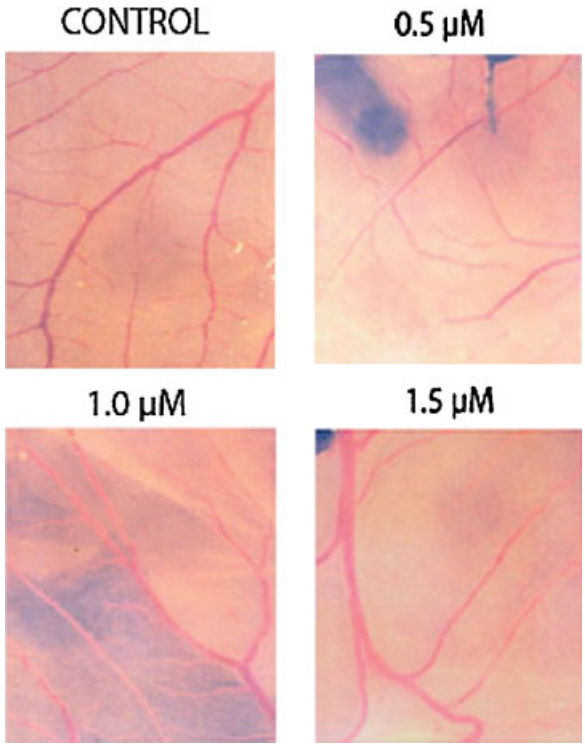

Fig. 5. Effect of Agkistin-s on the chick CAM angiogenesis: After treated as indicated, 10-day-old chick embryos were incubated for 48 h and then photographed with a stereomicroscope.

incubated with 100 μl of Annexin V-FLUOS labeling (Roche) for 15 min at room temperature in the dark and then flow cytometric analysis was performed immediately with FACScan.

BAEC Adhesion Assay

Glass coverslips were coated with gelatin or agkistin-s (0.5, 1.0, 1.5 μM) for 16 h at 4°C. Excess protein was washed off and then incubated for 1 h with 1% heat-denatured bovine serum albumin to block remaining protein-binding sites. BAECs were suspended in DMEM supplemented with 20% FBS and plated onto coverslips for 12 h. Unattached cells were removed by washing with PBS. Cell densities were determined every 12 h with a Coulter counter.

RESULTS

Expression and Purification of Agkistin-s

Recombinant agkistin-s protein was rapidly purified to near homogeneity by a simple one-step Ni^{2+}-affinity purification procedure. The SDS–PAGE and Western blotting results proved the expression of agkistin-s in the *E. coli* (Fig. 1).

Inhibition of BAECs Proliferation

To examine the effect of agkistin-s on angiogenesis, we used BAECs proliferation assay system that was developed for the study of tumor angiogenesis [O'Reilly et al., 1994]. As shown in Figure 2, agkistin-s was able to inhibit the proliferation of BAECs in a dose-dependent manner. One-half inhibition of BAECs proliferation was observed with agkistin-s concentration of 0.5–1.5 μM. In addition, it was found that inhibitory effect enhanced as the incubation time was increased. This effect was also correlated with a change in cell morphology. Compared to normal cells (Fig. 3A), cells treated with agkistin-s for 12 h became fibroblast-like morphology (Fig. 3B) and after 24 h, cells began to shrink into round and detached from the matrix (Fig. 3C).

Inhibition of BAECs Migration

In this experiment, we studied the migration of confluent BAECs grown on gelatin into a scratch area. Complete coverage of the scratch area by PBS-treated BAECs (Fig. 4A) was

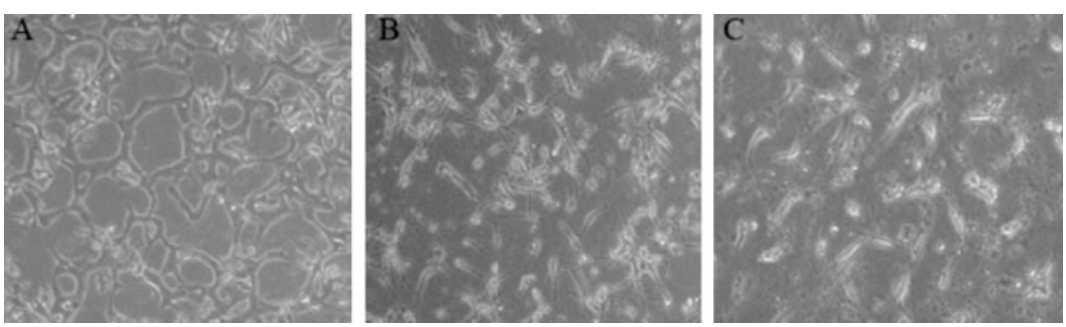

Fig. 6. Tube formation in collagen gel. Representative images captured after incubation for 2 and 4 days. (**A**) On culture day 4, tube-like structures appeared in control cell. (**B**) Cells treated with 0.5 μM agkistin-s for 2 days. (**C**) Cells treated with 0.5 μM agkistin-s for 4 days.

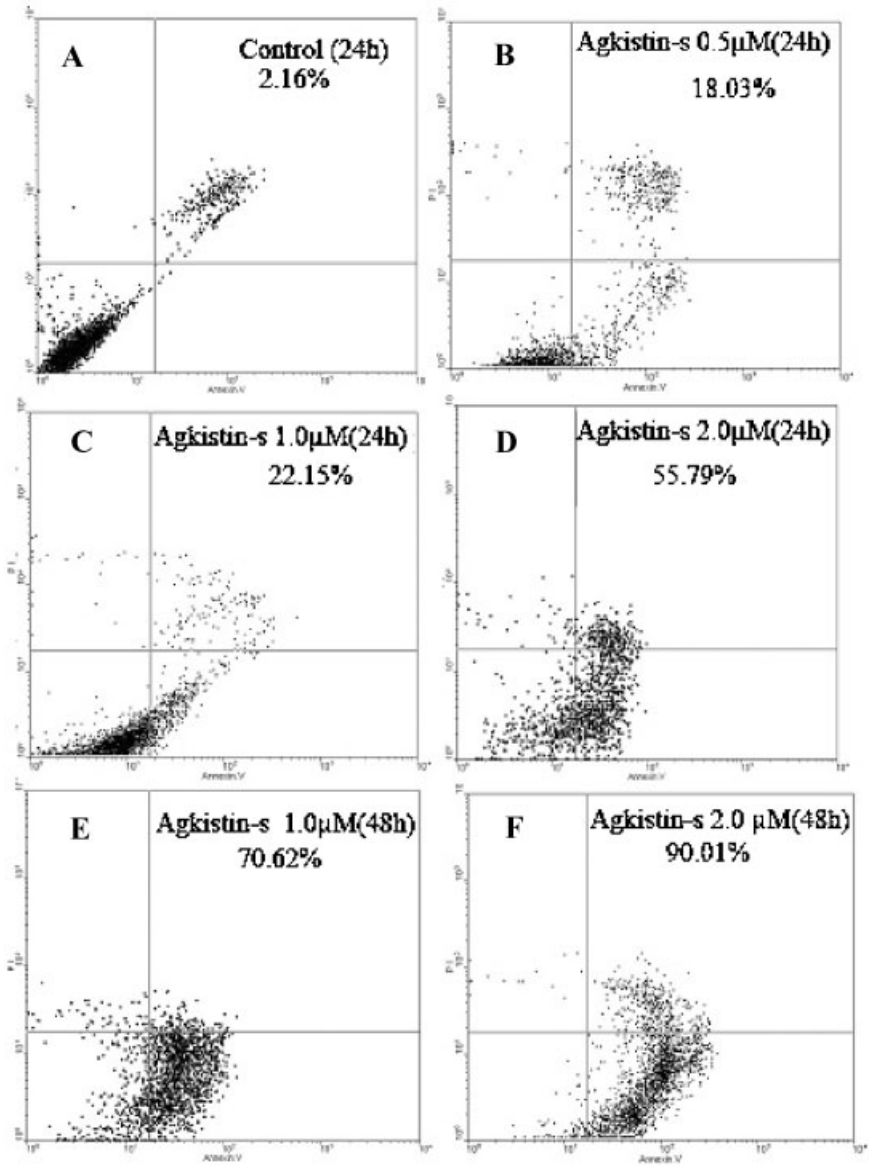

Fig. 7. Agkistin-s induces apoptosis in BAECs. BAECs were cultured in 60 mm dishes and incubated with PBS, agkistin-s, for 24: (**A**) control, 24 h; (**B**) 0.5 μm agkistin-s, 24 h; (**C**) 1.0 μm agkistin-s, 24 h; (**D**) 2.0 μm agkistin-s, 24 h; (**E**) 1.0 μm agkistin-s, 48 h; (**F**) 2.0 μm agkistin-s, 48 h. Then cell were stained with annexin V and PI and analyzed by FACS. **Bottom left quadrant**, PI^- and Annexin V^- (viable) cells; **bottom right quadrant**, PI^- and Annexin V^+ (early apoptotic) cells; **top right quadrant**, PI^+ and Annexin V^+ (late apoptotic and necrotic) cells.

observed after 24 h incubation, while a scratch on a plate of agkistin-s-pretreated BAECs remained unfilled and the inhibitory effect was enhanced in dose-dependent manner (Fig. 4B,C,D).

In Vivo Angiogenesis Assays

The chick embryo CAM assay is one of the most common in vivo models used to study the role of cell adhesion molecule in angiogenesis [Bischoff, 1997; Brooks et al., 1999]. To assess the effect of agkistin-s on angiogenesis we observed whether agkistin-s is capable of interrupting the angiogenesis in CAM. Upon dissection of the CAM of 10-day-old chick embryo, the spontaneous angiogenesis was markedly reduced in the presence of agkistin-s. However, a control PBS showed little effect on the new vessel formation (Fig. 5). After its topical application for 48 h, agkistin-s inhibited the spontaneous angiogenesis in a dose-dependent manner. It is also noteworthy to observe that agkistin significantly inhibited angiogenesis without affecting preexisting blood vessels.

Inhibitory Effect of Agkistin-s on Tube Formation by BAECs

To examine the effects of agkistin-s on tube formation, we performed tube formation assay in vitro model in which BAECs are cultured in sandwich collagen gels. Control cells exhibited tube-like structure on day 4, as evidenced by morphological changes (Fig. 6A). By contrast, treatment with agkistin-s strongly prevented tube formation, as shown in Figure 6B,C. In a concentration study, agkistin-s reduced tube formation in a dose-dependent manner.

BAECs Apoptosis

Apoptosis was evaluated after 24 h treatment of agkistin-s. While in such conditions the percentage of apoptotic cells increased in BAECs. These data suggest that the apoptotic effects of agkistin-s are prominent on endothelial cell lines. As showed in Figure 7, control was in a typical cell cycle stage, however, (0.5–2.0 μM) agkistin-s-pretreated BAECs apoptosis in a dose-and time-dependent manner.

Agkistin-s is not Cytotoxic to BAECs In Vitro

To determine whether agkistin-s induces apoptosis in BAECs by a direct cytotoxic effect, we cultured BAECs on agkistin-s-coated glass coverslips and compared them to cells grown on gelatin-coated coverslips. The result showed after 12 h BAECs seeded onto agkistin-s-coated coverslips proliferated normally, however, cells became little fibroblast-like morphology (Fig. 8B), compared to seed on a gelatin coated (Fig. 8A). When changing with new complete DMED medium, the cells seeded on agkistin-s-coated coverslips turn normal morphology. It may because the residual agkistin-s in coverslips dissolved into medium and effected on the cells. The growth curve (Fig. 9) indicates that after changing the medium that cells in the presence of agkistin-s proliferated equally as well as in the presence of gelatin. This result indicates that agkistin-s itself is not cytotoxic, and BAECs can proliferate by using agkistin-s as an ECM protein.

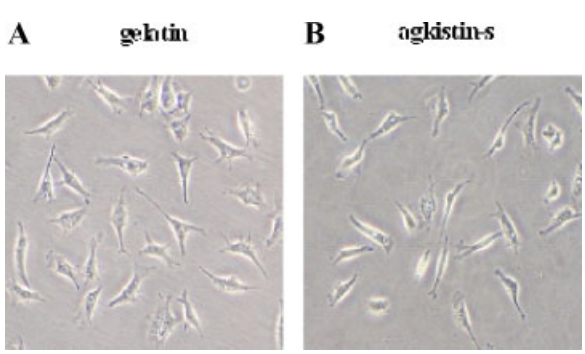

Fig. 8. BAECs proliferated on agkistin-s-coated glass coverslips. BAECs were seeded onto glass coverslips coated with either gelatin (**A**) or agkistin-s (**B**) and incubated in DMEM for 6 h. Cell attachment and proliferation on each coverslips were observed by microscopy.

DISCUSSION

This report provides evidence that agkistin-s effectively prevents BAECs proliferation, migration, and induces BAECs apoptosis in a dose- and time-dependent manner. The anti-proliferating effect of agkistin-s on BAECs is related to the induction of apoptosis as evidenced by flow cytometric analysis. BAECs seeded onto agkistin-s-coated coverslips proliferated normally, as compared to proliferation on gelatin-coated plate, suggesting that agkistin-s is not cytotoxic, which is consistent with the previous observations that disintegrin (i.e., salmosin) induce an apoptotic effect toward BAECs [Kang et al., 1999]. Recent reports demonstrated that most of disintegrins containing RGD sequence induce endothelial cell apoptosis by blocking the function of integrin $\alpha_v\beta_3$, $\alpha_5\beta_1$, or $\alpha_{2b}\beta$ [McGill et al., 1997; Brassard et al., 1999; Yeh et al., 2001]. Agkistin-s prevents proliferation and induced apoptosis might by competing for integrin binding sites on

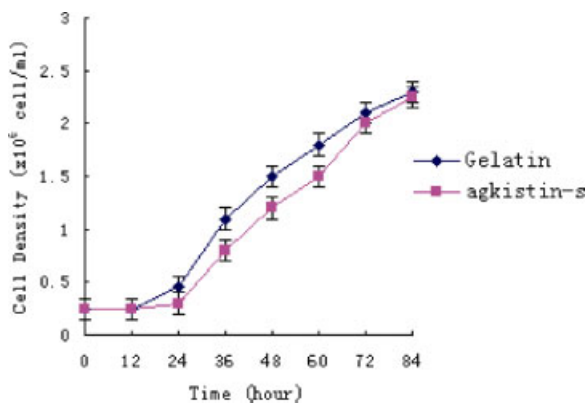

Fig. 9. Effect of agkistin-s on proliferation of BAECs. Coverslips coated with gelatin or agkistin-s (1.5 μM) was seeded with BAECs (0.25×106/ml) in complete DMEM medium. Cell density was determined every 12 h. Growth curves of BAECs on coverslip coated with gelatin and with agkistin-s are illustrated. Each point is expressed as mean (cells/ml) from duplicates.

ECM, which destroys focal contacts and causes cellular detachment from the matrix.

Agkistin-s is an effective inhibitor of angiogenesis in vivo (Fig. 5). Upon dissection of the CAM of 10-day-old chick embryo, the spontaneous angiogenesis was markedly reduced in the presence of agkistin-s in a dose-dependent manner. The result showed that agkistin-s significantly inhibited angiogenesis without affecting preexisting blood vessels. In addition, agkistin-s was observed to inhibit tube-like structure formation. Our data suggest the mechanism of agkistin-s in suppressing angiogenesis appear to be related to induce endothelial cells apoptosis.

In conclusion, the study shows that agkistin-s effectively induce BAECs apoptosis and inhibit angiogenesis. We also found that disintegrin primarily affects the newly forming vessels but not the preexisting large vessels. This study provides new insights into the biologic function of agkistin-s as an angiogenesis inhibitor. Further investigations will provide useful information in understanding the functional mechanism and biological significance of agkistin-s.

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