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High-throughput screening of cell death inducible short peptides from TNF-related apoptosis-inducing ligand sequence

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Abstract Therapeutic peptides and small molecules, rationally designed to trigger cell death have attracted strong attention. Cell death inducible peptides were screened from amino acid sequence of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Using Fmoc solid phase synthesis, cellulose membrane-bound octameric peptide library of TRAIL scan was prepared and cell viability assay was directly performed on peptide disk with Jurkat cells. Six peptide sequences that could induce cell death were found. Peptide sequence with RNSCWSKD ($TRAIL_{227–234}$) that exist in the zinc-binding site revealed high cell death inducible activity. Apoptotic cell death was observed when cells were treated with soluble synthesized peptide.

Keywords: Peptide array; Tumor necrosis factor-related apoptosis-inducing ligand; Cell death inducible peptide

1. Introduction

Peptide ligands that interact with cell surface receptors for cell signaling can be excellent leads for antagonist development. Screening of small peptides or small molecules that modulate protein–protein interaction continues to be major challenge for drug discovery since these molecules enable to provide targeted therapy. Numbers of therapeutic peptides and small molecules have been designed to trigger cell death and entering the clinic against cancer [1–4].

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a member of the TNF family, is particularly interesting for its unique ability to induce cancer cell death while sparing most normal cells, which implies its use as a potent anti-cancer agent [5]. TRAIL is a type II transmembrane protein that exists as a homotrimeric jelly roll proteins [6]. TRAIL has been shown to interact with the cell surface through five distinct receptors, death receptor (DR) 4, DR5, decoy receptor DcR1, DcR2, and osteoprotegrin. DR4 (TRAIL-R1) and DR5 (TRAIL-R2) possess an intracellular tail containing a conserved motif known as the death domain and allow interaction with the downstream adaptor proteins to initiate apoptosis signals. In contrast, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2) contain an incomplete non-functional death domain and act as decoys, inhibit signaling through TRAIL-R1 and -R2 [7]. Osteoprotegrin is a secreted decoy receptor that binds to TRAIL with lower affinity than other receptors. Since TRAIL and its receptors are constitutively expressed in almost tissues and induction of apoptosis does not depend on p53 statuses that are considered to be critical factor in cancer therapies, TRAIL attracted considerable attention as a potential therapeutic to human cancers.

From our previous experiments [8], cell death peptide has been screened from human Fas antigen ligand sequence using peptide array. A novel 5mer peptide, CNNLP (Cys-Asn-Asn-Leu-Pro) that exist in the Fas ligand–receptor interaction site revealed cell death effect. Peptide array has been utilized to identify active peptides and explore analogue peptides with improved activity. The method for synthesizing large numbers of peptides on cellulose membrane was developed by Frank [9] using the Fmoc solid phase peptide synthesis. The SPOT technology enables to synthesize variety of peptides from the C to the N-terminus in one membrane in a parallel manner and provides versatile tools for molecular recognition and screening of biologically active peptides [10–13]. Screening of peptide ligands for cell surface receptors will extend peptide array applications from probing protein–protein interactions to imitating cell signaling.

In the present study, high-throughput screening of cell death inducible peptides was performed with peptide-scan of the TRAIL extracellular domain that interacts with its receptor. The direct cell assay of cell death inducible peptides was performed using peptide array. Techniques that rapidly assess biomolecular interactions between receptors on the surfaces of mammalian cells and synthetic ligands of extracellular matrix proteins are critical in the fields of biomaterials, medicals, and tissue engineering.

2. Materials and methods

2.1. Preparation of peptide arrays covering the amino acid sequence of TRAIL

Peptide library of 8mer peptides overlapping by 6 residues of TRAIL sequence (position 39–281 that is annotated as extracellular domain) was prepared by automated spot synthesis. The cellulose membrane (Whatman, Maidstone, UK) was activated using β-alanine

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Abbreviations: Calcein AM, calcein acetoxymethyl ester; MAP, multiple antigenic peptide; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand

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to introduce amino group and Fmoc-amino undecanoic acid was introduced as the spacer. Standard Fmoc-chemistry was used in accordance with the manufacturer's instructions (Intavis, Köln, Germany), with some modifications. The activated amino acids were spotted using a peptide auto-spotter (model ASP222, Intavis). Activated amino acids at a concentration of 0.25 M were spotted at 80 mm intervals. The membrane was washed with *N*,*N*′-dimethyl formamide, and deprotected with 20% piperidine. Additional washes were performed with *N*,*N*′-dimethyl formamide, followed by washing with methanol. These steps were repeated in this order at every elongation step. The removal of the side-chain protecting group was carried out with 50% trifluoroacetic acid, 3% tri-isopropylsilane, 2% water in dichloromethane for 3 h. After removal of protecting group, membrane was washed several times with diethyl ether and methanol, and was allowed to dry.

2.2. Cell assay with peptide array

The synthesized peptide array was washed for more than 12 h with phosphate buffered saline, and then soaked in methanol for sterilization. After the array allowed drying under sterile condition, peptide spots were punched out and settled in the 96-well plate. Jurkat cells at cell concentration of 2.0×10^5 cells/ml were inoculated and cells were incubated for 48 h on peptide bound membrane without injection of serum. One hour before the time point of measurement, calcein acetoxymethyl ester (calcein AM, Molecular Probes Inc., Eugene, OR, USA) that is an indicator for living cells was applied to a final concentration of 5 μM. Fluorescent intensity was measured by fluorescence plate reader (type 374, Fluoroskan Ascent, Labsystems, Helsinki, Finland). For each peptide, triplet spots were synthesized on an array. Relative fluorescent intensity was calculated using the no-peptide bound membrane as the control.

The effectiveness of screened peptides was also examined with soluble peptides. Synthesized linear peptide (purity above 80%) and multiple antigenic peptide (MAP) were obtained from Operon Biotechnologies Inc. (Tokyo, Japan). Tetra-branching MAP is a multimerized peptide with carboxyl terminal linkages of poly-lysine and used to higher peptide density interact with cell surface. Fluorescent intensity of cells after incubation for 24 and 48 h was measured using calcein AM.

2.3. Cell death analysis

Apoptotic cells were detected by DNA staining using Hoechst 33258. Jurkat cells (1.0×10^5 cells/ml) were incubated with 2 mM peptide No. 95 and 0.1 mM MAP form of peptide No. 95 for 5 h. The cells were collected and resuspended in 10 μM Hoechst 33258 dissolved in phosphate buffered saline. Apoptotic cell stain was also performed using annexin V-Alexa Fluor 488 binding to phosphatidylserine expressed on the cell membrane in the early phase of apoptosis using Vybrant Apoptosis kit #2 (Molecular Probes Inc.). The cells were collected and resuspended in annexin-binding buffer containing calcium, annexin V-Alexa Fluor 488, and propidium iodide. After 15-min incubation, cells were observed using fluorescent microscope (model IX71, Olympus, Tokyo, Japan). TRAIL (100 ng/ml) was used as a control apoptotic cells.

2.4. Compositional characterization of cell death inducible peptides

Characterization of cell death inducible peptides was performed with five site that show decreased fluorescent intensity with 13 peptide (Nos. 6–9, 18–20, 66, 81, and 93–96). The number of occurrences of each amino acid in the sequence of $TRAIL_{39–281}$ (extracellular) and in the site that revealed cell death effect was enumerated.

3. Results

3.1. Screening of cell death inducible peptide using 8mer peptide library of TRAIL peptide-scan

Octameric peptide library covering extracellular domain sequence of TRAIL was prepared with and without introducing Fmoc-amino undecanoic acid as a spacer. Significant decrease in relative fluorescent intensity of Jurkat cells stained with calcein AM after 48-h incubation on peptide disk was not observed without introducing the spacer. In contrast, relative fluorescent intensity distinctly decreased at some peptides. These results indicate that screening of cell death inducible peptides can be performed by the direct cell assay using peptide array when introducing the spacer that would increase the flexibility of short peptides bound to cellulose membrane.

Detailed cell assay was performed with 8mer peptide library of TRAIL peptide-scan. The average fluorescent intensity of 106 peptides that did not reveal cell death effect (negative peptides) was 0.86 ± 0.22. Table 1 shows the sequence of peptides with relative fluorescent intensity below 0.50. Five sites that revealed low fluorescent intensity were found; peptide number 6–9 at $TRAIL_{49–62}$ (YSKSGIACFLKEDD), Nos. 18–20 at $TRAIL_{73–84}$ (MNSPCWQV KWQL), No. 66 at $TRAIL_{169–175}$ (LRNGELVI), No. 81 at $TRAIL_{198–206}$ (NTKNDKQM), and Nos. 93–96 at $TRAIL_{223–236}$ (MKSARNSCWSKDAE) (Fig. 1). Among these peptides, relative fluorescent intensity of peptide Nos. 7, 8 and 95 were below 0.42 (mean value ± 2S.D.) and regarded as statistically low. Peptide No. 95 was 0.21 ± 0.03 and showed the strongest effect. It was shown that cell death inducible peptides were screened from the amino acid sequence of TRAIL using peptide array.

3.2. Soluble peptide effect on Jurkat cells

Fig. 2 shows the effect of screened peptide No. 95 with soluble form. When cells were incubated with soluble RNSCWSKD peptide at 2 mM, cell growth was not observed. With RERGPQRV peptide used as a negative control peptide (No. 39) that revealed no effect in Fig. 1, viable cell number increased after 24- and 48-h incubation.

To further examine the effect of peptide No. 95, cells were incubated with MAP form with four anchoring peptides. With 0.5 mM MAP form of No. 95, the viable cell concentration decreased as with TRAIL (Fig. 3). The effect was further high compared to the 2.0 mM free peptide in Fig. 2. Cell growth was not observed even at 0.01 mM MAP form of No. 95. It was indicated that the MAP form will allow interaction of cells at higher density and thus affected cells effectively. Therefore, it was shown that the screened peptide No. 95 induced cell death in the soluble form and worked effectively in the MAP form.

3.3. Apoptosis analysis of Jurkat cells treated with linear and MAP form of peptide No. 95

To evaluate whether the peptide No. 95 (RNSCWSKD) can induce apoptosis, cells after 5-h incubation with linear and MAP form of peptide No. 95 and without peptide were observed by fluorescent microscope after DNA staining with Hoechst 33258. As shown in Fig. 4, stereotypic morphological changes in nucleus (chromatin condensation) were observed with the

Table 1
Sequence of screened 8mer peptide that induced cell death

Peptide number	Peptide sequence	Relative fluorescent intensity[a]
7.	KSGIACFL	0.39 ± 0.19
8.	GIACFLKE	0.41 ± 0.17
20.	CWQVKWQL	0.50 ± 0.20
81.	NTKNDKQM	0.48 ± 0.26
94.	SARNSCWS	0.45 ± 0.28
95.	RNSCWSKD	0.21 ± 0.03

The average fluorescent intensity of 106 peptides that did not revealed cell death effect (negative peptides) was 0.86 ± 0.22.
[a]Mean value of two different peptide array experiments containing triplicate spots.

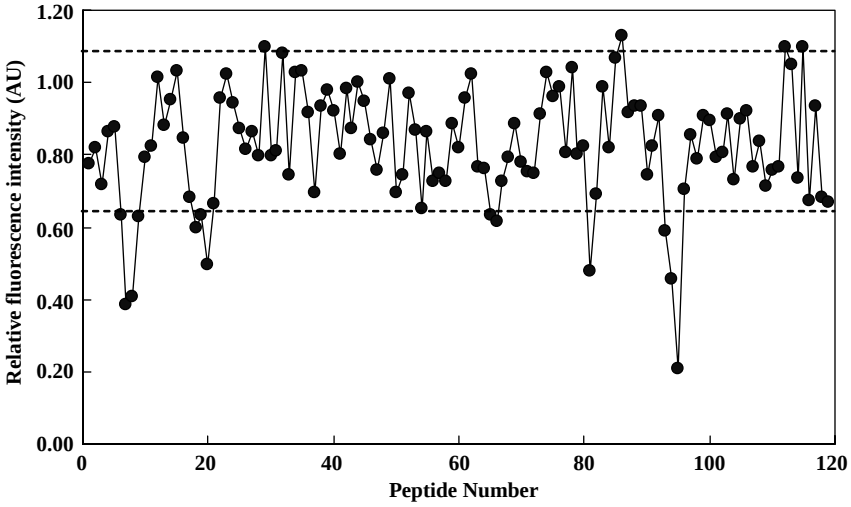

Fig. 1. Screening of cell death inducible peptides using 8mer peptide library of TRAIL-scan. Relative fluorescent intensity of Jurkat cells after 48 h of incubation on peptide disk was measured by staining with calcein AM. No peptide bound disk membrane was used as the control. Each point represents the average value of triplicate spots on two different peptide arrays. Broken lines indicate average relative fluorescent intensity ± S.D.

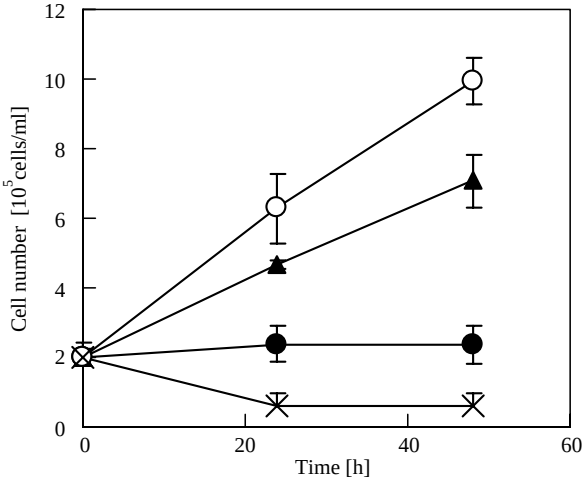

Fig. 2. Effect of synthesized soluble peptide on Jurkat cells. Jurkat cells (2×10^5 cells/ml) were inoculated with 2 mM peptide No. 95 (RNSCWSKD; ●), peptide No. 39 (RERGPQRV; ▲), 3 nM TRAIL (×), and without peptide addition (○).

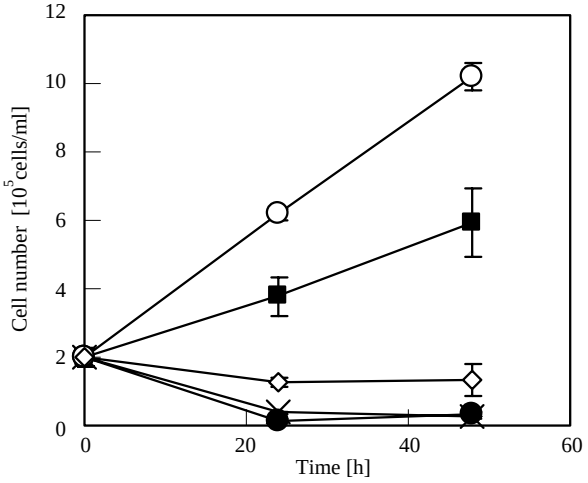

Fig. 3. Effect of RNSCWSKD MAP. Jurkat cells (2×10^5 cells/ml) were inoculated with MAP form (0.5 mM; ●, 0.01 mM; ◇) or linear form (■) of 0.5 mM RNSCWSKD. 3 nM TRAIL (×) and no peptide (○) condition was used as a positive and negative control, respectively.

cells treated with linear and MAP form of peptide 95 as with TRAIL treated cells. The number of cells stained with Hoechst 33258 was higher when cells were treated with MAP form of peptide. Also, phosphatidylserine externalization on the cells membrane was observed using annexin V. Most of cell treated with linear and MAP form of peptide No. 95 were stained with annexin V. Some of the cells were double stained with propidium iodide indicating these cells are in the later phase of apoptosis. These observations indicated that the peptide No. 95 induced apoptosis and its effect was higher in the MAP form.

3.4. Compositional characterization of cell death inducible peptides

Characterization of cell death inducible peptides was performed with 13 peptide sequences that show decreased fluorescent intensity (No. 6–9, 18–20, 66, 81, and 93–96). Fig. 5 shows the number of occurrences of each amino acid in the sequence of $TRAIL_{39–281}$ (extracellular) and in five sites that revealed cell death effect ($TRAIL_{49–62}$, $TRAIL_{73–84}$, $TRAIL_{169–175}$, $TRAIL_{198–206}$, $TRAIL_{223–236}$). From the enumeration, it was revealed that cell death inducible peptides were enriched in cysteine and tryptophan. All of three cysteine residue exists in the TRAIL sequence were included in these sites revealed cell death. Abundance of lysine and serine was high in these sites and $TRAIL_{39–281}$. Also, histidine was underrepresented (nil). Therefore, cysteine was indicated to play as a key residue in the 8mer cell death inducible peptides.

4. Discussion

In assessing the biological activities of small molecules, it is useful to consider not only protein binding but also phenotypic effects. Since peptides can be excellent leads for drug, peptide

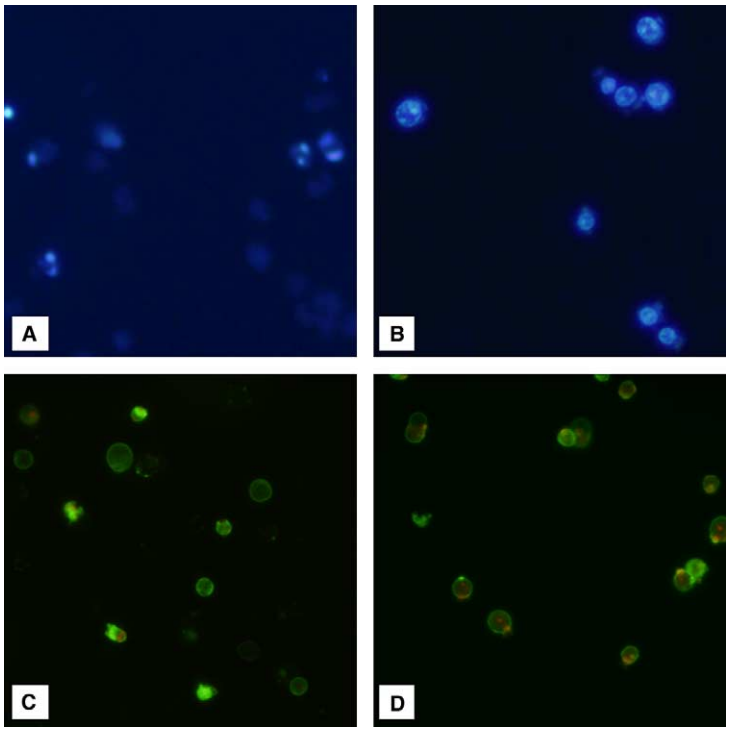

Fig. 4. Fluorescent microscopic observation of Jurkat cells treated with linear (A, C) and MAP form (B, D) of peptide No. 95. (A, B) DNA staining was performed with Hoechst 33258. (C, D) Phosphatidylserine externalization (annexin V-Alexa Fluor 488) and nuclear condensation of cells decreased membrane permeability (propidium iodide).

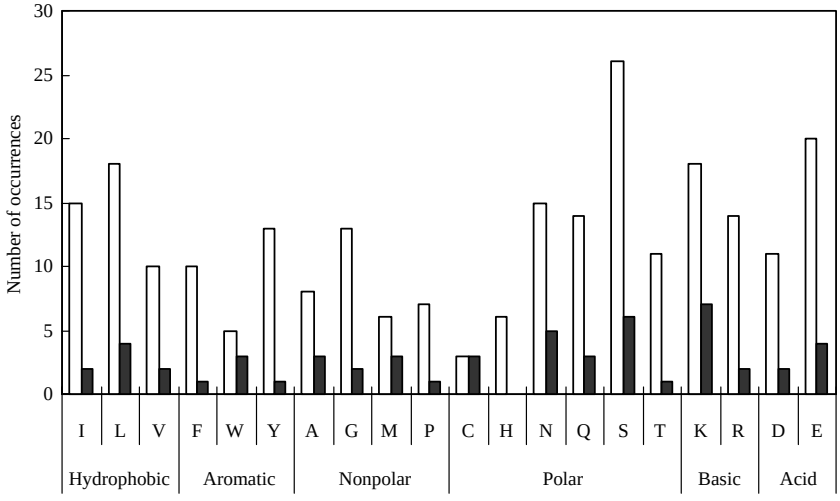

Fig. 5. Compositional characterization of cell death inducible peptides. The number of occurrences of each amino acid in the sequence of $TRAIL_{39–281}$ (□) and in the active site (No. 6–9, 18–20, 66, 81, and 93–96; ■) that revealed cell death effect were enumerated.

array might be an emerging technique for high-throughput screening by their ability to assay both protein interaction and phenotypic effects. Recently, large numbers of peptides synthesized by SPOT technology have been utilized to screen peptide variants with improved antimicrobial activity [13]. A high-throughput screening assay was performed with complete substitution library of the 12mer peptide of bovine bactenecin by cleaving peptides from cellulose support. In the present study, we have investigated the screening of cell death inducible peptides by the direct cell assay without cleaving peptides. Peptides bound at high density (usually approximately 300 nmol/cm^2) on a cellulose support effectively induced cell death. The screened peptide could also affect cells in a soluble form and worked more effectively in the MAP form since peptide density interact to cell surface is higher (Fig. 3).

Recent studies indicated that protein–protein interactions occur through small binding epitopes and small peptides that have high affinities to such binding epitopes may be sufficient for the regulation of large protein–protein interactions [14,15]. Exocyclic peptidomimetics or synthetic peptides corre-

sponding to ligand-binding region of death receptors, DR5, Fas, and TNFR were reported to function effectively and inhibit cell death [14,16,17]. The most effective cell death inducible peptide No. 95 (RNSCWSKD) that indicated apoptotic cell death include C230 exist in the zinc binding site. The zinc ion buried at the trimmer interface is required for maintaining the native structure, stability and biological activity of TRAIL [18–20]. In the site direct mutagenesis of C230A and C230S, apoptotic activity highly decreased. Also, peptide No. 81 (NTKNDKQM) in the AA loop showed deceased fluorescent intensity. The major extension of AA loop is the unique site of TRAIL within the TNF family and it is the site for interaction with DR5 receptor. Also, with our previous study for screening of cell death peptide from human Fas antigen ligand sequence, a 5mer peptide CNNLP that contained P206 reported to involve in the receptor–ligand interaction was found to induce cell death [8]. Therefore, the TRAIL derived peptide No. 95 indicated to induced apoptosis in this study might has the ability to interact with its receptors and can partly substitute for TRAIL function. Future study is necessary to investigate the interaction of TRAIL derived active peptides with DR4 and DR5 receptors and find out the mechanism of inducing apoptosis.

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