Purification of syncytia-producing peptide as a potential therapeutic agent for cancer and viral infection

Michiko Koga¹*, Sokoku Tei¹, Manabu Watanabe¹, Sakiko Sato¹, Kazuhiro Tanabe²,³

¹Life Science Institute, Sunkokai Medical Corporation, Shizuoka, Japan
²Medical Solution Promotion Department, Medical Solution Segment, LSI Medience Corporation
³Kyushu Pro Search Limited Liability Partnership

*Correspondence: K.M. (m-kohga@sunkohkai.or.jp)
Virus-induced syncytium formation, or cell fusion, has been investigated as a potential therapeutic approach for cancer. HVJ virus infection or transfection of membrane glycoproteins of virions have been known to cause syncytium formation in affected cells. Also, it was known that other enveloped viruses such as Herpes viruses, leukaemia viruses induce cell fusion. However, substances or molecules that directly cause syncytium formation have not been identified to date. Here, we identify a peptide that efficiently induces syncytia and report its structure, as an actionable therapeutic agent for cancer and viral infection. We purified and identified the fusion factor from the exosomes of the cells infected with murine leukaemia virus but not producing viruses by column chromatography, mass spectrometry, and amino acid analyses. We confirmed the peptide purified from the cell culture media and synthesized peptides induce syncytia as well as the murine leukaemia viruses, or the membranes or exosomes of MuLV infected cell lines in RFL cells and several cancer cell lines leading to apoptosis. And this peptide suppresses in vivo growth of cancer cells significantly. Furthermore, we found the synthesized peptide can cause fusion of enveloped virions as well as virus infected cells or cancer cell lines. These results nominate the use of this peptide as a potentially promising therapeutic approach for cancer and viral infection through efficient induction of syncytium formation followed apoptosis.
Cell fusion occurs in wide varieties of essential biological phenomena such as endocytosis, exocytosis, phagocytosis, fertilization and maturation of striated muscles\textsuperscript{1}. Furthermore, cell fusion is very useful for making hybrid cells and genetic analyses, and has potential of further applications such as gene transfers\textsuperscript{2}. Although cell fusion can cause tumour progression through inducing aneuploidy and genomic instability\textsuperscript{1,3-7}, implication of the utility of cell fusion for cancer treatment exists\textsuperscript{1,8,9}. We previously reported that syncytium formation of RFL cells, which are fibroblasts derived from the lungs of WKA rat embryo, was induced syncytia by infection of murine leukaemia viruses (MuLVs)\textsuperscript{10,11}. Cell fusion induced by viruses which has envelopes composed of lipids and proteins, such as paramyxoviruses, orthomyxoviruses\textsuperscript{12}, herpesvirus\textsuperscript{13,14} and retroviruses(15-20), has been widely studied as well. It has been shown that this cell fusion activity was related to the glycoproteins in the envelope. However, the actual substances that induce cell fusion have not been identified\textsuperscript{20,21}.

**Cell lines**

RFL cell line, an established cell line derived from the embryonal lung of WKA rats, was obtained from R. Takaki, School of Medicine, Kyusyu University. RM4 cells were obtained from a clone of RFL cells infected with Moloney-MuLV which was obtained from N. Ida, Toyo
Kogyo Hospital. LLC, CW2, B1203L, and A549 cell lines were obtained from Cell Bank, RIKEN BioResource Research Centre. (Tokyo, Japan). RM4 cells induced syncytia with RFL cells, but don’t fuse each other, the RM4 cells resistant against fusion factor of themselves.

All cell lines were maintained in RPMI-1640 medium (Wako, 189-02025) supplemented with 5% FBS (Biosera, 0158S493) at 37°C in a humidified incubator with 5% CO2.

**Treatment with RM4 cell’s conditioned media and the RM4 plasma membrane.**

RFL cells were induced syncytium by cocultivation with the RM4 cells and cell membranes of RM4 cells and exosomes in the culture medium of RM4 cells. We tried to purify the fusion factor from the exosomes produced by RM4 cells\textsuperscript{12}.

**Purification of a syncytium-inducing peptide and determination of amino acids arrangement from the exosomes produced RM4 cells.**

Exosomes derived from RM4 cells were collected by filtration of the RM4-conditioned media through Amicon Ultra-15 filters (Merk Millipore LTD.). The collected exosomes were treated with 70% acetone at room temperature and the supernatant which showed the activity of syncytium formation was concentrated by evaporation. The supernatant was applied on QAE
Sephadex C-25 anion exchanger and SP Sephadex C-25 cation exchanger (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Then the solubilized fraction which has the activity of syncytium formation was applied on Hitachi LaChrom Elite HPLC system Pump L-2130 (Hitachi, Co. Ltd, Tokyo, Japan) using X Bridge 18 column (Waters, Ireland) and eluted by 10% Acetonitrile. The fraction which has the activity of syncytia formation was further analysed by mass spectrometry (Shimadzu LC-10A). The LC-MS data were acquired on a liquid chromatography system (Agilent HP1200, Agilent Technologies, Palo Alto, CA) equipped with a C18 column (CAPCELL PAK C18 IF, 2 μm, 50 mm × 2.0 mm ID, Shiseido, Tokyo, Japan) coupled with an electrospray ionization quadrupole time-of-flight (Q-TOF) mass spectrometer (Agilent 6520, Agilent Technologies, Palo Alto, CA, USA). Solvent A was 5 mM ammonium acetate in water, and solvent B was acetonitrile. The target peptides were eluted at a flow rate of 0.2 mL/min at 40°C with a following gradient program: 0 to 10 min, 10% to 100% solvent B; and 5 min hold at 100% solvent B. The mass spectrometer was operated in both positive and negative mode with the capillary voltage of 3500 V. The nebulizing gas pressure was 30 psi and the dry gas flow rate was 8 L/min at 350°C.

The injection volume was 10 μL. MS/MS spectra to identify the target peptides which were also obtained with QTOF mass spectrometer. Detected range was set from m/z 70 to 3000. Liquid chromatography (Fig.1a) and mass spectrometry (Fig.1b) of the concentrated supernatant of the
solubilized RM4-derived exosomes revealed that certain peptides were enriched in the samples.

We analysed the amino acid sequence of the enriched peptides and identified the peptide sequence as CH3-Pro-Ile-Val-Ser-Gln-Thr-Thr-Ala-Ile-Ala.

The synthetic peptide induces syncytia as well as purified peptide from culture media.

To validate this purified peptide is responsible for syncytium formation, we synthesized the peptide of the same sequence. The synthetic peptide as well as the plasma membrane or the exosomes derived from RM4 cells induced syncytia formation in RFL cells (Fig. 2a). RM4 cells and murine Lewis lung carcinoma (LLC) cells formed syncytia after treatment with this synthesized peptide (Fig. 2b). These results were complicated, because RM4 cells did not form syncytia with RM4 cells itself, cell membranes or exosomes derived from RM4 cells. The reason why only purified peptide or, synthesized peptide induced syncytia in RM4 cells and several cancer cells is unknown. There may be exist protective mechanism in the membrane to fuse each other or by exosomes of themselves.

Syncytium formation assay

For the syncytium formation assay, 8x10^4 of RFL or LLC cells per 0.25 ml of the media were plated in each well of 24-well plate (Iwaki 2820-024). After 30min, the media was replaced by
50 µL of the media containing the peptide (1µg/ml). After 16 to 24 hours, the cells were fixed with methanol (Wako, Japan) and Giemsa staining (Muto Chemistry, 15003) was performed according to the manufacturer’s instruction.

The peptide induces apoptosis after syncytium formation

It has been shown that viral-induced syncytium formation lead to apoptosis. Thus we investigated if the treatment with the synthetic peptide lead to apoptosis of the treated cells by measuring caspases and annexin V. Caspase 3/4 and annexin V were increased in RFL cells after treatment with the synthetic peptide (Fig. 2c-e). Induction of apoptosis by the peptides in dose dependent manner were confirmed in RFL cells and RM4 cells (Fig. 2f, g), as well as several cancer cells lines, such as LLC which is a mouse lung cancer cell, CW2 which is a human colon adenocarcinoma cell line, and B1203L which is a human lung squamous cell carcinoma cell line (Fig. 2h-j). The sensitivity of apoptosis followed by syncytium formation by the synthetic peptides were higher in cancer cell lines and RM4, which are viral infected cells, as compared with RFL cells, which are non-viral infected, non-cancer cells (Fig. 2f-j), suggesting existence of therapeutic windows of these synthetic peptides against cancer and viral infection.

The peptide induced fusion in viruses as well as cells
HVJ viruses, obtained Ishihara Sangyo Kaisya LTD, and RFL cells were observed with electron microscope (JEM-1400Plus; JEOL Ltd, Tokyo, Japan) after treatment by synthesized peptides. Fig. 4a shows that HVJ viruses fused each other after treatment with the peptide. In Fig 4b shows the syncytia of RFL cells after treatment of the peptide.

**Measurement of the apoptosis by the synthesized peptides.**

The cells were incubated in IncuCyte® SX1 Live-Cell Analysis System (Essen BioScience, Sartorius Company) at 37°C, 5% CO2. IncuCyte® Caspase-3/7 reagent is DEVD group. When an activated Caspase-3/7 cut the DEVD group reagent, the nuclei of apoptosis cells are put on green fluorescence. Each cell was plated in 96 well plates (Iwaki, 3869-096, Japan) as 5000 cells/well. After 1 hour, the medium of each well were removed and synthesized peptide and 1000 times diluted solution of Casparse-3/7 Green Reagent (SARTORIUS, 4440, Unit size: 20ul, 5mM/vial) with RPMI-1640 media (Wako, 189-02025) containing 5% fetal bovine serum (Chile Origin, USDA approved) (Biosella, Noailles, France).

**The peptide inhibits tumour growth in vivo**

To further evaluate in vivo anti-tumour efficacy of the synthetic peptides, we treated nude mice harbouring subcutaneous tumours of B1203L, a human lung cancer cell line. Tumour growth
was significantly suppressed in the peptide-treated group compared with the vehicle-treated group (Fig. 3a, b) without affecting the body weight of the animals (Fig. 3c). Histological examination of the tumours presented highly cellular tumours in the vehicle-treated group (Fig. 3d) and prominent necrosis in the peptide-treated group (Fig. 3e), suggesting \textit{in vivo} anti-tumour efficacy of this synthetic peptide.

\textbf{Treatment of subcutaneous tumour growth by synthesized peptide}

The antitumor experiment in mice were performed by HAMRI Co. LTD (Tsukuba, Japan). The human lung cancer cell line B1203L was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT/AMED, Japan. Male mice (CAnN.Cg-Foxnl\textsuperscript{nu} / CrlCrlj) were obtained from Japan Charles River Co.LTD. 4x10\textsuperscript{6} cells of B1203L cells suspended in 0.1 mL of phosphate buffered saline were injected in the right flank of six nude mice for each of the treatment group and the vehicle group. Either 25mg/kg of the peptide or the vehicle was administered intravenously from the tail veins every 3 days starting on 14 days after tumour inoculation. The volume of the subcutaneous tumours was measured by a vernier callipers.

The animals were euthanized 38 days after tumour implantation and the subcutaneous tumours were excised and weighed. The body weight of all the animals were measured until the end of the experiment. The body weight was not affected by peptide treatment, suggesting that the peptide has not toxicity. The peptide- treated group exhibits necrosis, but vehicle-treated
group does not exhibit necrosis., and there are many live cancer cells. The cancer growth in the nude mice was significantly suppressed in the peptide-treated group.

Discussion

In this study we have identified the substance that induce syncytium formation in several cell lines including viral infected cells and cancer cell lines from the exosome in the culture media of MuLV infected cells. Our findings reveal that the short peptide derived from the MuLV-infected cells, which can be artificially synthesized, is an actionable factor that induce syncytium formation and apoptosis in several cell lines with higher sensitivity in cancer and virus-infected cells. In RFL cells MuLV, RM4cells, cell membranes of RM4 cells, exosomes in culture medium of RM4 cells and purified fusion peptide and synthesized peptide, all can induce syncytia. But in RM4cells or cancer cells only this peptide can induce syncytia.

The reason why RM4 cells and HVJ virions which has fusion factor in the membranes did not fuse each other, and only this peptide can induce syncytia is under investigation. This Peptide may subversive the harmony of the membranes of RM4 cells and cancer cells/.

The background mechanisms of this variability in the sensitivity against this peptide among different cell lines are further to be investigated. Nonetheless, this peptide significantly
suppressed the tumour growth in the nude mice. Consequently, this peptide could serve as a tool for future therapeutics of neoplastic diseases and viral infections.

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Author contributions

M.K. conceived the study design. M.K., S.T., and M.S. performed experiments, analysed and interpreted data. M.K. wrote the manuscript, and all authors edited and approved the manuscript.

Competing interests

M.K. is founder, and S.T. and M.W. are employees of Sunkokai Medical Corporation.

Correspondence and request for materials should be addressed to M.K.
Fig. 1

Fig. 1a Purification and identification of a fusion factor.

Liquid chromatography of purified fusion factor.

Fig. 1b Liquid chromatography and mass spectrometry (LC-MS).

EIC chromatogram

MSMS chromatogram
Fig. 2

a. Control RF cells

b. Syncytia in RFL cells

c. Control RM4 cells

d. Syncytia in RM4 cells

e. LLC

f. RFL Cells after inoculation of peptide caspase 3-7

g. RFL

h. RM4

i. LLC

j. CW2

k. B1203L
Fig. 2 The synthetic peptide induces syncytium formation and apoptosis. a, b, Giemsa staining of RFL cells (a), and LLC cells (b), 18 hours after treatment with the synthetic peptide. c, A representative image of caspase 3/7 and annexin V detection in the RFL cells treated with the synthetic peptide for 18 hours. Green and red indicate caspase 3/7, and annexin V, respectively. d, e, Time course quantification of caspase 3/7 (d) and annexin V (e) in RFL cells up to 72 hours after application of the synthetic peptide. f-j, Time course quantification of caspase 3/7 in RFL (f), RM4 (g), LLC (h), CW2 (i), and B1203 (j) cell lines up to 72 hours after application of the synthetic peptide.
Fig. 3 The synthetic peptide inhibits tumour growth in vivo.  

a, Volume of A549 subcutaneous tumour with vehicle and the synthetic peptide administration.  
b, Weight of the A549 subcutaneous 38 days after tumour inoculation in the vehicle-treated and the peptide-treated group.  
c, The weight change of animals treated with vehicle and the synthetic peptide.  
d, e, Haematoxylin Eosin staining of the tumour sections from the vehicle-treated (d) and the peptide-treated group (e).
Fig. 4 The syncytium formation of RFL cells and fusion of viral membrane of HVJ viruses by the peptide.

a, electron micrograph of HVJ viruses treated with the peptide. b, electron micrograph of syncytia of RFL cells treated by the peptide for 1 hour.