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Semaphorin 3A lytic hybrid peptide binding to neuropilin-1 as a novel anti-cancer agent in pancreatic cancer

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ABSTRACT

We previously reported that novel targeted "hybrid peptide" in which epidermal growth factor receptor (EGFR) binding peptide was conjugated with lytic-type peptide had selective cytotoxic activity to EGFR expressing cancer cells. In this study, we have generated a novel type hybrid peptide, semaphorin 3A lytic (Sema3A-lytic), which is composed of two functional amino acid domains: a sequence derived from Sema3A that binds to neuropilin-1 (NRP1) and a cytotoxic lytic peptide. We found that this hybrid peptide had cytotoxic activity against NRP1-positive pancreatic cancer cell lines such as BxPC-3 and Panc-1, whereas the peptide did not affect the viability of normal cells in vitro. It was also found by affinity analysis that Sema3A peptide binds to NRP1, and two arginines (372R and 377R) in Sema3A peptide are involved in the interaction with NRP1 protein. In addition, confocal microscopy analysis revealed that Sema3A-lytic peptide could not penetrate normal cells regardless of the presence of NRP1 mRNA, suggesting that the ability of Sema3A-lytic peptide to concentrate adjacent to the cell membrane by binding to NRP1 with the target-binding moiety contributes to its selective cytotoxic activity. These results indicate that Sema3A-lytic hybrid peptide would be a possible anti-cancer agent for treatment of human pancreatic cancer.

1. Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the USA [1] and the fifth leading cause of cancer death in Europe [2]. It has a dismal 5-year survival rate of about 5% [3], mainly due to the fact that disease-specific symptoms occur late in the course of the disease [4]. The median survival time for patients with unresectable pancreatic cancer is 4–6 months. Treatment is further complicated by the predisposition of pancreatic cancers to show early metastasis and resistance to conventional chemotherapeutic and radiation treatments [5]. Therefore, a considerable number of patients with pancreatic cancer need additional treatment as the disease progresses.

Numerous studies have demonstrated the expression of neuropilin-1 (NRP1) in various human tumours, including prostate, breast, lung, and colon cancer, and astrocytoma and pancreatic adenocarcinoma [6–11]. It was previously reported that elevated levels of NRP1 mRNA were detected in pancreatic cancer and were clearly associated with less differentiated tumours [12]. It was also found using immunohistochemical analysis that NRP1 was expressed in all 12 of 12 human pancreatic adenocarcinoma specimens but was absent in non-malignant pancreatic tissue specimens. NRP1 is a multifunctional non-tyrosine kinase receptor that binds to class three semaphorins, and was initially characterized as an axonal membrane glycoprotein involved in neuronal guidance and development [13]. The semaphorins are a large family of secreted and membrane-bound proteins, which contain an approximately 500-amino acid extracellular domain called the sema domain at their N-terminus, and which are highly conserved from invertebrates to mammals [14,15]. Class 3 semaphorins are secreted proteins and comprise seven members (Sema3A–3G). They bind to signalling receptors of the plexin A family but also require NRPs as binding co-receptors [15,16]. NRP1 consists of an 860-amino acid extracellular glycoprotein region, a 22-amino acid transmembrane region, and a 40-amino acid intracellular region [17]. The extracellular region consists of five domains: the C-terminal MAM (meprin, A5, μ-phosphatase) domain, the a1 and a2 (CUB) domains, and the b1 and b2 (CF V/VIII) domains [17]. NRPs have a very short cytoplasmic domain that cannot transduce intracellular signals, and plexins form complexes with NRPs and act as the signal-transducing components of the semaphorin–NRP–plexin ligand–receptor complex [16,18,19].

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Novel approaches to cancer therapy developed in recent decades include immunotoxins, monoclonal antibodies, or ligands that selectively target all cancer cells conjugated to plant or bacterial toxins [20–23]. A number of novel bacterial toxin-based immunotoxins have been reported, such as the interleukin-2–diphtheria toxin (IL-2-DT; Ontak™), interleukin-4–Pseudomonas exotoxin, and interleukin-13–Pseudomonas exotoxin fusion proteins [20,24–26]. Although it has been suggested that the targeting moiety of these immunotoxins binds to a cell-surface receptor and then permeates the cell to induce cell death, they have several limitations, such as liver toxicity and immunogenicity caused by the bacterial toxin and their large molecular size prevents them from intracellular permeation [20,24,25]. To overcome these issues, we have developed new lytic-type peptide containing D- and L-cationic-rich amino acids to form amphipathic partial α-helices that disrupts the cancer cell membrane selectively and are stable when combined with a cancer-targeting moiety [27]. In this study, we have developed a novel Sema3A-lytic hybrid peptide targeting NRP1-expressing pancreatic cancer cells. Here we describe the effectiveness and selectivity of Sema3A-lytic hybrid peptide and discuss the possible mechanism underlying the selective cytotoxic activity of this peptide.

2. Materials and methods

2.1. Cells and cell culture condition

The human pancreatic cancer cell line BxPC-3 was purchased from the European Collection of Cell Cultures (ECACC, Salisbury, Wiltshire), and CFPAC-1, SU.86.86., and Panc-1 cell lines were purchased from the American Type Culture Collection (ATCC, VA). The human normal pancreatic epithelium cell line PE was purchased from DS Pharma Biomedical. Cell lines were cultured in RPMI 1640 (BxPC-3, SU.86.86.), DMEM (Panc-1), IMDM (CFPAC-1) or CS-C medium (PE) containing 10% fetal bovine serum (FBS), 100 μg/ml penicillin, and 100 μg/ml streptomycin.

2.2. Anti-lytic antibody

The anti-lytic peptide antibody was obtained from custom antisera by Sigma Genosys (Sigma–Aldrich Japan, Ishikari), and characterized the specificity for antigen as described previously [28].

2.3. Peptide synthesis

The following peptides used in this study were synthesized by Invitrogen (Carlsbad, CA, USA) and American Peptide Company, Inc. (Sunnyvale, CA, USA). Bold and underlined letters are D-amino acids.

Lytic peptide: KL**L**LK**L**L**KK**LLK**L**LKKK.
Sema3A-lytic peptide: NYQWVPYQGRVPYPRGGGKL**L**LK**L**L**KK**L LK**L**LKKK.
Peptides were dissolved in water and buffered to pH 7.4 as described previously [27].

2.4. Binding assay

Binding assay was performed as described previously [29]. Briefly, Sema3A wild and several deletion peptides labelled with FITC were incubated with BxPC-3 cells. After the incubation, cells were washed with PBS three times, and then binding peptides were detected by FACS calibur (BD, NJ, USA). Binding activity was calculated from the fluorescence intensity.

2.5. Cell viability assay

Cell viability assay was performed as described previously using WST-8 solution (Nacalai Tesque, Kyoto) [27].

2.6. Membrane potential analysis

Transmembrane potential of cancer and normal cells were assayed as described previously using diS-C3-5 (Molecular Probes) [30].

2.7. Biomolecular interaction analysis

Surface plasmon resonance (SPR) experiments were performed using a Biacore T100 system (GE Healthcare, NJ) as described previously [31]. All data analysis was performed using Biacore T100 Evaluation Software version 2.0.2 (GE Healthcare, NJ).

2.8. RT-PCR analysis

Total RNA from cultured cells was extracted using a NucleoSpin RNA kit (Qiagen, Hilden). For reverse transcription, RNA samples of 0.5 μg were used and the reaction was performed using the Rever TraAce RT kit (Toyobo, Osaka) according to the manufacturer's protocol. cDNA (0.05 ng) was used as a template in a total volume of 50 μl for PCR. The following primers are used.

NRP1 For: 5′-CTCCATCGAAGACTTCCACGTAGT-3′
NRP1 Rev: 5′-AAATGCGAATGGCTGATTCAG-3′
NRP2 For: 5′-GAGATTCGGGATGGGGACAGTGAATC-3′
NRP2 Rev: 5′-CCAAGTTGTGTGGATACTTCTCAG-3′
Plexin A1 For: 5′-CCTTGACTACCGGACATATGCCATGCG-3′
Plexin A1 Rev: 5′-GTTCTTGCTCTCCAGGTTCTTCTCGATG-3′
Plexin A2 For: 5′-GGAGAACAAGAACCACCCCAAGCTGCTAC-3′
Plexin A2 Rev: 5′-GGCATCAAGAATCTTCTCCTTGACCTG-3′
VEGFR2 For: 5′-CCCAGTCTGGGAGTGAGATG-3′
VEGFR2 Rev: 5′-GGATTGGTAAGGATGACAGTG-3′
GAPDH For: 5′-GTCTTCACCACCATGGAGAAGGCT-3′
GAPDH Rev: 5′-CATGCCAGTGAGCTTCCCGTTCA-3′

GAPDH was used as an internal control. Quantitative real-time PCR was carried out using SYBR Green Real-time PCR Master Mix Kit (Toyobo, Osaka) on the Mx3000P Real-Time QPCR System (Stratagene, CA, USA). The primers were the same as for RT-PCR analysis.

2.9. Flow cytometry analysis

Flow cytometry analysis by Annexin V-Flurorescein Staining kit (Wako, Osaka) was performed as described previously [27].

2.10. Immunocytochemistry

Immunocytochemistry was performed as described previously using anti-lytic peptide antibody [28].

3. Results

3.1. Design of the Sema3A-lytic hybrid peptide

It was previously reported that Sema3A binds to NRP1 by the sema domain, which is located at the N-terminus of Sema3A, and the basic C-terminal tail of Sema3A [32–34]. It was also suggested that the sema domain is important for selectivity of binding to NRP1, whereas the semaphorin tail does not directly contribute

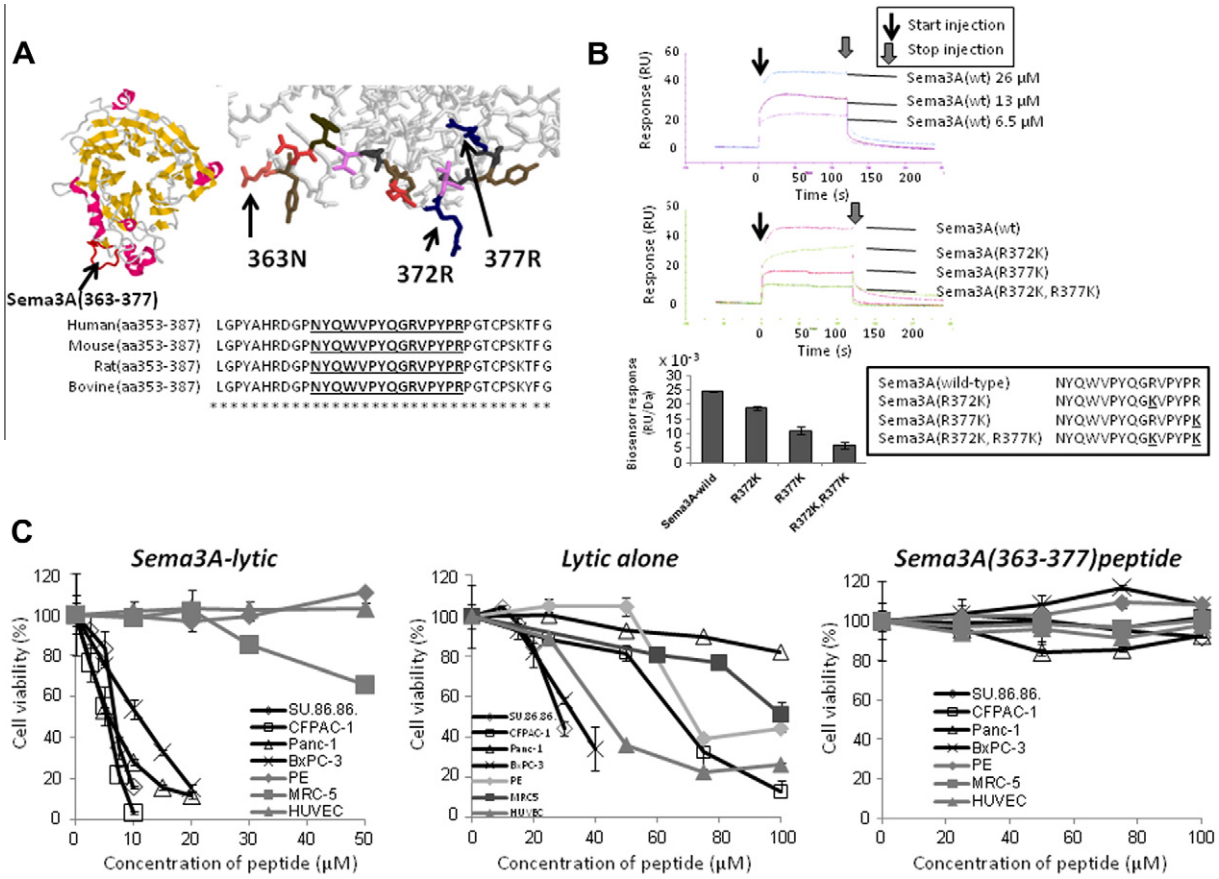

Fig. 1. Design and characterization of novel Sema 3A and Sema 3A-lytic peptide. (A) Structure of the sema domain of mouse Sema3A viewed from the "top" face of the molecule, and residues 363–377 are highlighted in red (left diagram). The enlarged stick model structure of mouse Sema3A (363–377) sequence viewed from the "side" face of the molecule (right diagram). Residues 363–377 are highlighted in colour. The information about structure was obtained from Protein Data Bank (1q47), and stick model is shown using Ras Mol software. The sequences of sema domain (353–387) were aligned using Clustalw. (B) Interaction analysis of wild-type and mutant Sema3A (363–377) with NRP1 by Biacore. Samples of various serially diluted concentrations of Sema3A (363–377) wild-type peptide (6.5, 13, and 26 μM) were analyzed on parallel sensor surfaces (top graph), and samples of wild and various Sema3A mutant peptides (26 μM) were analyzed on parallel sensor surfaces (middle graph). Binding ability of various Sema 3A peptides by biosensor analysis was compared (bottom bar graph). Biosensor response (RU/Da) at 26 μM of each peptide concentration was calculated as described in Section 2. Inset indicates the sequences used in this analysis. (C) Cytotoxic activity of Sema3A-lytic peptide in various cell lines. Pancreatic cancer and normal cells were cultured with various concentrations of the peptides for 48 h, and cytotoxic activity was assessed as described in Section 2. The results are represented as means ± SD (bars) of triplicate determinations, and the assay was repeated three times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to receptor specificity, but increases the affinity of the molecule for its membrane-bound receptors [32–34]. The crystal structure of the sema domain has shown that it broadly conforms to the typical architecture of a seven-blade β-propeller structure (Fig. 1A), containing approximately 500 amino acids, and mutational studies have demonstrated that at least two regions (residues 252–260 and 359–366) are involved in binding to NRP1. Furthermore, Shirvan et al. have showed that peptides derived from residues 363–380 of Sema3A can mimic the biological activity of full-length Sema3A at 180 μM on neuronal cells, inducing apoptosis [35,36]. According to the stick model of the sema domain of mouse Sema3A, as shown in Fig. 1A, it was confirmed that the Sema3A (363–377) region stands out from the sema domain structure. Furthermore, this region was shown by sequence analysis to be highly conserved among mammals (Fig. 1A). Binding assay using Sema3A (363–377) and several deletion peptides labelled with FITC also demonstrated that Sema3A region was capable of binding to BxPC-3 cancer cells and significant for the binding activity (Supplementary Fig. S1 A and B). Based on these information, we extracted the Sema3A (363–377) sequence as a targeting moiety for the novel hybrid peptide, which could be covalently linked to a modified lytic-type peptide.

3.2. Binding of the Sema3A peptide to NRP1 protein

To confirm the binding properties of Sema3A peptide to NRP1, NRP1 protein was immobilized on sensor chips and its interaction with Sema3A peptide, which is amino acids 363–377 of human Sema3A (Fig. 1B), was analysed by Biacore system. As shown in Fig. 1B, Sema3A peptide bound to the immobilized NRP1 in a concentration-dependent manner, with a K_D value of 2.3×10^{-6} M. We next examined the affinity of various mutant peptides in which arginine residues were replaced with lysine (Fig. 1B). As shown in Fig. 1B, mutant Sema3A peptides (R372K, R377K) showed decreased binding affinity compared with the wild-type peptide, indicating that these arginines are important for the interaction with NRP1. Moreover, the binding ability of Sema3A (R377K) to NRP1 was less than that of Sema3A (R372K) (Fig. 1B).

3.3. Selective cytotoxic activity on pancreatic cancer cells

Based on the analysis of interaction of Sema3A peptide with NRP1, we next examined cytotoxic activity to assess the selectivity of Sema3A-lytic hybrid peptide, lytic peptide alone, and NRP1-binding portion of Sema3A (amino acids 363–377) against

Table 1
Cytotoxic activity of Sema3A-lytic peptide in various cell lines.

Cell line	$IC_{50}(\mu M)^*$	
	Sema 3A-lytic	Lytic
Cancer cells		
SU.86.86.	5.2 ± 0.4	28 ± 0.5
CFPAC-1	6.7 ± 0.1	67 ± 0.9
Panc-1	13 ± 2.7	>100
BxPC-3	5.7 ± 2.3	32 ± 1.6
Normal cells		
Pancreatic epithelium	>50	71 ± 0.5
HUVECs	>50	42 ± 3.2
MRC5	>50	>100

* Results are means of three independent experiments and each was performed in triplicate.

pancreatic cancer cell lines (SU.86.86., CFPAC-1, Panc-1, and BxPC-3) and normal cell lines (PE, HUVECs, and MRC5). It was revealed that Sema3A-lytic peptide had remarkable cytotoxic activity and selectivity against cancer cell lines, acting in a concentration-dependent manner, but that lytic peptide alone did not have selective characteristic (Fig. 1C). Sema3A (363–377) peptide exhibited no significant cytotoxic activity against these cell lines (Fig. 1C).

The IC_{50} values of the peptides are shown in Table 1, indicating that 5–15 μM of Sema3A-lytic hybrid peptide induced more than 50% cell death in all cancer cell lines tested, whereas the same concentration of the lytic peptide alone could not induce sufficient cell killing of cancer cells. Sema3A-lytic peptide did not kill normal cells at the same concentration (Fig. 1C).

3.4. Expression levels of NRP1 in the cancer and normal cells

We then investigated the expression of NRPs in the cancer and normal cell lines. As shown in Fig. 2A, NRP1 and NRP2 mRNAs were detected in all four pancreatic cancer cell lines used in this study, whereas PE cells were negative for both NRPs. Because NRP1 and NRP2 are co-receptors of plexin receptors, to which Sema3s bind, and vascular endothelial growth factor (VEGF) receptors (VEGFRs), to which VEGF binds, we next investigated the mRNA expression of plexin A1, plexin A2, and VEGFR2 in these cells. All four pancreatic cancer cell lines and PE cells were positive for the plexin A1 receptor, whereas plexin A2 was clearly identified in pancreatic cancer cells except for Panc-1 cells, and VEGFR2 was detected at low levels in BxPC-3 cells. Primary HUVECs were also positive for mRNA for all of these genes (NRPs, plexin A1, plexinA2, and VEGFR2).

Quantitative PCR analysis also demonstrated that NRP1 was expressed at the highest level in SU.86.86. cells, followed by CFPAC-1, Panc-1, and BxPC-3 cells, whereas the expression level of NRP1 was low on normal PE cells, in concordance with the results of the RT-PCR analysis (Fig. 2B).

3.5. Characterization of cancer cell death by Sema3A-lytic peptide

We used flow cytometry to monitor the action of Sema3A-lytic peptide. It was found that after a 3 hr exposure to the peptide (20 μM), the translocation of phosphatidylserine (PS) to the external cell surface occurred in cancer cells but not in normal cells. The result suggests that Sema3A-lytic hybrid peptide induces cancer cell death partially via an apoptotic mechanism (Fig. 3A). We also confirmed by fluorescence microscopy using an anti-lytic antibody that Sema3A-lytic peptide can penetrate cancer cells, whereas this peptide does not penetrate normal cell membranes. Sema3A-lytic peptide was clearly identified by vesicular-like staining in BxPC-3 cells, whereas it was not detected in normal cells (PE cells and HUVECs) (Fig. 3B and Supplementary Fig. S2 A). At 3 min after addition

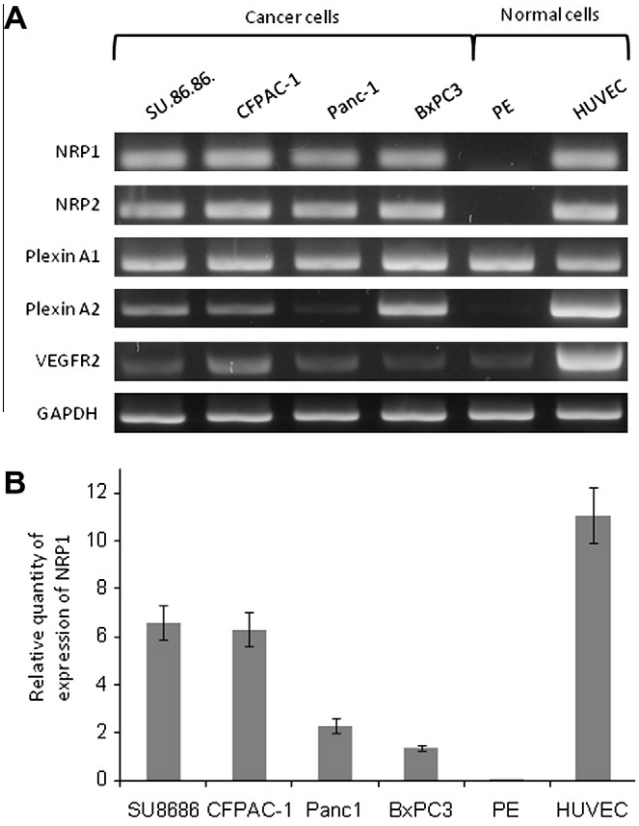

Fig. 2. Expression of mRNA of Sema3A-related receptors. (A) Expression of class 3 semaphorin receptors (NRPs, plexin A1 and A2) and VEGFR2 in pancreatic cancer cells (BxPC-3, CFPAC-1, Panc-1, SU.86.86.) and pancreatic epithelial cells (PE), and HUVECs (positive control), was analyzed by RT-PCR. (B) Quantitative PCR analysis of NRP1 in these cell lines.

of Sema3A-lytic peptide the staining was visible at low levels in BxPC-3 cells, indicating that Sema3A-lytic peptide forms an assembly adjacent to the cell membrane, and that this occurs quickly, but only in cancer cells (Fig. 3C). Between 3 and 30 min of incubation staining of the Sema3A-lytic peptide in BxPC-3 cells became progressively more intense in a time-dependent manner (Fig. 3C). When we checked the membrane potential of cancer (BxPC-3) and normal (HUVEC) cells using intrinsic fluorescence analysis, the difference of membrane potential between these cells was confirmed (Supplementary Fig. S2 B). These results suggest that Sema3A-lytic hybrid peptide have selectivity for discriminating the difference of property between cancer and normal cell membrane to induce cancer cell death.

4. Discussion

In this study we designed and characterized Sema3A-lytic hybrid peptide as a novel anti-cancer agent modelled on the binding interface between NRP1 and the sema domain of Sema3A. The sequence of interest in Sema3A peptide was extracted based on a previously reported critical sequence of Sema3A's interaction with NRP1. The crystal structure of the sema domain shows this sequence extruding from the surface of the protein's three-dimensional structure [34] (Fig. 1A). The N-terminal sema domain of the semaphorin family is conserved among mammals [14,15], and it was also confirmed by alignment analysis that the area around amino acids 363–377 of Sema3A is highly conserved, suggesting that the NRP1-targeted hybrid peptide plays an important role in the interaction of Sema3A-lytic protein with NRP1 (Fig. 1A).

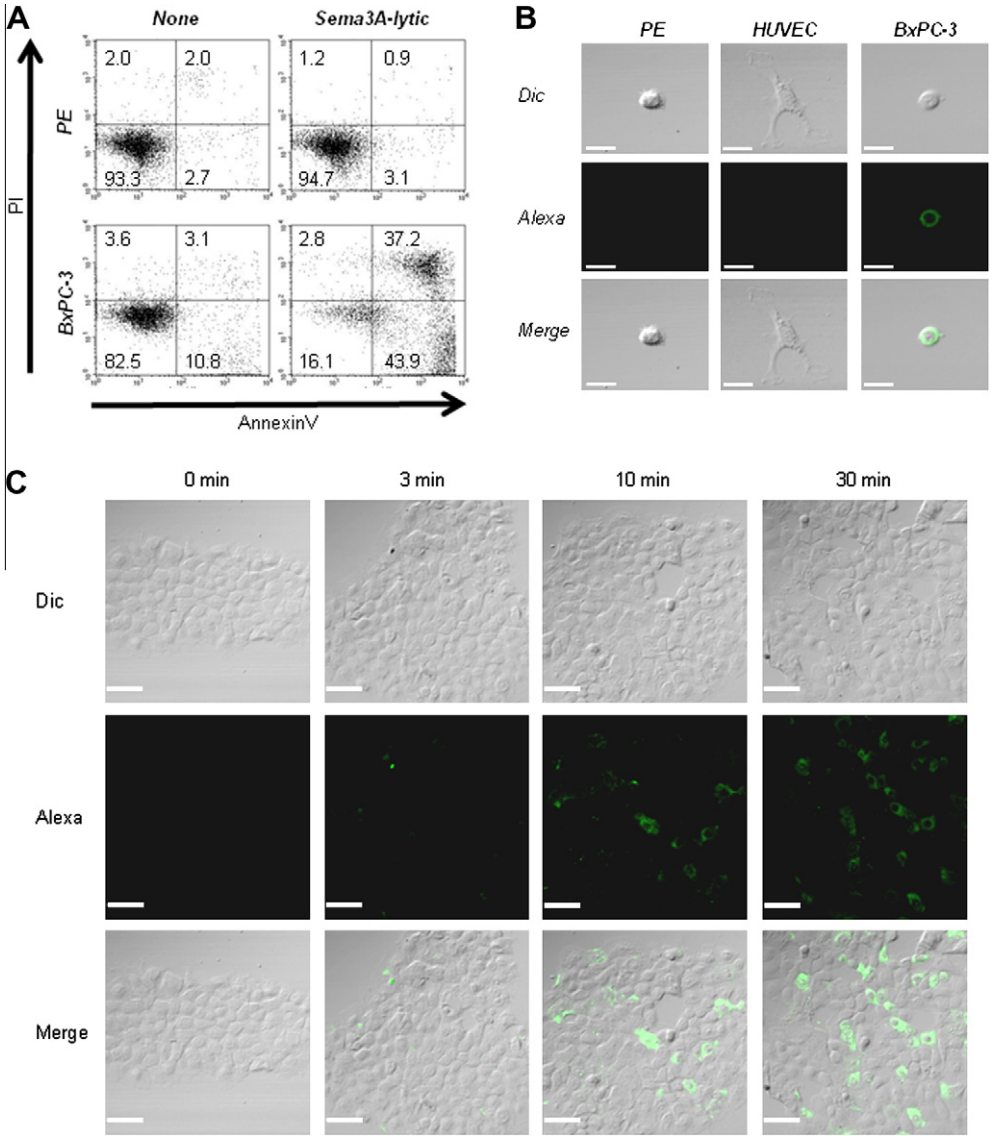

Fig. 3. Effect of Sema3A-lyic peptide on pancreatic cancer cell. (A) Induction of membrane translocation by Sema3A-lytic peptide. PE (top panels) and BxPC-3 (lower panels) cells were incubated with Sema3A-lytic peptide (20 μM) for 3 h at 37 °C, and then analyzed by dual-colour flow cytometry for annexin V labelling. (B) Confocal laser scanning microscopy of pancreatic cancer cell line (BxPC-3) and normal cell lines (PE cells and HUVECs) treated with Sema3A-lytic peptide (10 μM) for 30 min. Cells treated with Sema3A-lytic peptide were incubated with anti-lytic antibody and Alexa Fluore 488 goat anti-rabbit IgG (Alexa). Scale bars, 20 μm. (C) The presence of Sema3A-lytic peptide was detected in a time-dependent manner. BxPC-3 cells were incubated with Sema3A-lytic peptide (10 μM) as the indicated time (min), and then stained with anti-lytic antibody and Alexa Fluore 488 goat anti-rabbit IgG. Scale bars, 50 μm.

We also investigated binding ability of Sema3A peptide to cancer cell surface and the affinity of this peptide with the targeting receptor (Supplementary Fig. S1 and Fig. 1B). As a result, Sema3A peptide could bind to cancer cells, and this peptide also bound to immobilized NRP1, as seen by affinity analysis using Biacore, and analysis of various mutants showed that two arginines in the Sema3A (363–377) peptide significantly contributed to binding of this peptide to NRP1, suggesting that R377 contributes to binding to cell-surface NRP1 more significantly than R372 (Fig. 1A and B).

We also showed that Sema3A-lytic peptide has selective cytotoxic activity in vitro towards NRP1-positive pancreatic cancer cells (Table 1, Fig. 1C). The most important result in this study was that Sema3A-lytic peptide remarkably induced selective cell death, discriminating between cancer and normal cells, and that Sema3A-lytic peptide was less active in HUVECs, despite that all the genes for NRP1, NRP2, plexin A1, plexin A2, and VEGFR2 are expressed in this cell line (Fig. 2). The mechanisms underlying these findings can be explained partially by our immunocytochemical results, whereby in vitro fluorescence experiments revealed the difference between Sema3A-lytic peptide assembly in cancer cells and normal cells (Fig. 3B and Supplementary Fig. S2 A). This suggests that the ability of Sema3A-lytic peptide to concentrate and bind to its target, surface-expressed NRP1 on the cell membrane of cancer cells, could contribute selectivity to its cytotoxic activity. According to our results, there are two points involved in a reasonable explanation for this selective activity. First, Sema3A-lytic peptide concentrates by dint of the electrostatic attraction to the cytoplasmic membrane of cancer cells which contains more acidic components, including PS, and/or by the interaction between the target moiety derived from the Sema3A binding site and NRP1 expressed on the cell surface. Second, the density of Sema3A-lytic peptide adjacent to the cell membrane increases and then it makes a pore, leading to the cell death.

There have been numerous reports concerning anti-microbial peptides that contain not only basic amino acids but also hydrophobic amino acids and form positively charged amphipathic

α-helices at neutral pH [37–39]. The action of these peptides is mostly explained by the Shai–Matsuzaki–Huang model [40]. According to this model, these peptides interact with the membrane by electrostatic and hydrophobic forces [40]. It is also known that the microbial cytoplasmic membrane is composed predominantly of negatively charged acidic phospholipids, whereas the surface membrane of normal mammalian cells has no net charge. Thus anti-microbial peptides can show microbial selectivity by binding with a negative charge. We also previously reported that epidermal growth factor receptor (EGFR)-lytic hybrid peptide had selectivity for small unilamellar vesicles (SUVs) containing PS, which is exposed specifically on cancer cell membranes [27]. Since it was found that the permeation was not detected with HUVECs (Fig. 3B and Supplementary Fig. S2 A), it is suggested that surface membrane characteristics of normal cells is different from that of cancer cells. The difference of membrane potential between cancer (BxPC-3) and normal (HUVEC) cells was also confirmed (Supplementary Fig. S2 B). Taken together with our current study, the permeation rate of Sema3A-lytic hybrid peptide is superior in cancer cells and the selectivity of its cytotoxic activity is explained by its affinity for both targeting moiety to receptor and lytic moiety to acidic membranes components.

Furthermore, we used flow cytometry analysis to elucidate the movement of the cell membrane after treatment with Sema3A-lytic peptide. We found that translocation of PS to the external cell surface occurs in cancer cell lines only, which is coincident with the Sema3A-lytic peptide staining clearly identified by vesicular-like staining in BxPC-3 cells but not in normal cells (Fig. 3A). Although an apoptotic mechanism is suggested by flow cytometry analysis, the detailed mechanism underlying cancer cell death induced by Sema3A-lytic peptide is still not fully understood.

In summary, new cancer therapies involving peptide-based drugs offer a number of advantages, including easy preparation by synthesis using solid-phase chemical synthesis techniques, low manufacturing costs, lower risk of an immune response, and improved tumour penetration when compared to antibody-based therapies. Thus, the Sema3A-lytic peptide might provide a new strategy for efficient pancreatic cancer therapy. The findings of this study will assist the further elucidation of pancreatic cancer treatments targeting NRP1.

5. Competing financial interests

Koji Kawakami is a founder and stock holder, and Masayuki Kohno is an employee of Upstream Infinity, Inc. The other authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.09.021.

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