Published OnlineFirst November 14, 2011; DOI: 10.1158/1535-7163.MCT-11-0363

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Mol Cancer Ther 2012;11:235-243. Published OnlineFirst November 14, 2011.

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Preclinical Development

Targeting Interleukin-4 Receptor α with Hybrid Peptide for Effective Cancer Therapy

Liying Yang[1], Tomohisa Horibe[1], Masayuki Kohno[1,2], Mari Haramoto[1], Koji Ohara[1], Raj K. Puri[3], and Koji Kawakami[1]

Abstract

Interleukin-4 receptor α (IL-4Rα) chain is highly expressed on the surface of various human solid tumors. We designed a novel hybrid peptide termed IL-4Rα–lytic peptide that targets the IL-4Rα chain. The IL-4Rα–lytic peptide contains a target moiety to bind to IL-4Rα and a cellular toxic lytic peptide that selectively kills cancer cells. The anticancer activity of the IL-4Rα–lytic peptide was evaluated *in vitro* and *in vivo*. It was found that the IL-4Rα–lytic peptide has cytotoxic activity in cancer cell lines expressing IL-4Rα, determined by quantitative real-time PCR. The IC_{50} ratios of the lytic peptide to the IL-4Rα–lytic peptide correlated well with the expression levels of IL-4Rα on cancer cells ($r = 0.80$). In addition, IL-4Rα–lytic peptide administered either intratumoraly or intravenously significantly inhibited tumor growth in xenograft model of human pancreatic cancer (BXPC-3) in mice. These results indicate that the IL-4Rα–lytic peptide generated in this study has a potent and selective anticancer potential against IL-4Rα–positive solid cancers. *Mol Cancer Ther; 11(1); 235–43. ©2011 AACR.*

Introduction

By increasing knowledge of unique or overexpressed cell-surface antigens or receptors on tumor cells as targets, immunotoxin, one of the form of cancer therapy drug, has been developed over the last 3 to 4 decades. Immunotoxins are proteins that are composed of a target binding moiety (an antibody or growth factor that binds specifically to target cells) and a toxin moiety (a plant or bacterial toxin; ref. 1). Some immunotoxins have been tested in clinical trails and they exhibited some efficacy in most tested patients (2–5). An agent ONTAK that contains human interleukin-2 and truncated diphtheria toxin has been approved for use in cutaneous T-cell lymphoma (6).

However, there are concerns of immunogenicity and hepatotoxicity caused by the immunotoxins (7, 8). Moreover, due to their larger molecular sizes compared with chemical compounds or fragment antibody drugs, many immunotoxins might have difficulty in penetration into human tumor mass (6). To reduce immunogenicity caused by the immunotoxins, several approaches have been used for the design of immunotoxins, such as chemical modification with polyethylene glycol (PEGylation) or fusion with a single-chain Fv of an antibody (9, 10). PEGylation not only blocks immunogenicity but also prolongs half-life. But these immunotoxins still have larger molecular weight and are rather difficult to produce in larger scale.

To overcome these problems, a new hybrid peptide drug, which has a similar concept with immunotoxin but smaller molecular weight, has been developed (11). Anticancer hybrid peptide (type I) contains target-binding amino acids and toxic amino acid sequences. These molecules are chemically stable, small, and can be synthesized by simple peptide chemistry (12).

In the toxic part of the hybrid peptide, we have used a new lytic peptide (11), which is stable when combined with targeting peptide with less toxic to normal cell lines when compared with original lytic peptide composed of a 15 amino acid diastereomer composed of D- and L-amino acids (13).

High-affinity interleukin-4 receptor (IL-4R) is highly expressed on the surface of various human solid tumors including renal cell carcinoma, melanoma, breast carcinoma, ovarian carcinoma, glioblastoma, AIDS-related Kaposi's sarcoma, and head and neck squamous cell carcinoma (14–20). IL-4R–targeted protein-based immunotoxin was being tested in the clinic for the treatment of human solid tumors (3, 21, 22). The significance of expression of IL-4R on cancer cells still remains obscure. However, these receptors are able to mediate biological responses in cancer cells such as regulation of

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Note: Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

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doi: 10.1158/1535-7163.MCT-11-0363

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intercellular adhesion molecule-1 and major histocompatibility complex antigen expression, inhibition of cell growth, and induction of apoptosis (23). The IL-4R system exists in 3 different types. Type I IL-4Rs are consisted of a major protein (IL-4Rα) and the IL-2Rγ chain (24, 25). Type II IL-4Rs are composed of IL-4Rα and IL-13Rα1 chains. Type III IL-4Rs express all 3 chains. The IL-4Rs in solid tumor cells are composed of IL-4Rα and IL-13Rα1 chains (type II IL-4Rs; refs. 26–28).

These results prompted us to design a new hybrid peptide targeting IL-4Rα–overexpressing cancer cells, comprising of an IL-4Rα–binding moiety and the cellular membrane lytic moiety, termed IL-4Rα–lytic hybrid peptide. In this study, we examined the selective cytotoxicity of IL-4Rα–lytic hybrid peptide to cancer cells *in vitro* and antitumor activity of the peptide *in vivo*.

Materials and Methods

Cells and cell culture conditions

Human pancreatic cancer cell line (BXPC-3) was purchased from the European Collection of Cell Cultures. Human glioblastoma (T98G and A172), head and neck cancer (KB), pancreatic cancer (SU.86.86.), lung cancer (H322), and breast cancer (MDA-MB-231) cell lines were purchased from the American Type Culture Collection. Human pancreatic epithelium (PE) cell line was purchased from the DS Pharma Biomedical. No authentication of cell lines was done by the authors. Cells were cultured in RPMI-1640 (BXPC-3, A172, MDA-MB-231, KCCT873, SU.86.86., and H322), minimum essential medium (T98G and KB), or CS-C (PE), respectively, and supplemented with 10% FBS (BioWest), 100 units/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque). Cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C.

Peptides

The following peptides were purchased from Invitrogen:

1. Lytic peptide: KL**L**LK**L**L**KK**LLK**L**LKKK (bold and underlined letters are D-amino acids.)
2. IL-4Rα–lytic hybrid peptide: KQLIRFLKRLDRNGG KL**L**LK**L**L**KK**LLK**L**LKKK

All peptides were synthesized by use of solid-phase chemistry, purified to homogeneity (i.e., >80% purity) by reversed-phase high-pressure liquid chromatography, and assessed by mass spectrometry. Peptides were dissolved in water and buffered to pH 7.4.

Cell viability assay

Cells were seeded into 96-well plates at 3×10^3 cells per well in 50 μL medium and incubated at 37°C for 24 hours. The peptides diluted in 50 μL culture medium were added to the cells. After 72 hours of incubation, cell viability determinations using WST-8 solution (Cell Count Reagent SF) were carried out according to the instructions of the manufacturer.

Reverse transcriptase PCR analysis

Total RNA of cells was isolated using NucleoSpin RNA Kits (Macherey-Nagel). Each 0.5 μg of the RNA samples was used for an RT reaction. The reaction was carried out in a final volume of 10 μL of reaction mixture with Rever TraAce RT Kit (TOYOBO). Each 1 μL aliquot of the cDNA samples was amplified in a final volume of 25 μL of PCR mixture containing 12.5 μL Primix (Takara) and 1 μL each of the human IL-4Rα primers (forward 5′-CTGACCTGGAGCAACCCGTATC-3′ reverse 5′-GCAGACGGACAACACGATACAG-3′) or each of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (forward 5′-GTCTTCACCACCATGGAGAAGGCT-3′ reverse 5′-CATGCCAGTGAGCTTCCCGTTCA-3′). GAPDH was used as an internal control. PCR was carried out for 35 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. PCR product was run in a 1% agarose gel for ultraviolet analysis.

Quantitative real-time PCR analysis

Quantitative real-time PCR was carried out using SYBR Green Real-time PCR Master Mix Kit (TOYOBO) at Mx3000P Real-Time QPCR System (Stratagene). Amplification was carried out under the following conditions: 45 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 45 seconds. The primers are same with RT-PCR analysis.

Annexin V analysis

Cells (PE and SU.86.86.) were cultured in 6-well plates for 15 hours and then were treated for 2 hours at 37°C with or without lytic peptide alone or IL-4Rα–lytic peptide at 10 μmol/L. Cells were washed, collected, and the flow cytometry (Becton Dickinson) analysis was conducted with Annexin V–Fluorescein Staining Kit (Wako). Data were analyzed by CellQuest software.

Cell-cycle analysis

Cell-cycle analysis was conducted as described previously (29). Briefly, SU.86.86. cells were seeded into 6-well plates overnight. The cells were then treated with or without IL-4Rα–lytic peptide. Then the cells were collected, washed with PBS, and fixed in ice-cold 70% ethanol at −20°C overnight. After washed twice with PBS, the cell pellet was resuspended in 0.25 mg/mL RNase A (Nacalai Tesque) for 30 minutes at 37°C and in 50 μg/mL propidium iodide (Nacalai Tesque) for 30 minutes at 4°C. The cells were next analyzed with FACS Calibur flow cytometry and Cell Quest software (Becton Dickinson).

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Terminal deoxynucleotidyl transferase–mediated dUTP end labeling assay

SU.86.86. cells were cultured in 6-well plate overnight. After incubation with or without IL-4Rα–lytic peptide, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay was conducted by MEBSTAIN Apoptosis Kit Direct (MBL) using flow cytometry according to the manufacture's instructions.

Antitumor activity of IL-4Rα–lytic in tumor xenografts *in vivo*

Six to 7-week-old female athymic nude mice (BALB/c nu/nu) were obtained from SLC. Human breast tumors were established in nude mice by s.c. injection of 5×10^6 BXPC-3 or MDA-MB-231 cells in 150 μL of PBS into the flank. After 5 days, mice were randomized into 3 groups, and saline (control) or IL-4Rα–lytic peptide (2 or 5 mg/kg) was injected intratumorally (i.t.) or i.v. (50 μL per injection) 3 times a week for 3 weeks. Tumors were measured with a caliper, and the tumor volume was calculated by the following formula: (length of the tumor) × (width of the tumor)^2/2 (11). The significance of differences between groups was determined by Student t test. $P < 0.05$ was considered statistically significant.

Toxicity assessment

For serum chemistry and organ toxicity, serum and tissue samples were obtained 1 day after last injection of the IL-4Rα–lytic hybrid peptide. Organs from these experimental animals were fixed with 10% formalin. Tissue sections (5 μm) prepared from paraffin-embedded blocks were stained with hematoxylin and eosin (H&E). Microscopy analysis was carried out by Olympus DP25 microscopy.

Results

Design of IL-4Rα–lytic peptide

It is known that various solid tumor cells highly express type II IL-4Rs that are composed of IL-4Rα and IL-13Rα1 chains (Fig. 1A) and IL-4Rα chain binds IL-4 with high affinity (K_d 20 to 300 pmol/L). Figure 1B shows the structure in the contact interface between human IL-4 and IL-4Rα. It was previously shown that 5 positively charged residues (K77, R81, K84, R85, and R88) and a neighboring residue (N89) are important for binding of human IL-4 to IL-4Rα (30–32). In addition, it was also reported that in the murine IL-4, the main binding site is 79QRLFRAFR86 and the residues R80, R83, and R86 play a crucial role for binding to IL-4Rα (33). Three arginine

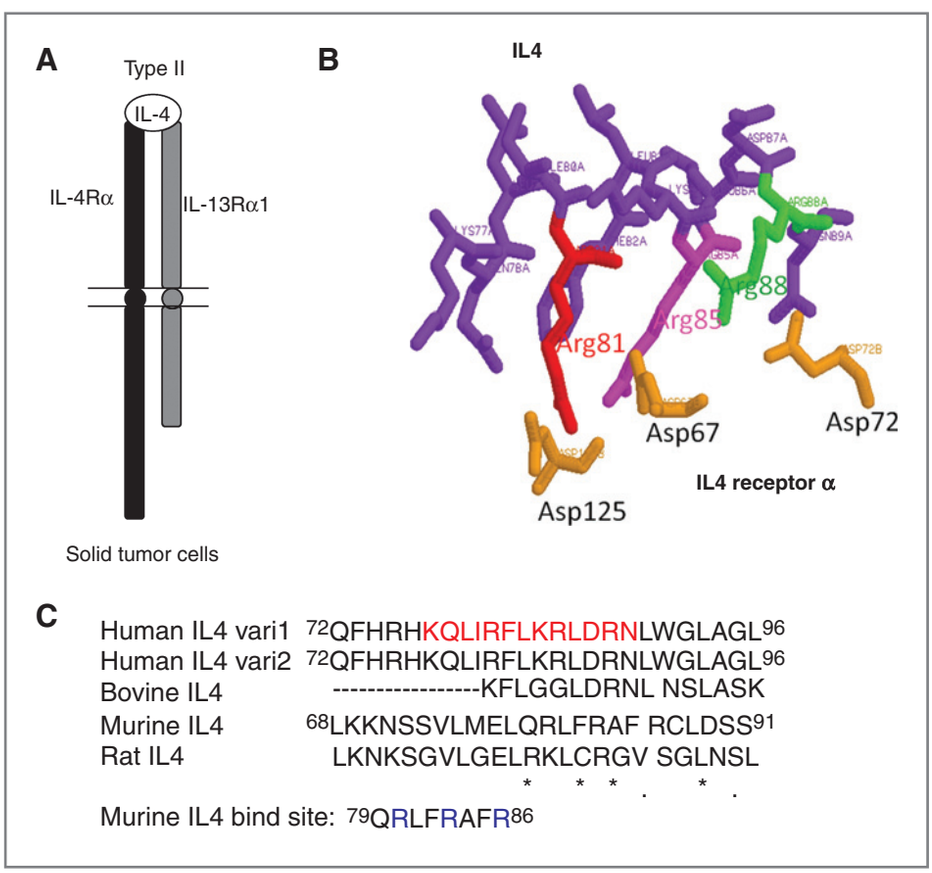

Figure 1. Structures of human IL-4 and IL-4Rα and aligned sequences of mature IL-4. A, schematic model of IL-4R on solid tumor cells. B, structure in the contact interface of human IL-4 and IL-4Rα. Significant residues R81, R85, and R88 of IL-4 for the binding to IL-4Rα are indicated as red, magenta, and green color. The information about structure was obtained from Protein Data Bank (1iar), and stick model is shown using Ras Mol software. C, aligned sequences of mature IL-4. The sequences for mature IL-4 were aligned using the program ClustalW. The red letters are the important sequences for human IL-4 binding to IL-4Rα. The blue letters are the critical residues of murine IL-4 binding to IL-4Rα.

Published OnlineFirst November 14, 2011; DOI: 10.1158/1535-7163.MCT-11-0363

residues R81, R85, and R88 of human IL-4 may mimic those arginine residues of mouse IL-4 in binding to IL-4Rα as shown in the alignment among human, bovine, murine, and rat IL-4 sequences (Fig. 1C). From these results, we have designed an IL-4 peptide, 77KQLIRFLKRLDRN89, which includes the critical amino acids R81, R85, and R88. SPR analysis showed that the designed peptide can bind to recombinant IL-4Rα with the K_d value of 2.90×10^{-4} mol/L by BIACORE system (data not shown). We then produced IL-4Rα–lytic peptide, which contains lytic sequence (11) including 3 glycine as a spacer. Mutation analysis of IL-4Rα–binding peptide also showed that the sequence shown here was the best to achieve the cytotoxic activity to IL-4Rα–expressing cancer cells, as assessed by WST-8 assay (data not shown).

Expression levels of IL-4Rα in normal and cancer cell lines

Normal pancreatic cell line PE and 7 cancer cell lines including BXPC-3 and SU.86.86. pancreatic cancer, KB head and neck cancer, T98G and A172 glioblastoma, H322 lung cancer, and MDA-MB-231 breast cancer were examined for mRNA expression of IL-4Rα by RT-PCR analysis (Fig. 2A). It was found that the normal cell line PE did not express IL-4Rα mRNA. On the contrary, 6 cancer cell lines expressed different levels of IL-4Rα mRNA. To further compare the expression levels of IL-4Rα mRNA in these cells, quantitative real-time PCR was carried out and it was shown that BXPC-3, SU.86.86., T98G, A172, H322, and MDA-MB-231 cell lines expressed high levels of IL-4Rα. On the other hand, KB cells expressed low level of IL-4Rα, and mRNA of IL-4Rα was not found in the normal PE cells (Fig. 2B).

Selective killing of cancer cell lines by IL-4Rα–lytic peptide

To assess the cytotoxic activity of IL-4Rα–lytic peptide, WST-8 assay was conducted with normal and cancer cell lines treated with lytic peptide alone or IL-4Rα–lytic peptide. As shown in Fig. 2C, both lytic peptide and IL-4Rα–lytic peptide induced a concentration-dependent cytotoxicity to BXPC-3 and SU.86.86. cancer cells. Less than 10 μmol/L dose of IL-4Rα–lytic peptide sufficiently induced more than 80% of cell death of BXPC-3 and SU.86.86. Whereas, the same concentration of this peptide did not induce cell killing of normal cells (PE). These results suggest that IL-4Rα–lytic peptide has selective cytotoxic activity to distinguish between normal and cancer cells.

Enhancement of the IL-4Rα–lytic peptide–induced cytotoxicity correlates well with the expression levels of IL-4Rα

The cytotoxic activity of lytic and IL-4Rα–lytic peptides is as shown in Table 1. The cytotoxic activity of hybrid peptide was enhanced when compared with that

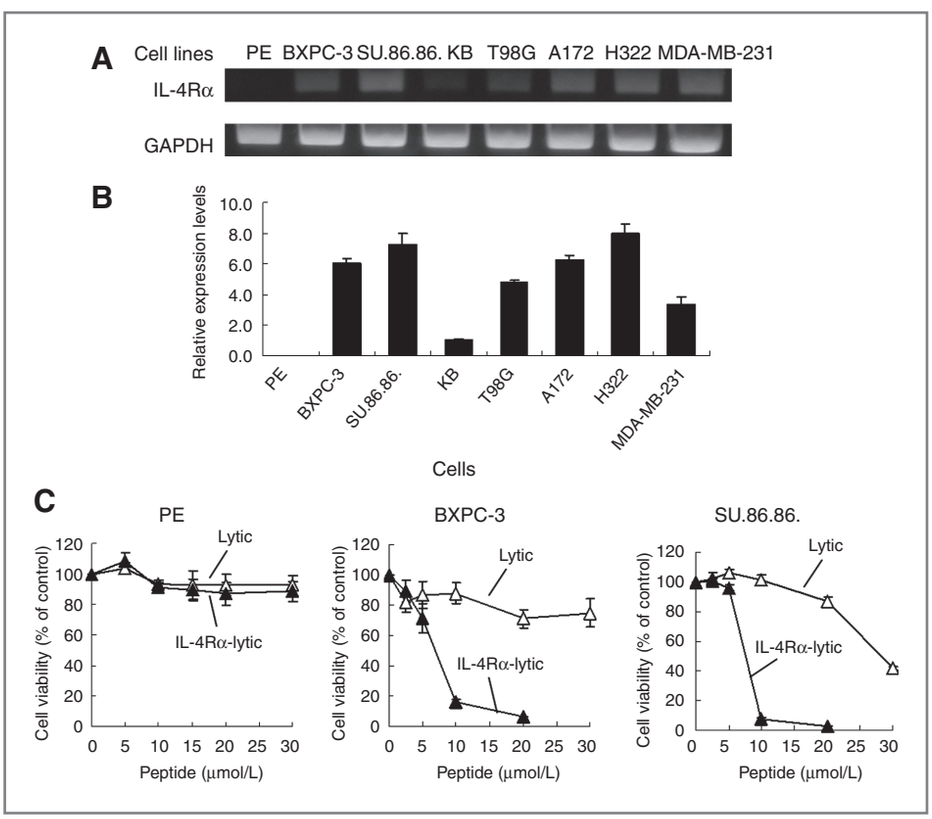

Figure 2. The expression levels of IL-4Rα on normal and cancer cell lines and the cytotoxic activity of IL-4Rα–lytic hybrid peptide. Total RNA from normal (PE) and cancer (BXPC-3, SU.86.86., KB, T98G, A172, H322, and MDA-MB-231) cell lines were reverse transcribed to cDNA, and then assessed by PCR (A) or real time quantitative PCR (B) using IL-4Rα–specific primers. GAPDH served as an internal control. C, cytotoxic activity of IL-4Rα–lytic hybrid peptide to normal and cancer cell lines. The data were expressed as a percentage of the untreated control cells (% of control). The experiments were conducted at least 3 times.

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Table 1. Cytotoxic activity of peptide to various cell lines and IL-4Rα expression

Cell lines	IC_{50} (μmol/L)		IC_{50} ratio lytic/ IL-4Rα–lytic	IL-4Rα relative expression[a]
	lytic Mean ± SD	IL-4Rα–lytic Mean ± SD		
Normal cells				
PE	70.3 ± 2.4	35.2 ± 1.7	2.0	N.D.
Cancer cells				
BXPC-3	37.1 ± 0.7	6.8 ± 0.3	4.7	6.0
SU.86.86.	28.0 ± 0.5	7.5 ± 0.4	3.7	7.3
KB	37.4 ± 0.9	13.2 ± 0.8	2.8	1.0
T98G	77.3 ± 1.7	18.5 ± 0.7	4.2	4.8
A172	30.5 ± 0.9	6.8 ± 0.4	4.5	6.3
H322	18.5 ± 0.4	3.6 ± 0.5	5.1	8.0
MDA-MB-231	27.1 ± 1.5	5.7 ± 0.4	4.8	3.4

Abbreviation: N.D., not detected.

[a]The relative expression was determined by quantitative real-time PCR.

of lytic peptide alone. The IC_{50} (peptide concentration inducing 50% inhibition of control cell growth) of IL-4Rα–lytic peptide improved 2.0- to 5.1-fold. We then examined whether the enhancement of cytotoxicity of IL-4Rα–lytic peptide was correlated with the expression levels of IL-4Rα in the cells. The expression levels of IL-4Rα in cells was correlated well with IC_{50} ratio of lytic peptide to IL-4Rα–lytic peptide ($r = 0.80$, data not shown), indicating the enhancement of cytotoxic activity due to the targeting (IL-4Rα) moiety of hybrid peptide. These results suggest that the increase in cytotoxic activity depends on the expression levels of IL-4Rα in cell.

IL-4Rα–lytic peptide induces rapid killing of cancer cells

We next examined the time course of IL-4Rα–lytic peptide to induce loss of viability of PE normal and BXPC-3 cancer cells. As shown in Fig. 3, 10 μmol/L of IL-4Rα–lytic peptide induced 50% of cancer cell death within 5 to 10 minutes, and 80% of cells were killed by this hybrid peptide after 1 hour, but the same concentration of lytic peptide alone did not induce cytotoxic activity (Fig. 3A). On the contrary, neither lytic peptide alone nor IL-4Rα–lytic peptide did induce optimal cell killing to PE (Fig. 3B). These results suggest that IL-4Rα–lytic hybrid peptide can rapidly and selectively kill cancer cells.

Characterization of cancer cell death mechanism by IL-4Rα–lytic peptide

To reveal the mechanism of cancer cell death induced by IL-4Rα–lytic hybrid peptide, flow cytometry analysis was conducted using Annexin V. As shown in Fig. 4A, Annexin V–positive cells were found when 10 μmol/L of IL-4Rα–lytic peptide was added to SU.86.86. cells. The percentage of Annexin V–positive and PI-negative cells (19.78%, lower right region) of SU.86.86 cells treated with IL-4Rα–lytic peptide was higher than that of the control (11%), and the percentage of Annexin V- and PI-positive cells (59.54%) was remarkably higher than the control (3.67%). However, the percentage of Annexin V–positive cells of SU.86.86 cells treated with lytic peptide was not significantly different from that of the control. Treatment

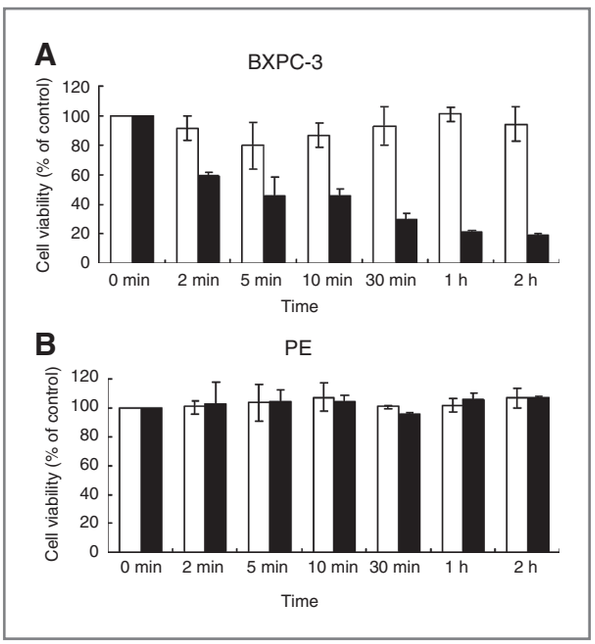

Figure 3. Rapid killing of cancer cells by IL-4Rα–lytic hybrid peptide. BXPC-3 (A) or PE (B) cells were treated with IL-4Rα–lytic hybrid peptide (10 μmol/L; black columns) or lytic peptide alone (10 μmol/L; white columns) for indicated time periods. Cell viability was determined by WST-8. The results are represented as means ± SD (bars).

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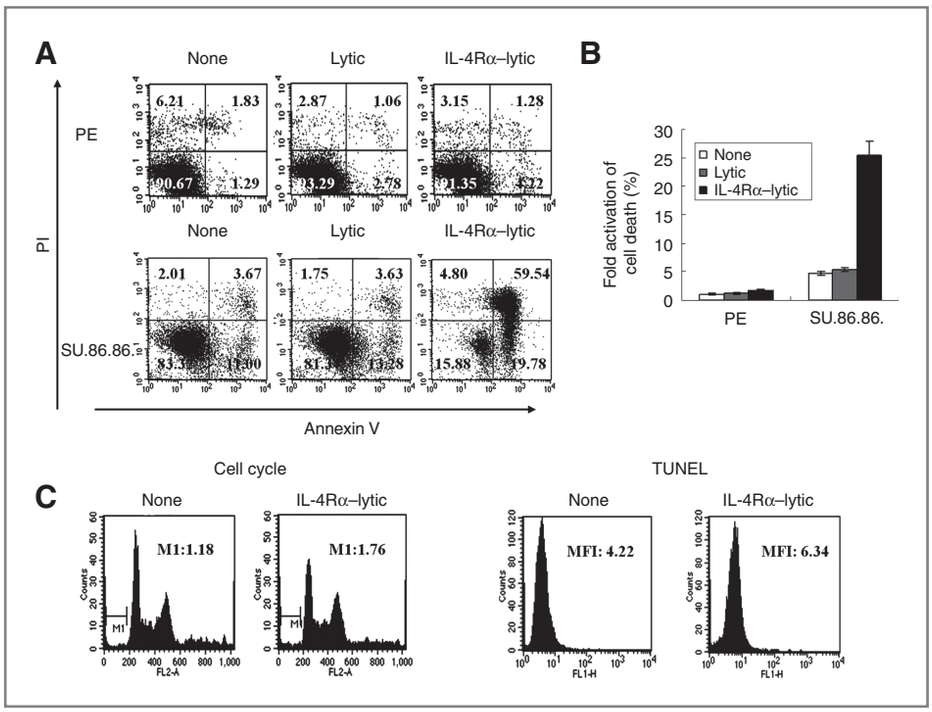

Figure 4. Characterization of cancer cell death mechanism by IL-4Rα–lytic hybrid peptide. A, Annexin V assay. PE or SU.86.86. cells treated with or without lytic or IL-4Rα–lytic peptide (10 μmol/L) for 2 hours were analyzed by flow cytometry for Annexin V and propidium iodide (PI) staining. The percentage of cells in each quadrant is as indicated. B, fold activation of cell death compared with untreated PE cells. The results were represented as the fold increase of untreated dead PE cells (Annexin V–positive cells) as control. C, cell-cycle and TUNEL assay. SU.86.86. cells were treated with or without IL-4Rα–lytic peptide (3 μmol/L) for 1 hour. The percentage of cells in the sub-G_1 population of the cell cycle and the intensity of TUNEL signal (MFI) were determined by flow cytometry.

of PE cells with lytic peptide alone or IL-4Rα–lytic peptide (10 μmol/L) for 2 hours did not increase Annexin V–positive cells at all. The ratio of Annexin V–positive cell percentage by either lytic peptide alone or IL-4Rα–lytic peptide when compared with untreated normal cells (PE) as control was 1.23 (PE) and 5.44 (SU.86.86.; lytic peptide alone), and 1.77 (PE) and 25.50 (SU.86.86.; IL-4Rα–lytic peptide), respectively (Fig. 4B). To further clarify the characterization of cancer cell death induced by IL-4Rα–lytic peptide, cell-cycle analysis and TUNEL assay were conducted. When SU.86.86. cells were treated with IL-4Rα–lytic peptide for 16 hours, the percentage of sub-G_1 was increased from 6.78% to 19.17%, and the mean fluorescent intensity (MFI) was increased from 2.19% to 42.54%, compared to untreated cells (Supplementary Fig. S1A). On the other hand, there was no significant difference on the percentage of sub-G1 and the value of MFI between treated and untreated cells after 1 hour exposure to the same concentration of IL-4Rα–lytic peptide (Fig. 4C). Furthermore, it was found that cell viability was quickly decreased within 1 hour after the treatment with IL-4Rα–lytic hybrid peptide (Supplementary Fig. S1B). Taken together with these results, it is suggested that the increases in the percentage of sub-G_1 population and the MFI value of TUNEL assay were induced by secondary effect after rapid cancer cell death with IL-4Rα–lytic hybrid peptide, and the apoptotic cell death induced by IL-4Rα–lytic hybrid peptide is not primary.

Antitumor activity of IL-4Rα–lytic peptide *in vivo*

In vitro experiments indicated that IL-4Rα–positive cancer cell lines are highly sensitive to IL-4Rα–lytic peptide. To assess antitumor activity of this hybrid peptide *in vivo*, nude mice received s.c. injections of 5×10^6 BXPC-3 cells. The efficacy of IL-4Rα–lytic peptide, when administered by different routes, was evaluated in these mice. Intratumoral administration of IL-4Rα–lytic peptide (2 or 5 mg/kg, 3 times a week) significantly inhibited tumor growth. As shown in Fig. 5A (left graph), tumors in saline-treated control mice grew aggressively, reached 979 mm^3 by day 59. On the contrary, animals treated with IL-4Rα–lytic peptide showed significant tumor regression at both dosages, the mean tumor sizes were 553 mm^3 (2 mg/kg) and 234 mm^3 (5 mg/kg) in the treated mice on day 59. Intravenous treatment also showed antitumor activity. As shown in Fig. 5A (right graph), the effect of IL-4Rα–lytic peptide was clearly dose dependent. Two mg/kg dose of the treatment was less effective against BXPC-3 tumor growth. The mean tumor volume was 752 mm^3 on day 59, which is smaller than control tumor volume (1,182 mm^3, $P < 0.05$). Five mg/kg dose of IL-4Rα–lytic peptide exhibited superior antitumor activity. The mean tumor volume of treated tumors was 402 mm^3, which is significantly smaller than the control tumor at day 59 ($P < 0.05$).

To preliminarily assess peptide drug-related organ toxicities, analyses of complete blood counts and blood serum chemistry, and pathology experiments of major organs (including liver and kidney) were conducted with animals receiving i.t. or i.v. administration of IL-4Rα–lytic peptide. Samples were obtained 1 day after the last injection of drug. In blood examination, animals treated with 5 mg/kg dose of IL-4Rα–lytic peptide by i.v. route showed a minor decline of white blood cell count compared with the control mice. No other abnormality was observed in mice

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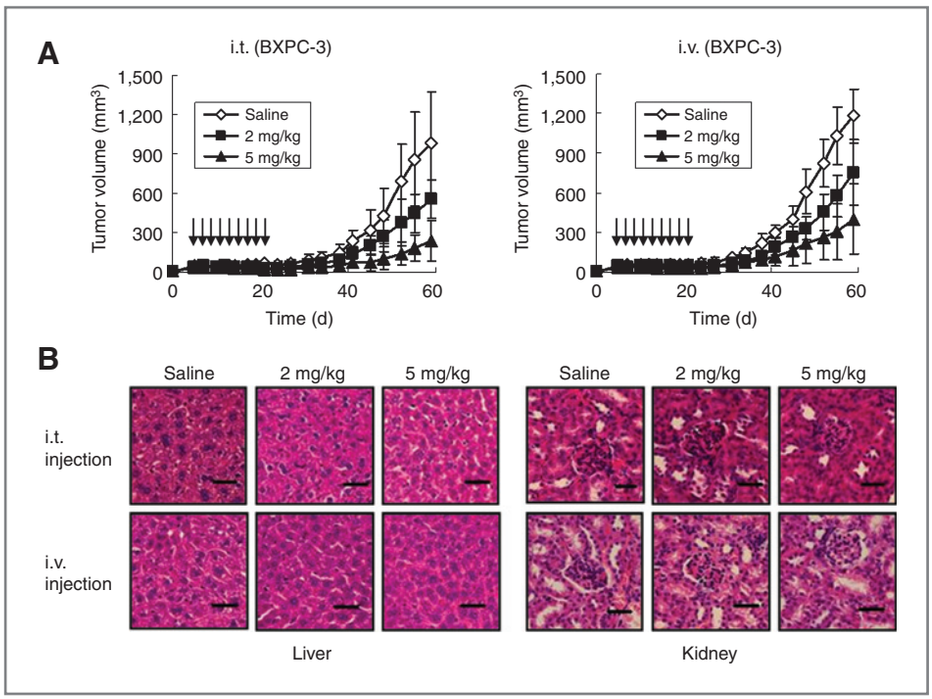

Figure 5. Antitumor activity of IL-4Rα–lytic hybrid peptide *in vivo*. BXPC-3 pancretic cancer cells were implanted subcutaneously into athymic nude mice. Intratumoral (i.t.; A, left) or intravenous (i.v.; A, right) injection of either saline or IL-4Rα–lytic hybrid peptide (2 or 5 mg/kg) was provided 3 times per week from the 5th day for 3 weeks as indicated by the arrows. Each group had 6 animals ($n = 6$). Data are expressed as mean ± SD (bars). B, liver and kidney organs obtained from the mice treated as above were stained with H&E. Images (magnification, ×400) were obtained using light microscopy. Scale bars, 20 μm.

treated with IL-4Rα–lytic peptide either by i.t. or i.v. routes (data not shown). Microscopic analysis showed no abnormal changes in the major organs tissues obtained from the mice treated with IL-4Rα–lytic either by i.t. or i.v. routes (Fig. 5B shows pictures of liver and kidney). Remarkable loss of body weight was not observed.

On the mouse xenograft model of human breast cancer MDA-MB-231, IL-4Rα–lytic hybrid peptide also showed effective antitumor activity when administered by i.t. or i.v. injection (Supplementary Fig. S2A). No abnormal changes were observed in the major organs tissues obtained from the mice treated with IL-4Rα–lytic hybrid peptide (Supplementary Fig. S2B). In addition, no remarkable loss of body weight was observed.

These results indicate that the IL-4Rα–lytic hybrid peptide selectively targets to cancer cells inducing tumor regression without unexpected organ toxicities.

Discussion

It has been reported that several peptides composed of L-amino acids exhibited cytotoxic activity against cancer cell lines *in vitro* (34, 35), however, most of these L-amino acid–based peptides also affected normal cells, limiting clinical usage. Moreover, some of these L-amino acids peptides failed to exhibit desirable antitumor activity *in vivo*, because these peptides lose cytotoxic activity in serum in the body circulation due to enzymatic degradation and binding to serum components (36). Supporting these reports, in this study, we also found that both a lytic peptide, which is entirely composed of L-amino acids, and a hybrid peptide composed of IL-4Rα binding moiety and the lytic moiety composed of L-amino acids killed normal cell lines at a low concentration *in vitro*, and failed to show antitumor activity *in vivo* (data not shown). N. Papo and colleagues developed a novel lytic peptide composed of DL-amino acids, which selectively killed cancer cells *in vitro* and *in vivo* (13). However, because we found that this lytic sequence was not suitable to combine with targeting moiety, we modified the DL-amino acids sequence to appropriately induce modest cancer cells killing, with less toxicity to normal cells in a lower concentration (11). Similar to the lytic peptide previously reported, the new lytic sequence has positive charge and binds to negatively charged membranes (37) and subsequently lyses them (38). It is known that the outer membrane of cancer cells contains a slightly more negatively charged phosphatidylserine than that of normal cells (39). This fact probably, at least partly, contributes to the selectively killing cancer cells of lytic peptide.

By using peptide phage display and molecular modeling, G. Yao and colleagues have showed that in murine the amino acid residues spanning from 76 to 86 (QRLFRAFR) especially the residues R80, R83, and R86 play a crucial role in binding to the IL-4Rα chain (39). It has also been shown that residues K77, R81, K84, R85, R88, and N89 are important for binding of human IL-4 to IL-4R (31, 32). Three arginine residues R83, R85, and R88 on human IL-4 may mimic arginine residues R80, R83, and R86 on mouse IL-4 in binding to IL-4Rα. Taken together, we hypothesize that 77KQLIRFLKRLDRN89 peptide, the IL-4Rα moiety designed in this study, can specifically bind to IL-4Rα on cells. As shown in Fig. 2, IL-4Rα–lytic peptide exhibited enhanced cytotoxic activity to cancer

Published OnlineFirst November 14, 2011; DOI: 10.1158/1535-7163.MCT-11-0363

cells expressing IL-4Rα *in vitro* when compared with lytic peptide alone. The enhancement of the cytotoxic activity against cancer cells depended on the expression levels of IL-4Rα on the cells. Normal cell PE without expressing IL-4Rα is found not sensitive to IL-4Rα–lytic peptide. These results suggest that the binding moiety peptide designed in this study can specifically bind to IL-4Rα in cells.

Although increase in the percentage of sub-G_1 population and TUNEL-positive cells were found after 16 hours treatment with IL-4Rα–lytic peptide, these positive cells were not almost found after 1-hour treatment with this peptide (Fig. 4C and Supplementary Fig. S2A). Because IL-4Rα–lytic peptide quickly induced cancer cell death (Fig. 3 and Supplementary Fig. S2B), it is suggested that these apoptotic positive cells were induced by secondary effect, however, the detail mechanism of cancer cell death induced by IL-4Rα–lytic hybrid peptide is still obscure.

It was also found that IL-4Rα–lytic peptide exhibited high cytotoxic activity against cancer cells expressing IL-4Rα *in vitro* (Fig. 2C) and that i.t. administration of this peptide dramatically inhibited the growth of pancreatic cancer BXPC-3 (Fig. 5A) or breast cancer MDA-MB-231 (Supplementary Fig. S2A) tumors *in vivo*. In addition, i.v. administration of this peptide induced dose-dependent regression of BXPC-3 (Fig. 5A) or MDA-MB-231 (Supplementary Fig. S2A) tumors. We assume that because i.t. administration holds the drug locally at higher concentration in tumor, the antitumor effect exhibited was superior. No specific toxicity was found by either i.t. or i.v. administration. Taken together, IL-4Rα–lytic peptide might be a potent anticancer drug to IL-4Rα–expressing solid tumor, under the condition of local administration or the systemic administration in combination with the suitable drug delivery system.

In conclusion, in this study, we described the IL-4Rα–lytic hybrid peptide targeting IL-4Rα in cancer cells. Further analyses to this drug including cytotoxic mechanisms, detailed safety profiles in animals, and justification of the appropriate usage in clinic will be necessary. These researches are currents ongoing in our laboratory.

Disclosure of Potential Conflicts of Interest

Koji Kawakami is a founder and stock holder of Upstream Infinity, Inc. Masayuki Kohno is an employer of Upstream Infinity, Inc. No potential conflicts of interest were disclosed by the other authors.

Acknowledgments

The authors thank Dr. Oumi Nakajima and Jun Suzuki, Department of Pharmacoepidemiology and Department of Medical chemistry, Kyoto University, for valuable advice on animal and flow cytometry experiments, respectively, and also thank Ms. Ritsuko Asai, Nana Kawaguchi, Aya Torisawa, and Kumi Kodama of Kyoto University for technical assistance.

Grant Support

The study was conducted by a research fund from Olympus Corporation.

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Received May 18, 2011; revised October 13, 2011; accepted October 19, 2011; published OnlineFirst November 14, 2011.

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