Structure–activity relationships of the human prothrombin kringle-2 peptide derivative NSA9: anti-proliferative activity and cellular internalization

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The human prothrombin kringle-2 protein inhibits angiogenesis and LLC (Lewis lung carcinoma) growth and metastasis in mice. Additionally, the NSA9 peptide (NSAVQLVEN) derived from human prothrombin kringle-2 has been reported to inhibit the proliferation of BCE (bovine capillary endothelial) cells and CAM (chorioallantoic membrane) angiogenesis. In the present study, we examined the structure–activity relationships of the NSA9 peptide in inhibiting the proliferation of endothelial cells lines e.g. BCE and HUVE (human umbilical vein endothelial). N- or C-terminal truncated derivatives and reverse sequence analogues of NSA9 were prepared and their anti-proliferative activities were assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay. This cell proliferation assay demonstrated that both the N-terminal region and sequence orientation of NSA9 are important for inhibiting the proliferation of endothelial cells. In particular 2 C-terminal truncation derivatives of NSA9 [NSA7 (NSAVQLV) and NSA8

(NSAVQLVE)] inhibited cellular proliferation to a greater extent than did NSA9. The heptapeptide NSA7, was found to be more potent than NSA9 in inhibiting CAM angiogenesis, and tubular formation and migration of HUVE cells. In addition NSA9, NSA8 and NSA7 peptides exhibited considerable inhibitory effects on the proliferation of tumour cells such as B16F10 (murine melanoma), LLC and L929 (murine fibroblast). Also, cellular internalization studies demonstrated that NSA7 was internalized into both endothelial and tumour cells more easily than was NSA9. In conclusion, these results suggest that NSA7, residing within the full sequence of NSA9, contains the required sequence for anti-proliferative activity and cellular internalization.

Key words: anti-angiogenesis, derivative, endothelial cell proliferation, flow cytometry, NSA9, structure–activity relationship (SAR).

INTRODUCTION

Angiogenesis is the process of forming new blood vessels from pre-existing capillaries during various physiological and pathological processes, including embryonic development, menstruation, wound-healing, arthritis, diabetic retinopathy, inflammation and tumour growth [1]. Angiogenesis is tightly regulated by a balance of angiogenic factors and inhibitors [2,3]. Numerous angiogenic inhibitors such as angiostatin, TSP-1 (thrombospondin-1), endostatin, platelet Factor 4 and prothrombin fragment have been reported [4–9]. These protein-based, anti-angiogenic molecules reportedly inhibit proliferation, migration and adhesion of endothelial cells. Several drugs, such as endostatin, angiostatin, interferon- α 2a, and Vitaxin (an anti-integrin $\alpha_v\beta_3$ antibody) have already entered clinical trials for the treatment of tumour metastasis [10,11]. However, there are several problems concerning the therapeutic use of these anti-angiogenic protein drugs, such as metabolic instability, immunoreactivity, limited tissue distribution, and appropriate routes of administration due to their large molecular size. On the other hand, protein-derived peptides are much smaller in size and have less immunogenicity than protein-based drugs. Recently, many peptides have been developed as therapeutic agents [10–12].

To produce anti-angiogenic peptide drugs that have a lower therapeutic dose, greater efficacy and a lower molecular mass than their parent protein- or peptide-based drug, their SARs (structure–activity relationships e.g. functional key amino acid residues, chirality and sequence length) need to be investigated [13–15].

Mayo et al. reported that anginex, a 33-mer synthetic peptide, inhibits angiogenesis and tumour growth in mice [16–18]. One of the anginex mimetics, which was designed using information from anginex SARs, was found to be more potent than the anginex parent-peptide as an inhibitor of angiogenesis [19]. An arginine-rich ES-2 peptide (IVRRADRAAVP) derived from the N-terminus of endostatin interacts with β 1 integrin and heparin, and inhibits bFGF (basic fibroblast growth factor)-induced directional migration and tubular morphogenesis of microvascular endothelial cells [20]. In addition, several other groups have reported that endostatin-derived peptides (e.g. synthetic partial fragments of endostatin, N-/C-terminal-deleted derivatives of human endostatin, and a 27-mer peptide derived from the N-terminus of endostatin) have anti-angiogenic effects [21–25]. Small peptides from TSP-1, which is a naturally occurring inhibitor of angiogenesis blocked proliferation, tubular formation and migration of endothelial cells [26–28]. Additionally T3, T7, PTHrP_{1–10} and Flt2-11 peptides from tumstatin, parathyroid hormone-related peptide and vascular endothelial growth factor receptor respectively have been reported to inhibit angiogenesis [29–31].

Previously, we reported that recombinant human prothrombin kringle-1, -2 and -1-2 all have potent anti-angiogenic activities. They inhibit CAM (chorioallantoic membrane) angiogenesis, LLC (Lewis lung carcinoma) tumour growth and metastasis in mice [32]. We have previously constructed an overlapping synthetic peptide library that represents the entire sequence of human prothrombin kringle-2. Subsequent work demonstrated that the

Abbreviations used: BCE, bovine capillary endothelial; bFGF, basic fibroblast growth factor; CAM, chorioallantoic membrane; FBS, foetal bovine serum; HUVE, human umbilical vein endothelial; LLC, Lewis lung carcinoma; SAR, structure–activity relationship; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; TSP-1, thrombospondin-1.

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nonapeptide NSA9 (NSAVQLVEN), strongly inhibited the proliferation of BCE (bovine capillary endothelial) cells and CAM angiogenesis [33]. In the present study, we synthesized various derivatives of the NSA9 peptide derived from human prothrombin kringle-2 by truncation of amino acid residues from either the N- or C-terminus of NSA9, and the design of reverse sequence peptides. We investigated the primary sequence of NSA9 that is essential for its anti-proliferative activity against endothelial cells. Cellular internalization of these peptides was also studied by flow cytometry. The results demonstrated that one of the NSA9 derivatives, NSA7, was able to inhibit CAM angiogenesis, tube formation and cell migration more effectively than the original parent peptide.

EXPERIMENTAL

Peptide synthesis

The peptides used in this study were synthesized by solid-phase techniques based on a Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry method using p-methyl-benzhydrylamine resin and L-amino acids protected with Fmoc [34]. Removal of Fmoc was carried out using 20% piperidine in NMP (*N,N*-methylpyrrolidone). Coupling was achieved with DCC (*N,N*-dicyclohexylcarbodiimide) and racemization was prevented using HOBt (1-hydroxybenzotriazole). The reaction was repeated until the desired peptide sequence was achieved. FITC-labelled peptides were synthesized by coupling FITC to the N-termini of peptides while they were still attached to the resin. Peptides were cleaved from the resin in the presence of trifluoroacetic acid (94%), triisopropyl silane (3%) and octanedithiol (3%) for 2 h and purified by HPLC on a C18 column. Peptides were resuspended in DMSO and stored frozen at -20°C until further use.

Cell culture

HUVE (human umbilical vein endothelial) cells were purchased from Clonetics and maintained in Medium 199, supplemented with 20% FBS (foetal bovine serum) (Gibco BRL, NY, U.S.A.), 1% antibiotic-antimycotic, 2.2 g/l sodium bicarbonate, 2 mM glutamine and 5 units/ml heparin at 37°C in a humidified atmosphere of 5% CO_2 . BCE, LLC, murine fibroblast L929 and murine melanoma B16F10 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated FBS at 37°C in a humidified atmosphere of 10% CO_2 . HUVE and BCE cells were stimulated by the addition of bFGF, 3 ng/ml (R & D Systems, MN, U.S.A.).

Cell proliferation assay

Assessment of cell proliferation was performed according to the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay protocol [35]. Tumour cells were added to a 96-well plate at a density of 5000 cells/well and allowed to adhere overnight. Cells were treated with NSA9-derived peptides (1 μM) and incubated for 48 h. By contrast, BCE and HUVE cells were seeded on to a gelatin-coated, 96-well plate at a density of 5000 cells/well and allowed to adhere overnight. Cells were treated with NSA9-derived peptides (1 μM) for 30 min before stimulation with bFGF (3 ng/ml) and incubation for 48 h. Then, 50 μl of 2 mg/ml MTT solution was added to each well, and cells were incubated for 4 h at 37°C . After the supernatants were discarded, formazan crystals were dissolved in 100 μl of DMSO. Absorbance was measured at 570 nm on an ELISA reader.

CAM assay

The CAM assay was performed as previously described [17,30]. Fertilized chicken eggs (Pulmuone, Korea) were incubated for 3 days with the blunt pole uppermost, at 37°C in a humidified atmosphere. On day 4, a window (1 cm $\times$ 1 cm) was made in the eggshell, and egg albumin (3–4 ml) was removed. On day 6, a Thermanox coverslip (NUNC, Roskilde, Denmark), saturated with peptides or PBS, was applied to the top of the CAM of an individual embryo. Subsequently, the window was covered with tape to prevent dehydration and the embryos were incubated for another 72 h, at 37°C with humidity for the development of spontaneous angiogenesis. Finally, a 20% fat emulsion (Green cross, Korea) was added to the egg and the CAMs were photographed.

Tube formation assay

The tube formation assay was performed according to the procedures previously described [36]. Matrigel (BD Biosciences, CA, U.S.A.) was added to each well of a 96-well plate (50 μl /well) and allowed to polymerize. HUVE cells were seeded in each well at a density of 10000 cells/well with or without each peptide (5 μM). After 30 min, cells were stimulated with 10 ng/ml bFGF and incubated for 18 h at 37°C . Subsequently, cells were rinsed twice with PBS and tube formation was assessed using an inverted phase-contrast microscope.

Migration assay

The migration assay was performed as previously described [20]. Transwells (8 μm pore size, Corning, NY, U.S.A.) were coated with gelatin and incubated overnight at 37°C . Serum-starved HUVE cells were harvested, resuspended in Medium 199 containing 1% FBS, and added at a density of 20000 cells/well into the upper chamber. Peptides (10 or 20 μM) and bFGF (50 ng/ml) in Medium 199 containing 1% FBS were added into the bottom chamber. Cells were allowed to migrate for 6 h at 37°C . The membranes were removed, fixed and stained with Crystal Violet. Microscopic fields of view (10) were randomly selected and the cells migrating through the filter were counted.

Flow cytometry

To observe cellular uptake of fluorescently labelled peptides, cells were seeded on to a 6-well plate at a density of 10^6 cells/well. Cells were incubated with FITC-labelled peptides (1 μM) for 18 h at 37°C . After removal of the incubation medium, cells were washed twice with ice-cold PBS and detached by treatment with trypsin. Cells were then washed twice with PBS, resuspended in PBS and analysed by flow cytometry. Control cells that were not incubated with fluorescent peptides were also analysed.

Statistical analysis

Data are expressed as the means $\pm$ S.D. Statistically significant differences were assessed by Student's *t* test.

RESULTS

Identification of the NSA9 peptide sequence that is responsible for its anti-proliferative activity against endothelial cells

To identify the SARs of NSA9 responsible for anti-proliferative activity against BCE and HUVE cells, we synthesized several NSA9 N- and C-terminal truncated derivatives, and its reverse

Table 1 Primary sequences of human prothrombin kringle-2 derived peptides

Peptide	Sequence	Derivative
NSA9	Ac-NSAVQLVEN-NH ₂	(NSA9)
C-terminal truncated derivatives of NSA9	Ac-NSAVQLVE-NH ₂	(NSA8)
	Ac-NSAVQLV-NH ₂	(NSA7)
	Ac-NSAVQL-NH ₂	(NSA6)
	Ac-NSAVQ-NH ₂	(NSA5)
	Ac-NSAV-NH ₂	(NSA4)
	Ac-NSA-NH ₂	(NSA3)
N-terminal truncated derivatives of NSA9	Ac-SAVQLVEN-NH ₂	
	Ac-AVQLVEN-NH ₂	
	Ac-VQLVEN-NH ₂	
	Ac-QLVEN-NH ₂	
	Ac-LVEN-NH ₂	
	Ac-SAVQLV-NH ₂	
N-terminal truncated derivatives of NSA7	Ac-AVQLV-NH ₂	
	Ac-VQLV-NH ₂	
	Ac-NEVLQVASN-NH ₂	(RNSA9)
	Ac-VLQVASN-NH ₂	(RNSA7)
Reverse sequence of NSA9	Ac-NEVLQVASN-NH ₂	(RNSA9)
Reverse sequence of NSA7	Ac-VLQVASN-NH ₂	(RNSA7)

analogue (i.e. with its amino acids in reverse order). The sequences of the NSA9 derivatives are given in Table 1. These peptides were tested, at a concentration of 1 μ M using the MTT assay, for their ability to inhibit the proliferation of endothelial cells; the anti-proliferative activity of the NSA9 derivatives was compared with that of NSA9.

The results of anti-proliferative peptide activity against BCE cells are shown in Figure 1(A). The amount of proliferation of BCE cells that were stimulated by bFGF (3 ng/ml) was increased by 53 %. The original nonapeptide, NSA9, markedly inhibited the proliferation of BCE cells to a similar extent of proliferation as observed in bFGF-untreated control cells. Additionally, the octapeptide (NSA8) and the heptapeptide (NSA7), which were synthesized by deleting either the first (asparagine), or first and second (glutamic acid) residues from the C-terminus of NSA9, exhibited similar or more enhanced anti-proliferative activity than did NSA9 (Figure 1A, a). By contrast, further deletion of residues from the C-terminus of NSA9 decreased the anti-proliferative activity of the resulting peptides against BCE cells when compared with NSA9. In addition, when amino acid residues from the N-terminus of NSA9 were deleted, none of these peptides showed significant anti-proliferative activity against BCE cells, as NSA9 did (Figure 1A, b). These data suggest that the N-terminal region of NSA9 is important for the anti-proliferative activity of NSA9 against BCE cells.

To examine whether the N-terminal region of NSA7 is also important for inhibiting the proliferation of BCE cells, N-terminal-truncated derivatives of NSA7 were synthesized by deleting residues from the N-terminus of NSA7. Similar to the data obtained using the N-terminal truncated derivatives of NSA9, the NSA7 derivatives had no significant effect on the proliferation of BCE cells (Figure 1A, c). These data suggest that NSA7 contains a sequence that is required for the anti-proliferative activity of NSA9 against BCE cells.

To elucidate whether the sequence orientation in either NSA9 or NSA7 has an effect on their anti-proliferative activity, the peptides RNSA9 (NEVLQVASN) and RNSA7 (VLQVASN) were synthesized with sequences in reverse order to those in NSA9 and NSA7. The anti-proliferative activity of these reverse analogues was lower than that of either NSA9 or NSA7 respectively (Figure 1A, d). These data suggest that the sequence orientation in NSA9 and NSA7 also affects the inhibition of proliferation in BCE cells.

A. BCE

Figure 2 Anti-proliferative activity of NSA9, NSA8 and NSA7 against tumour cells

B16F10, LLC and L929 cells were incubated with each peptide (1 μ M) for 48 h. Cellular proliferation was assessed using the MTT assay. Control cells were incubated without peptides. Statistically significant differences were assessed by Student's *t* test. *, Indicates a statistically significant difference between control cells and peptide-treated cells (* $P < 0.05$, ** $P < 0.01$).

Anti-proliferative activity of NSA9, NSA8 and NSA7 against tumour cells

To examine whether NSA9, NSA8 and NSA7 also inhibit the proliferation of tumour cells such as L929, B16F10 and LLC, these cells were treated with each peptide (1 μ M). The proliferation of cells treated with these peptides was compared with that of untreated control cells.

As shown in Figure 2, NSA9 (1 μ M) inhibited the amount of proliferation in B16F10, LLC and L929 cells by approx. 20 % of that in untreated control cells. NSA8 and NSA7 clearly showed greater increased anti-proliferative activity (30–40 %) against B16F10 and LLC cells than did NSA9, whereas the anti-proliferative activity of NSA8 and NSA7 against L929 cells was slightly increased over that of NSA9. Accordingly, the NSA9, NSA8 and NSA7 peptides were shown to considerably inhibit the proliferation of both endothelial and tumour cells, although the extent of their inhibitory effects on cell proliferation was cell line-dependent.

In vitro and *in vivo* anti-angiogenic activity of NSA9 and NSA7

To confirm the anti-angiogenic activity of NSA9 and NSA7 *in vivo*, the CAM assay was performed as previously described [17,30]. In control samples treated only with bFGF (30 ng/ml) numerous new microvessels were observed, compared with untreated samples (Figure 3, panels A and B). On the other hand, new microvascular formation was apparently inhibited without affecting the pre-existing vasculature in the presence of NSA7 or NSA9 (20 μ g/ml) (Figure 3, panels C and D).

Next, we examined the abilities of NSA9 and NSA7 to inhibit endothelial cell tube formation in matrigel. HUVE cells were seeded on matrigel, and tube formation was assessed. As shown in Figure 4, panels A and B, numerous tube formations were observed after treatment with bFGF (10 ng/ml), compared with the controls. However, NSA9 and NSA7 (5 μ M) dramatically decreased tube formation of HUVE cells induced by bFGF (Figure 4, panels C and D). Inhibition of tube formation by NSA9 and NSA7 is quantified in Figure 4(E). NSA7 particularly suppressed tube formation to a greater extent than did NSA9.

To observe anti-migratory activity of NSA9 and NSA7, the endothelial cell migration assay was performed as described in the Experimental section [20]. NSA9 and NSA7 (10 μ M) inhibited HUVE cell migration by approx. 11.9 and 61.9 % respectively, compared with bFGF-controls. In addition, the amount of

Figure 3 Inhibition of neovascularization in CAM by NSA9 and NSA7

The CAM of a 3-day-old chick embryo was treated with PBS, NSA9 and NSA7. After 72 h, microvascular formation was observed and photographed. (A) PBS-treated; (B) treated with bFGF (30 ng/ml), (C) treated with bFGF (30 ng/ml) and NSA9 (20 μ g/ml), and (D) treated with bFGF (30 ng/ml) and NSA7 (20 μ g/ml).

HUVE cell migration induced by bFGF (50 ng/ml) was greatly decreased by 142.4 and 131.4 % in the presence of 20 μ M NSA9 or NSA7 respectively (Figure 5). Collectively, these results suggest that both NSA9 and NSA7 are potent angiogenic inhibitors.

Sequence requirement in NSA9 for its internalization into endothelial and tumour cells

To determine the sequence in NSA9 that is essential for cellular uptake, fluorescently labelled peptides were synthesized and their internalization into endothelial and tumour cells was measured. NSA9 and NSA7 have potent anti-proliferative activities, and NSAV and LVEN, the N- or C-terminal sequence peptides of NSA9, exhibited no anti-proliferative activity (Figures 1

Figure 4 Inhibition of tube formation by human prothrombin kringle-2-derived peptides NSA9 and NSA7

HUVE cells were seeded on to a matrigel-coated plate in the presence of NSA9 or NSA7. Tube formation was evaluated after 16 h using an inverted phase-contrast microscope. (A) untreated control; (B) treated with bFGF (10 ng/ml) only; (C): treated with bFGF (10 ng/ml) and NSA9 (5 μ M); (D) treated with bFGF (10 ng/ml) and NSA7 (5 μ M). (E) The inhibition of tube formation by NSA9 and NSA7 peptides. Tube-length was measured. Tube formation was expressed as a percentage of untreated control cells. Statistically significant differences were assessed by Student's *t* test. *, Indicates a statistically significant difference between bFGF control cells and peptide treated cells (* *P* < 0.05, ** *P* < 0.01).

Figure 5 Effect of the human prothrombin kringle-2-derived peptides NSA9 and NSA7 on HUVE cell migration

Peptides (10 and 20 μ M) were placed in the bottom chamber together with bFGF (50 ng/ml). HUVE cells were added to the upper chamber and incubated for 6 h. The cells that migrated were counted. Control cells were allowed to migrate in the absence of bFGF and peptide. Data are the number of cells counted/well. Statistically significant differences were assessed by Student's *t* test. *, Indicates a statistically significant difference between bFGF-control cells and peptide-treated cells (* *P* < 0.05).

and 2). These peptides were labelled with FITC as previously described. Cells were incubated with FITC-labelled peptides at a concentration of 1 μ M for 18 h and their cellular internalization was quantified by flow cytometry. The cellular uptake was

Table 2 Relative mean fluorescence of the cellular uptake of NSA9 and its derivatives

Results are the means for relative fluorescence uptake of NSA9 and its derivatives versus controls.

Peptide	Mean relative fluorescence uptake (nm)				
	BCE	HUVEC	B16F10	L929	LLC
Control	1.00	1.00	1.00	1.00	1.00
NSA9	1.75 $\pm$ 0.03	1.81 $\pm$ 0.10	1.41 $\pm$ 0.06	1.79 $\pm$ 0.13	1.60 $\pm$ 0.01
NSA7	1.88 $\pm$ 0.02	5.22 $\pm$ 0.19	3.73 $\pm$ 0.14	1.85 $\pm$ 0.12	2.29 $\pm$ 0.04
NSAV	1.18 $\pm$ 0.02	1.00 $\pm$ 0.04	1.19 $\pm$ 0.03	1.03 $\pm$ 0.03	1.06 $\pm$ 0.05
LVEN	1.35 $\pm$ 0.07	1.37 $\pm$ 0.05	1.22 $\pm$ 0.01	1.23 $\pm$ 0.05	1.22 $\pm$ 0.03

expressed as the mean relative fluorescence for FITC-labelled peptides versus untreated controls.

Quantification of the cellular internalization of FITC-labelled peptides is shown in Table 2. NSAV and LVEN showed little cellular internalization regardless of the cell line used. On the other hand, NSA9 and NSA7 were internalized into both endothelial and tumour cells. NSA7 exhibited greater uptake 2.88-, 2.64- and 1.43-fold into HUVE, B16F10 and LLC cells respectively, when compared with NSA9. The extent of NSA7 and NSA9 cellular internalization appeared to be similar in BCE and L929 cells. Therefore the cellular uptake data suggest that NSA7 contains the requisite internalization sequence for uptake of the NSA9 peptide into endothelial and tumour cells.

DISCUSSION

Numerous endogenous protein fragments have been identified as angiogenic inhibitors. For the development of angiogenic inhibitors with enhanced half-lives and efficacy, and decreased production costs and immunogenic reactions, studies of their SARs have been actively pursued. For example, several groups have shown that peptides derived from endostatin have anti-angiogenic effects [20–25]. A recent report has shown that amino acids 6–49 and 134–178 from four endostatin-derived peptides (residues 6–49, 50–92, 93–133 and 134–178) inhibited cell proliferation and cell migration with a potency and efficacy greater than that of full-length endostatin [22–23]. The peptides, including amino acids 50–92 and 93–133 of endostatin exhibited no activity. By contrast, Wickström et al. reported that an 11-mer, arginine-containing peptide (amino acids 60–70) that was derived from residues 50–92 of endostatin showed heparin-binding activity and was responsible for the anti-angiogenic activity of endostatin [20]. In another study, an N-/C-terminal deletion mutant (residues 31–140) of human endostatin, lacking the Zn-binding site, was shown to have anti-angiogenic and anti-tumour activities [24]. In addition, a 27-amino acid peptide (residues 1–27), corresponding to the N-terminal domain of endostatin, was reported to be responsible for Zn-binding and to have anti-tumour and anti-migration activities [25]. John et al. identified the existence of four endogenous proteolytic variants of endostatin present in the human circulation. Endostatin products (two) were characterized as short internal fragments (amino acids 47–86 and 47–90) of the entire molecule and two additional forms were O-glycosylated variants, such as V¹³-S¹⁸²-GalNAc-Gal₂-NANA and V¹³-S¹⁸²-GalNAc-Gal₂ [37].

Human prothrombin kringle-2 has been reported to inhibit CAM angiogenesis of chick embryos and LLC tumour growth and metastasis in mice [32]. The human prothrombin kringle-2 NSA9 peptide derivative, was found to markedly inhibit the proliferation of BCE cells and CAM angiogenesis [33]. We have previously

reported that NSA9 is internalized by endocytosis- and energy-dependent pathways, and exerts its anti-proliferative activity against BCE cells [38]. In the present study we investigated the SARs of NSA9 peptides derived from the human prothrombin kringle-2 for anti-proliferative activity against endothelial cells. The required sequence in NSA9 for cellular internalization was identified.

The research of SARs in bioactive peptides has been performed to determine structural requirements for their activities and to better understand their biological functions and specific interactions with receptors or binding proteins. For this purpose, various analogues of parent peptides were prepared by using a stepwise truncation from either the N- or C-termini of peptides, synthesis of reverse enantiomeric (D-amino acid substitution) and reverse-enantiomeric analogues, replacement of amino acids by a non-charged amino acid such as alanine, and transformation of peptide bonds into peptide bond surrogates such as *N*-methylated (CONCH₃) and reduced amide (CH₂NH) bonds [14,15,27,39–44]. Furthermore, the activity of parent peptides can be increased by other modifications, such as acetylation or amidation at the N- or C-terminus and cyclization [27,28,39,44]. These modifications increase the resistance of these peptides to proteolytic cleavage. We synthesized several NSA9 derivatives, including N- and C-terminal-truncated derivatives and a reverse sequence analogue of NSA9. All of the peptides used in our study were terminated by an acetylated residue at the N-terminus and by an amidated residue at the C-terminus, which is expected to increase their stability. As shown in Figure 1, most derivatives of NSA9 failed to inhibit the proliferation of BCE and HUVE cells, as compared with NSA9. However, an octapeptide, NSA8 (NSAVQLVE), and a heptapeptide, NSA7 (NSAVQLV), which were synthesized by deleting 1/2 residues from the C-terminus of NSA9, showed more enhanced anti-proliferative activities against these cells than did NSA9. RNSA9 and RNSA7, the reverse sequence peptides of NSA9 and NSA7 respectively, exhibited a greater decrease in anti-proliferative activity against endothelial cells than did NSA9 or NSA7. Therefore these results suggest that the N-terminal region and sequence orientation of the NSA9 peptide are necessary for inhibiting the proliferation of endothelial cells.

In the cell proliferation assay (Figure 1), we did not expect the reverse sequence peptides to exert anti-proliferative activity. However, RNSA9 and RNSA7 showed some anti-proliferative activity against endothelial cells, although they exhibited a greater decrease in activity than the parent peptides. Several studies using reverse peptides have suggested that these peptides may or may not have equipotent activity to the parent peptide. For example, the IKVAV peptide from laminin 1 chain has been shown to promote cell adhesion, neurite outgrowth and tumour growth. On the other hand, its reverse and reverse-enantio peptides were inactive [41]. In the case of C-peptide derived from processing proinsulin to insulin, the peptide has been known to prevent diabetes- and hyperglycemia-induced vascular and neural dysfunction in animal models of diabetes. The reverse peptide has also shown biological activity similar to that of the native C-peptide. This similarity in results has been reported for many antimicrobial peptides such as cecropins, mangainins and dermaseptins [42,45,46].

Reverse peptides have the same sequence as their parent peptides, with a reversed amide bond. The chirality of the side-chain position of a reverse peptide is equivalent to that of a D-peptide. D-peptides have the mirror image structure of their corresponding, parent L-peptide. Thus a reverse peptide might be topologically equivalent to the mirror image of the structure of its parent peptide. The reverse peptide is expected to be inactive or less active if both the sequence/side-chain and amide-bond direction contribute to its biological activity [43,47].

Until now, evidence regarding NSA9 receptors, and its exact internalization mechanism has been scarce. However, in our previous report, we showed that NSA9 is internalized into BCE cells by endocytosis and energy-dependent pathways, and thus exerts its anti-proliferative activity [38]. Accordingly, several reasons may be related to the low activity of RNSA9 and RNSA7. First, the reverse peptides might not be as successfully internalized as NSA9 and NSA7 due to a requirement for the N-terminal region of NSA9 and NSA7, or the reverse amide-bond direction, resulting in loss of their activity. On the other hand, RNSA9 and RNSA7 could share a common pathway with NSA9 and NSA7 for cellular internalization. However, the internalized reverse peptides might not be sufficient for the activation of cellular signalling molecules. Therefore further detailed studies are necessary to reveal the SAR of the reverse peptides.

In the presence of NSA9, NSA8 and NSA7 at 1 μ M, a clear inhibition (20–40%) of the amount of proliferation of tumour cells such as B16F10, LLC and L929 was observed (Figure 2). However, when the anti-proliferative activity of these peptides against endothelial and tumour cells was compared, it appeared that their activity is greater towards endothelial cells than tumour cells. Thus the human prothrombin kringle-2 peptide derivatives NSA9, NSA8 and NSA7 inhibit the proliferation of endothelial cells to a greater extent than tumour cells. Also, these observations support the anti-angiogenesis assay results, which show that NSA9 and NSA7 significantly inhibit angiogenesis *in vitro* and *in vivo* e.g. CAM angiogenesis, tube formation and cell migration (Figures 3, 4 and 5). Therefore these results suggest that NSA7, as well as the original NSA9 parent-peptide are effective as angiogenic inhibitors.

Next we evaluated the sequence necessary for the internalization of NSA9 into endothelial and tumour cells. As shown in Table 2, the cellular internalization efficiency of peptides decreased in the following order: NSA7 (NSAVQLV) > NSA9 (NSAVQLVEN) $\gg$ LVEN > NSAV. In particular NSA7 was internalized more readily than NSA9. Thus NSA7, the C-terminal truncated derivative of NSA9, contains the essential sequence for cellular internalization of the NSA9 peptide derived from human prothrombin kringle-2. In addition, NSA7 exhibited more efficient anti-proliferative activity and cellular internalization than NSA9. The reason for the enhanced anti-proliferative activity and cellular internalization of NSA7 is unclear at present. One possibility is that the presence or absence of a negative charge on NSA9 or NSA7 may affect their cellular internalization and anti-proliferative activity. A negative charge on glutamic acid is absent from NSA7 (NSAVQLV), compared with NSA9 (NSAVQLVEN). NSA7 may have better access to the negatively charged cellular membrane or membrane components and be internalized into cells more easily than NSA9. Accordingly, NSA7 may inhibit cell proliferation more effectively than NSA9. However, both peptides showed similar amounts of uptake into either endothelial or tumour cells, except for greater uptake of NSA7 into HUVEC and B16F10 cells. Therefore further studies are needed to identify whether the NSA9 peptide derivatives are internalized into endothelial and tumour cells via the same mechanism, and the relationships between cellular internalization and the action mechanism of these peptides.

Many anti-angiogenic studies of various peptides derived from anti-angiogenic proteins have not provided consistent results or definitive mechanisms of action. However, these studies suggested that various factors, including the length and secondary structure of peptides, degree of sample aggregation in peptide preparation, and optimal concentration and administration route of peptides might influence the anti-angiogenic and anti-tumour activity of the peptides. Moreover, it is possible that novel unidentified

domains could be related to the anti-angiogenic activity of proteins known as angiogenic inhibitors. Therefore we cannot exclude that in addition to the NSA9 peptide, another region derived from human prothrombin kringle-2 may have anti-angiogenic and anti-tumour activities. Also, for assessment of the anti-tumour activity of the NSA9 peptides, it was necessary to investigate the optimal concentrations for administration and cellular entry routes of the human prothrombin kringle-2 peptide derivatives through pharmacokinetics studies, and to identify anti-tumour activity in these peptides using animal models.

Taking these results together, we conclude that the N-terminal region and sequence orientation of the human prothrombin kringle-2 NSA9 peptide derivative are necessary to inhibit the proliferation of endothelial cells. Significantly NSA7, a C-terminal truncated derivative of NSA9, was found to be superior in both anti-angiogenic activity and cellular uptake, compared with NSA9. These features indicate that NSA7, as well as NSA9, could serve as effective inhibitors of angiogenesis.

This work was supported by grant R01-2005-000-10179-0 from the Basic Research Program of the Korea Science and Engineering Foundation and by the Brain Korea 21 Project in 2005.

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Received 9 August 2005/14 November 2005; accepted 3 January 2006

Published as BJ Immediate Publication 3 January 2006, doi:10.1042/BJ20051300