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Biochemical and Biophysical Research Communications 310 (2003) 153–157
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Angiogenic activity of osteopontin-derived peptide SVVYGLR

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Received 28 August 2003

Abstract

Angiogenesis plays an important role in various pathological conditions as well as some physiological processes. Although a number of soluble angiogenic factors have been reported, extracellular matrix also has crucial effect on angiogenesis through interaction with endothelial cells. Since recent reports showed osteopontin had some angiogenic activity, the effect of the SVVYGLR peptide, novel binding motif in osteopontin molecule, on angiogenesis was examined in this study. Synthetic peptide SVVYGLR did not have proliferative effect on endothelial cells but adhesion and migration activity to endothelial cells. Furthermore, SVVYGLR had as potent activity for tube formation in three-dimensional collagen gel as vascular endothelial growth factor which is known to be the strongest angiogenic factor. Electron microscopical analysis showed a number of microvilli on the endothelial luminar surface and tight junction formation in the luminar intercellular border between endothelial cells, indicating SVVYGLR induced cell porarity and differentiation of endothelial cells. This small peptide might be expected to stimulate angiogenesis to improve some ischemic conditions in the future because of some advantages due to smaller molecular weight.

Keywords: Angiogenesis; Differentiation; Endothelial cells; Osteopontin; SVVYGLR; Tube formation

The establishment of vascular supply is fundamental for organ development and differentiation during embryogenesis as well as for wound repair and reproductive function in the adult [1,2]. The development of new blood vessels, a process known as angiogenesis, also plays an important role in a variety of pathologic processes, including proliferative retinopathies, rheumatoid arthritis, psoriasis, and cancer [3–5]. In the process of angiogenesis, endothelial cells digest the basement membranes of blood vessels, migrate, proliferate, and form tube-like structures. Subsequently the basement membranes of newly formed vessels are surrounded by pericytes [6]. Many studies have shown that these cellular responses are tightly regulated by signals from various growth factors and cytokines including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and interleukin 8 [7–18]. Other studies have reported that extracellular matrix plays crucial roles on the regulation of each process [19]. Developing microvessels during angiogenesis produce heterogeneous extracellular matrix composed of different collagen types, glycoproteins, and proteoglycans. Osteopontin, one of the extracellular matrix proteins, is a phosphoric acid protein containing a large quantity of sialic acid and is distributed widely in bone tissues, kidneys, placentae, ovaries, brains, skins, and so on [20]. Osteopontin has been considered multifunctional; osteopontin participates in bone metabolism, mediates inflammatory responses, and is related to malignant transformation and cancer progression [21–24]. Recently there have been some reports on the importance of osteopontin in angiogenesis [25–27]. Although the

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0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.09.001

Arg–Gly–Asp (RGD) motif in the central portion of OPN was supposed to be important for the functions of osteopontin, novel binding sequence Ser–Val–Val–Tyr–Gly–Leu–Arg (SVVYGLR) was found [28] and might be important in the pathological conditions as SVVYGLR, adjacent to the RGD sequence in an osteopontin molecule, was exposed by thrombin cleavage.

In this study, we demonstrate SVVYGLR functions as potent angiogenic factor as VEGF.

Materials and methods

Synthesis and characterization of the peptide SVVYGLR. The peptide SVVYGLR synthesized with a high efficiency solid-phase method by using an automatic peptide synthesizer (PSSM-8, Shimadzu, Kyoto, Japan) was a single ingredient and matched to a theoretical mass value by high performance liquid chromatography–mass spectrometry (HPLC/MS, Shimadzu, Kyoto, Japan).

The peptide was immobilized on the polyethylene glycol (PEG) resin and its function was tested with an adhesion assay. The ultrastructural analysis of the endothelial cell adhesion was examined in a Hitachi S-800 scanning electron micrographs (SEM).

Cell culture. Transformed rat lung endothelial cells (TRLECs) were kindly supplied by Dr. Tsurufuji (The Institute of Cytosignal Research, Tokyo, Japan) [29] and were used as endothelial cells in this experiment. TRLECs were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin–streptomycin 1000 IU/ml and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Endothelial cell proliferation assay. The effect of SVVYGLR on endothelial cell proliferation was assessed by the WST-1 assay (Dojindo Laboratories, Kumamoto, Japan) [30] based on the cleavage of tetrazolium salt WST-1 by mitochondrogenase in viable cells. TRLECs were seeded in 96-well plates coated by SVVYGLR (2, 0.2, and 0.02 μg/ml) at 37 °C for 2 h. The number of TRLECs was evaluated after 1, 2, or 3 days as the following: 10 μl of the working solution containing WST-1 was added to each well; then the plate was reincubated for 3 h in the 5% CO_2 incubator. The absorbance of each well was measured at 475 nm, with the reference wavelength at 630 nm by MTP-32 microplate reader (CORONA Electric, Ibaraki, Japan).

Endothelial cell adhesion assay. A 96-well plate was coated by SVVYGLR (2, 0.2, and 0.02 μg/ml) at 37 °C for 2 h. TRLECs were incubated on the plate in serum-free DMEM in the 5% CO_2 incubator for 1 h. After washing several times, adherent cells on the plate were stained with 0.04% crystal violet and the absorbance was measured at 550 nm with a reference wavelength at 650 nm by MTP-32 microplate reader.

Endothelial cell migration assay. The effect of SVVYGLR on endothelial cell migratory activity was examined by wound assay. The petri dish was coated by SVVYGLR (2, 0.2, and 0.02 μg/ml) at 37 °C for 2 h and TRLECs were seeded on the dish and were allowed to grow to confluence. Then the complete medium used for the cells was replaced with serum-free DMEM. One linear scar was drawn in the monolayer. A set of digital photographs was taken at the time of scarring and the denuded area was marked using NIH image analysis software. The dishes were washed and fresh serum-free medium containing 0.1% BSA and 0.01 μg/ml VEGF was added. After 3 h, the second set of photographs was taken. Photographs were superposed on the first photograph set to measure the migration of the cells. The wound healing area was measured with pixel units. Each condition was tested in duplicate in two independent experiments.

Tube formation assay. Tube formation was evaluated by three-dimensional collagen gel assay. Suspension of TRLECs in collagen gel was placed as the middle layer between collagen layer as the bottom layer and culture medium layer with SVVYGLR (0.02 μg/ml) or VEGF (0.01 μg/ml) as the top layer.

The tube formation ability of TRLECs at the middle layer was observed for 14 days by a light microscope (Nikon Diaphot 200). At day 14, the sample was fixed by immersion for 16 h at 24 °C in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). After three washes in phosphate buffer, the samples were postfixed for 1 h in 1% osmium tetroxide in phosphate buffer at 4 °C, dehydrated through ascending grades of alcohol, and embedded in Epon. Semithin sections were stained with 0.1% toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7100 transmission electron microscope.

Statistical analysis. Quantitative tests were carried out in quadruplicate and mean values with standard deviations were calculated. Statistical analysis of data was accomplished by one-factor analysis of variance. Scheffe's F test was used for comparison at the 95% confidence interval.

Results

Synthesis and binding activity of SVVYGLR

SVVYGLR is a small peptide which consisted of 7 residues of amino acid, and such a low molecular weight peptide can be synthesized with high purity by high efficiency solid-phase methods on the Fmoc resin. The peptide (purity 99%<) was synthesized by performing separation refining by HPLC strictly and used in this study (data not shown). Since this peptide should have a binding ability to endothelial cells for various functions, cell adhesion assay was tested. TRLECs were adhered well and spread in good shape on SVVYGLR immobilized on PEG resin while very few cells were bound in the control group (PEG resin) (Fig. 1).

Effects of SVVYGLR on endothelial cell proliferation, adhesion, and migration

Since endothelial cells were bound to SVVYGLR, the effects of SVVYGLR on proliferation, adhesion, and migration were examined. In WST assay, we found that SVVYGLR had little or no effect on cell proliferation for TRLECs (Fig. 2A). In cell adhesion assay, we found that TRLECs were adhered well on SVVYGLR, but there was no difference in the ability of adhesion between different peptide concentrations (Fig. 2B). In wound assay, cell migratory activity of SVVYGLR increased dose dependently (Fig. 2C). Thus, we found that SVVYGLR peptide did not influence the endothelial cell ability of proliferation but could be adhered to endothelial cells sufficiently and also potentiate migratory activity.

Effects of SVVYGLR on tube formation of endothelial cell

The ability of tube formation was evaluated by three-dimensional collagen assay, in which endothelial cells

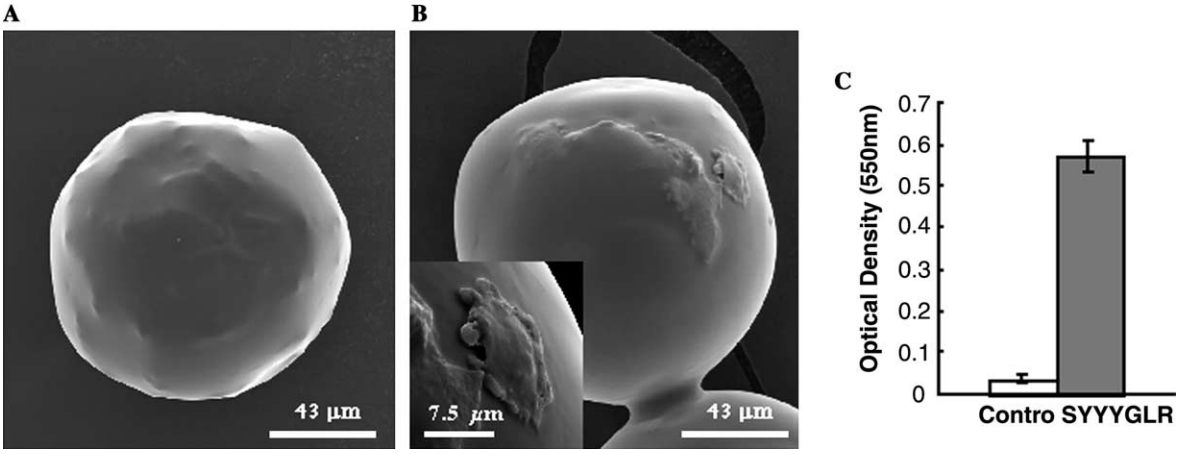

Fig. 1. Binding activity of the synthetic peptide SVVYGLR to endothelial cells. (A) Scanning electron micrographs of TRLECs on the PEG resin particles (control). (B) Scanning electron micrographs of TRLECs on the PEG resin particles coated with SVVYGLR peptide. (C) Endothelial cell adhesion assay on PEG resin with or without SVVYGLR. TRLECs were adhered and spread in good shape on SVVYGLR-immobilized PEG resin (B and C) while very few cells were adhered in the control group (A).

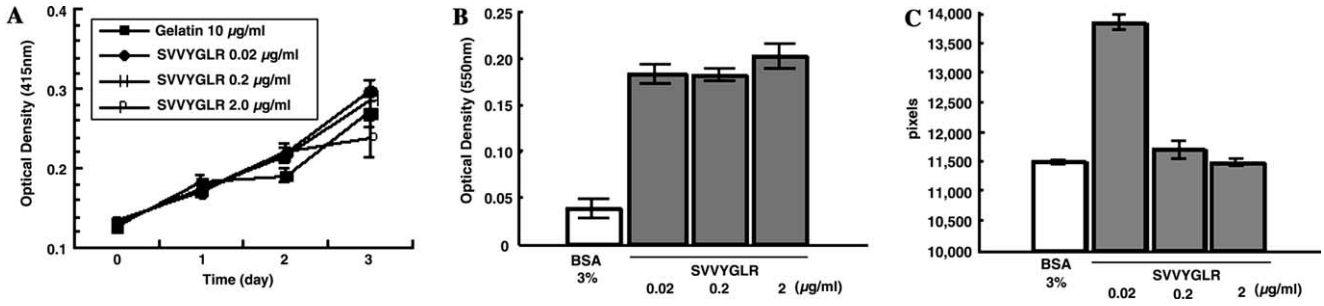

Fig. 2. (A) Effect of SVVYGLR on endothelial cell proliferation: TRLECs were inoculated on a plate coated with SVVYGLR. The number of cells was counted at days 1, 2, and 3 after inoculation. No effects of cell attachment to SVVYGLR were observed on proliferation ability. (B) Endothelial cell adhesion to SVVYGLR: TRLECs were inoculated on a plate coated with SVVYGLR. Adhered cells were counted 1 h after inoculation. TRLECs were adhered well to any concentrations of peptide. (C) Effect of SVVYGLR on endothelial cell migration (wound assay): one linear line was drawn to exfoliate a part of confluent TRLECs on a petri dish coated with SVVYGLR. The area without TRLECs which was diminished by cell migration was measured after 3 h. TRLEC stimulated cell migratory activity in a dose dependent manner.

could be differentiated to acquire polarity and make tube-like structure. We compared the effect of SVVYGLR with that of a known angiogenic factor, VEGF. As a result, both VEGF and SVVYGLR made tube formation after 1 or 2 weeks, while no tube-like structure was not observed in the control group (collagen gel only) (Fig. 3A). In electron microscopical examination a few endothelial cells were arranged to make tube-like structure, and furthermore a number of microvilli were observed on the luminar surface and tight junctions were detected at the luminar intercellular junctions of the endothelial cells (Fig. 3B). These findings implied the polarity of endothelial cells, thus demonstrating both VEGF and SVVYGLR made endothelial cells differentiate appropriately. VEGF has been known to be the strongest angiogenic factor, yet our data show small peptide SVVYGLR is as potent as VEGF at least in vitro angiogenesis assay.

Discussion

Most cells are surrounded by the extracellular matrix and exist in interaction with matrix proteins. Cells are not only bound to matrix proteins but also the interaction with matrix proteins influences various cell functions including cell differentiation, migration, and proliferation [19]. There have been numerous reports that matrix-derived synthetic peptides, corresponding to active sites in proteins, demonstrate biological activity. So far RGD peptide has been investigated most frequently since RGD sequence is contained in many extracellular matrix proteins such as fibronectin, collagen, and laminin [31].

Angiogenesis plays a key role in a variety of physiologic and pathologic conditions [32]. Therefore regulation of angiogenesis is expected to cure or improve a number of diseases. Angiogenesis inhibitors will be promising therapy of cancer or other angiogenic diseases

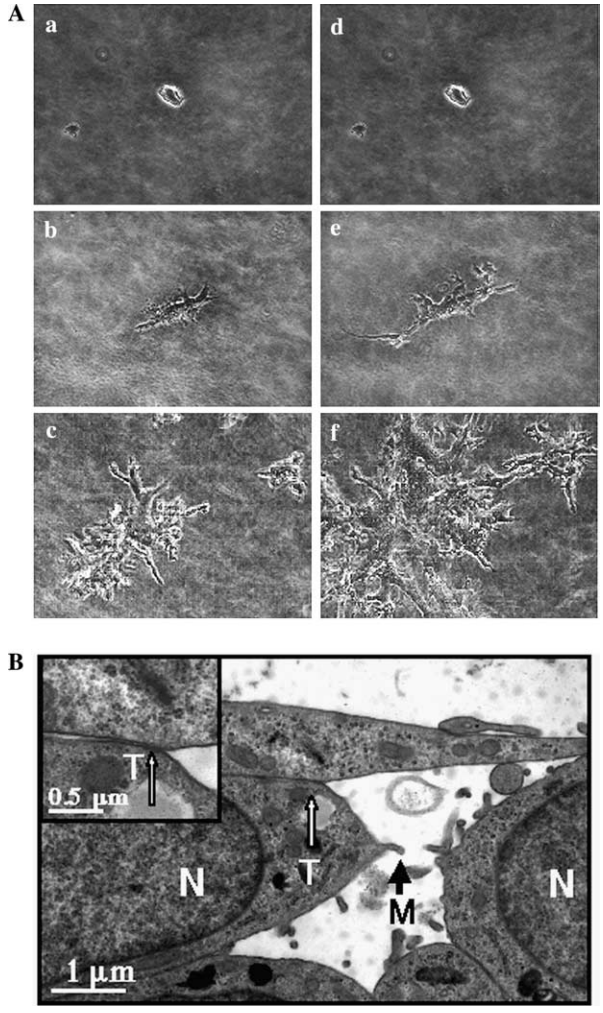

Fig. 3. (A) Effect of SVVYGLR on tube formation in collagen gel: TRLECs in collagen gel were placed as the middle layer between collagen layer as the bottom layer and culture medium layer with SVVYGLR (0.02 μg/ml) or VEGF (0.01 μg/ml) as the top layer. The tube formation ability was observed for 14 days with light microscope. While no tube-like structure was found in the control group (collagen gel only) after 14 days at all, both SVVYGLR and VEGF induced tube formation in the same after seven days. (a–c) Seven days after treatment. (d–f) Fourteen days after treatment. (a and d) Gelatin only. (b and e) VEGF (0.01 μg/ml). (c and f) SVVYGLR (0.02 μg/ml). (B) Electron microscopical examination (700×, 1500×); ultrastructural analysis was performed on the fourteenth day by transmission electron microscope. The large amount of microvilli (M) was observed on luminar surface of the tubes and tight junctions (T) were detected in luminar intercellular junction between two endothelial cells. T, tight junctions; M, microvilli; and N, nucleus.

such as diabetic retinopathy. On the other hand gene therapy for stimulating angiogenesis would be expected to heal ischemic diseases such as myocardial infarction. Several clinical trials have been tested using VEGF, hepatocyte growth factor (HGF) or FGF. Angiogenesis is composed of multiple steps such as basement membrane degradation, endothelial cell migration, proliferation, tube formation, and blood vessel maturation [6]. As interaction with extracellular matrix proteins is considered in various steps, functional peptide in matrix protein might influence angiogenesis. Since osteopontin was reported to have ability to induce angiogenesis recently, the effect of novel binding motif SVVYGLR in osteopontin has been examined.

In the present study, SVVYGLR did not have activity for endothelial cell proliferation but it had the binding capacity and stimulated migration of endothelial cells. Furthermore, SVVYGLR induced tube formation in three-dimensional collagen gel as potently as known angiogenic factor VEGF. Since tube formation implies endothelial cell polarity and differentiation, the importance of the interaction of endothelial cells with functional domain of extracellular matrix proteins is appreciated. Although activities of RGD containing peptides and laminin functional peptides, YIGSR and IKVAV [33], have been reported, SVVYGLR is supposed to have much stronger activity for angiogenesis as compared with the activity of VEGF.

When small peptides are used for medical treatment, they have several advantages of fast metabolism, low risk of immune response, and lower cost because of low molecular weight. Furthermore, it also may be possible to produce additional functional peptide using combinatorial chemical library for amino acid derivatives.

In conclusion osteopontin-derived synthetic peptide SVVYGLR does not only have binding and migratory activity for endothelial cells but also stimulate tube formation in three-dimensional collagen gel as potently as VEGF. Further investigation will be required to elucidate the mechanism of SVVYGLR to endothelial cell function.

Acknowledgments

The authors thank the members of the Department of Molecular Pathology, School of Medicine and Allied Health Sciences, Faculty of Medicine, Osaka University, and Division of Oromaxillofacial Regeneration, Osaka University Graduate School of Dentistry, and Department of Biomaterials Science, and Hipep Laboratories Inc., and Hiroshima University Faculty of Dentistry, and Department of Materials Science and Engineering, Osaka University Graduate School of Engineering for their kind advice and help.

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