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Anti-tumor Activity of an Antibiotic Peptide Derived from Apoprotein E

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Abstract. *Background: Recently, we found that a 30-mer peptide derived from apoprotein E (apoE) 133-162 has antibiotic activity that is comparable with the classic antibiotics and neutrophil-derived antibiotic peptide. In this study, we tested if apoE 133-162 also has anti-tumor activity against several cancer cell lines. Materials and Methods: Two gastric cancer cell lines (MKN-7, MNN-1), two pancreatic cancer cell lines (PANC-1, Paca-2) and one colon cancer cell line (COLO201) were used for MTT cytotoxic assay. Calcein leakage from artificial liposomes was also tested, varying the composition of liposome. Results: The apoE 133-162 peptide had cytotoxic activity against all tested human cancer cell lines in a dose-dependent manner. In the Paca-2 cell, an equivalent cytotoxic activity to 5-FU (10 μg/ml) was observed at about 40 μg/ml of apoE 133-162 peptide, but no synergistic effect of apoE 133-162 (40 μg/ml) with 5-FU (10 μg/ml), nor inhibitory effect by heparin(100 μg/ml), was observed. In the calcein leakage test, in the presence of 150 mM NaCl, the presence of cholesterol attenuated the membrane perturbation activity of apoE 133-162, and the more acidic membrane was susceptible to lysis. Conclusion: ApoE 133-162 has anti-tumor activity, probably through perturbation and formation of ion-permeable "pores" in membranes.*

Recently, we have found that an apoprotein E (apoE)-derived peptide has antibiotic activity (1). A 30-mer peptide derived from apoE 133-162 (LRVRLASHLRKLRK RLLRDADDLQKRLAVY) had antibiotic activity that was comparable with the classic antibiotics (Gentamycin) and neutrophil-derived antibiotic peptide (CAP18).

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ApoE 133-162 forms an amphiphilical cationic α-helix structure (1,2), which is a characteristic of antibiotic peptides (3). On the other hand, it has already been shown that some antibiotic peptides of this type can also discriminate between normal and tumor cells (4). Antibiotic peptides such as magainins (4,5) or artificial pro-apoptotic peptide (6) also possess anti-tumor activity.

In this paper, we report that apoE 133-162, with antibiotic activity, also has anti-tumor activity, against several cancer cell lines.

Materials and Methods

Peptide synthesis. The synthesis of peptides was carried out by Sawady Co.Ltd. (Tokyo, Japan) using a solid-phase method with a Shimazu automated peptide synthesizer and a tert-butyloxycarbonyl strategy. Deprotection and cleavage from the resin were achieved by treatment with anhydrous HF, and the crude peptides were purified by gel filtration and reverse phase high-performance liquid chromatography. The purity was more than 90%. The identity of the peptides was confirmed by amino acid analysis. ApoE 133-162 (LRVRLASHLRKLRKRLLRDADDLQKRLAVY) and its randomized sequence peptide (LRVLSLKRYARLDAHLQRDKL RVRADRLLK) were synthesized.

Cell culture. Two gastric cancer cell lines (MKN-7, MNN-1), two pancreatic cancer cell lines (PANC-1, Paca-2) and one colon cancer cell line (COLO201) were maintained in Dulbecco' modified Eagle medium (DMEM, Gibco BRL) supplemented with 10% fetal bovine serum (Sigma). These cell lines were grown in a humidified atmosphere (95% air and 5% CO_2) as monolayer at 37˚C in 24-well clustered culture dishes.

Cell growth inhibition assay. The MTT assay was performed essentially as described in ref. 7. In brief, for the study of cell growth inhibition, one milliliter of a $5x10^4$ cell suspension was incubated in a 96-well clustered culture dish (Coster, Cambridge, MA, USA) with variable concentrations (μg/ml) of apoE 133-162 (MW=4203.3). The cells were cultured for 72 hours and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT tetrazolium bromide, Sigma, Tokyo, Japan) was added to the culture medium at 0.5 mg/ml. The culture medium was changed and tumor cells were incubated for 4 hours with MTT tetrazolium bromide at 37˚C. At the end of

incubation, the culture medium was aspirated. Formazon crystal precipitates were dissolved in 200 μl of dimethyl sulfoxide (Sigma). Quantification of solubilized formazon was performed by obtaining absorption readings at 550 nm wavelength on an ELISA reader and measuring in a Multiscan Bichromatic (Labo System) at 570 nm. The MTT assay measures mitochondria NADPH-dependent dehydrogenase activity, and it is the most sensitive and reliable method for quantitating *in vitro* chemotherapy responses of tumor cells. The reported number represents the average cell count in quintuplet wells.

To examine the synergistic effect of 5-FU or the inhibitory effect of heparin, these agents were added at the same time as the apoE 133-162 peptide.

Hemolysis assay. The hemolytic activity of the apoE 133-162 peptide was assayed with human red blood cells. Red blood cells (10^8/ml) were incubated at 37˚C in distilled water for 100% hemolysis, in PBS (50mM sodium phosphate, 150 mM NaCl, pH7) as a control or in PBS containing various concentration of the peptide (up to 200 μg/ml) in a final volume of 0.2 ml. Release of hemoglobin was monitored, after centrifugation at 900xg, by measuring the absorbance of 100 μl of supernatant at 541 nm after 24 hours of incubation.

Calcein leakage. Large unilamellar vesicles (liposomes 1-3) were prepared and characterized, as described elsewhere (8). Briefly, a lipid film, after being dried under vacuum overnight, was hydrated with 70mM calcein solution (10mM HEPES/150mM NaCl/1mM EDTA/ adjusted to pH 7.4) and vortexed. The suspension was freeze-thawed for five cycles and then successively filtered through polycarbonate filters. Calcein-entrapped vesicles were separated from free calcein on a Sephadex G-50 column. The lipid concentration was determined by phosphorus analysis using a Phospholipid Test (Wako Pure Chemical Co., Osaka, Japan). Three kinds of liposomes were prepared for the experiments: Liposome 1; PC: PG=1:1; Liposome 2; PC: PG=7:3; Liposome 3; PC: PG: Cholesterol=7:3:1; where PC (phosphatidylcholine), PG (phosphatidylglycerol) and cholesterol were purchased from Avanti Polar Lipids (Birmingham, AL, USA).

The standard assay was done in 96-well FluoroNunc plates (Nunc). In a typical assay, a small volume of the peptide in 10mM HEPES buffer (pH 7.4) was added to 100 μl of calcein-containing large unilamellar vesicles in HEPES buffer and incubated at 23˚C. Leakage of calcein from the vesicles was monitored fluorometrically using a Millipore Cytoflutor 2300/2350 (excitation at 490 nm and emission at 530 nm). The fluorescence intensity corresponding to 100% calcein release was measured by the addition of Triton X-100 (final concentration 1% v/v). The increase in fluorescence from background to 100% leakage was more than nine-fold. Experiments were performed twice, each in triplet, depending on the peptide concentrations, the time from peptide addition and in the presence/absence of 150mM NaCl in 10mM HEPES buffer (pH 7.4).

Results

Cell growth inhibition assay. As shown in Figure 1, the apoE 133-162 peptide showed cytotoxic activity against all five human cancer cell lines in a dose-dependent manner. As a negative control, a randomized sequence of apoE133-162 was also tested, but it showed no cytotoxic effect (data not shown).

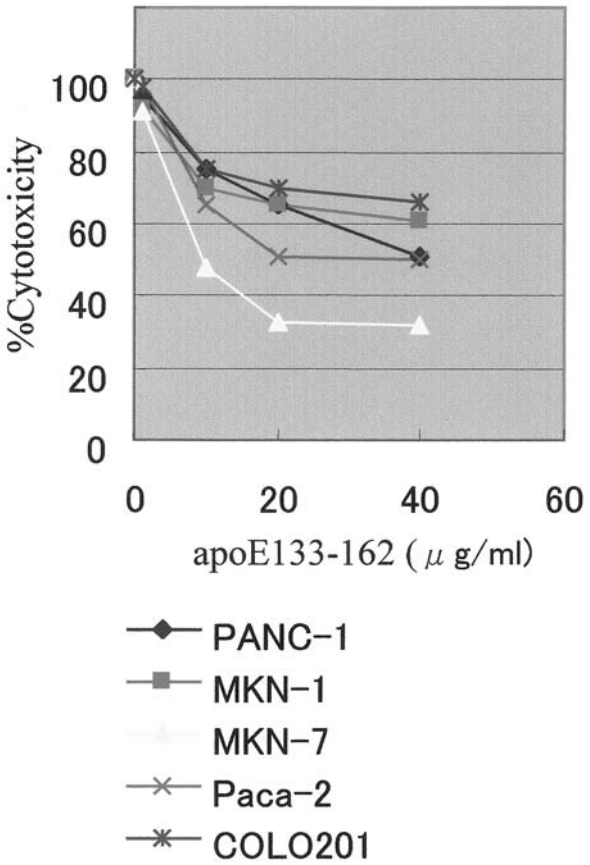

Figure 1. *Cell growth inhibition assay. ApoE 133-162 peptide showed cytotoxic activity against five human cancer cell lines (MKN-7, MNN-1, PANC-1, Paca-2, COLO201) in a dose-dependent manner (n=5, Standard Deviation was <15%.).*

In the Paca-2 cell , an equivalent cytotoxic activity to 5-FU (10 μg/ml) was observed at about 40 μg/ml of apoE 133-162 peptide. No synergistic effect of apoE 133-162 (40 μg/ml) with 5-FU (10 μg/ml) was observed (Table I).

Since it has been reported that peptides which are known to bind heparan sulfate, such as fibronectin, inhibit cell proliferation through suppressing certain growth factors' function (9), and it is known that apoE 133-162 binds heparan sulfate (10), we studied the effects of heparin on apoE 133-162 inhibitory effects. In the Paca-2 cell, no inhibitory effect of heparin (0, 1, 10, 100 μg/ml) was observed on the cytotoxic activity of apoE 133-162 (0, 20, 40 μg/ml) (data not shown).

Hemolysis assay. The apoE 133-162 peptide exhibited no hemolytic activity up to the concentration of 200 μg/ml(data not shown).

Calcein leakage. Three kinds of liposomes were prepared,

Table I. *Synergistic effect of apoE133-162 and 5-FU. In Paca-2 cell lines (n=5), an equivalent cytotoxic activity to 5-FU (10 μg/ml) was observed at about 40 μg/ml of apoE 133-162 peptide but no synergistic effect of apoE 133-162 (40 μg/ml) with 5-FU (10 μg/ml) was observed.*

Cell line (n=5)	Growth inhibition (%)		
	apoE 40 μg/ml	5-FU 10 μg/ml	apoE 40 μg/ml + 5-FU 10 μgml
PANC-1	48.7±3.0	91.9±2.0	49.9±2.4
Paca-2	52.4±2.5	61.9±1.5	51.1±3.2

according to the literature using magainins (5): PC/PG1:1; PC/PG 7:3; PC/PG/Cholesterol 7:3:1. Cholesterol is absent in bacterial walls, which is also one of the factors of selectivity of cytotoxic peptides.

In the absence of 150mM NaCl in 10mM HEPES buffer, calcein leakage by apoE 133-162 (after 30 min) was observed to the same extent in the three types of liposomes, in a peptide dose-dependent manner (Figure 2). The leakage increased and equalized 30 min after peptide addition, and no remarkable changes were observed after 60 and 120 min (data not shown).

In the presence of 150mM NaCl in 10mM HEPES buffer, calcein leakage was decreased (Figure 2), but a selectivity related to membrane composition was observed (Figure 3). More acidic membrane was susceptible to lysis (liposome 1 *vs* liposome 2) and the presence of cholesterol attenuated the membrane perturbation activity of apoE 133-162 (liposome 2 *vs* liposome 3), as can be speculated from the results of magainins (5). Here, also, the leakage increased and equalized by 30 min after peptide addition, and no remarkable changes were observed after 60 and 120 min (data not shown).

Discussion

The apoE 133-162 peptide with antibiotic activity (1) also has anti-tumor activity, like magainins (4) or artificial pro-apoptotic peptide (6), against five human cancer cell lines.

In the case of magainin II, it is proposed that the peptide interacts with (bacterial or tumor)cell membrane bilayers to produce dramatic structural changes, which lead to cell lysis (5). The amphiphilic cationic α-helical peptides may exert this effect by forming ion-permeable "pores" through membranes (4,5).

As shown by the dye leakage test, it is probable that apoE 133-162 may exert cytotoxic activity through membrane perturbation. On the other hand, apoE 133-162 does not lyse erythrocytes. The precise condition of discrimination of tumor cells (or bacterial walls) from normal cells by apoE

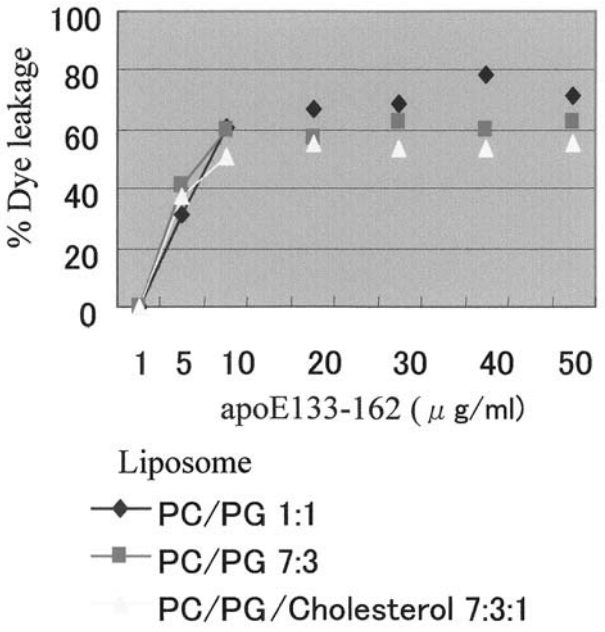

Figure 2. *Calcein leakage test (without 150mM NaCl). Calcein leakage after 30 min from the addition of apoE 133-162 peptide (0, 20, 30, 40, 50 μg/ml). Experiments were performed twice, each in triplet. Standard Deviation was <10%. No remarkable change of leakage was observed after 60 and 120 min.*

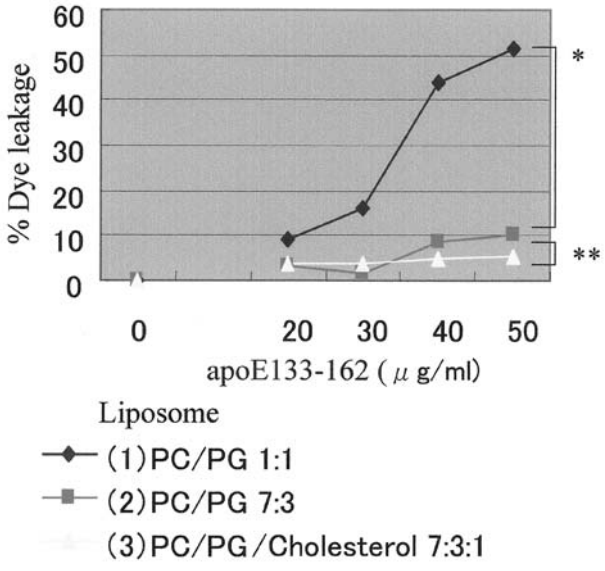

Figure 3. *Calcein leakage test (in presence of 150mM NaCl). Calcein leakage after 30 min from the addition of apoE 133-162 peptide (0, 20, 30, 40, 50 μg/ml). Experiments were performed twice, each in triplet. Standard Deviation was <10%. Significant differences between liposome 1 and liposome 2 (*p<0.01) or between liposome 2 and liposome 3 (**p<0.05) were found (the Student's t-test). No remarkable change of leakage was observed after 60 and 120 min.*

133-162 is not apparent, but it is proposed that the absence of acidic phospholipids and the abundant presence of cholesterol, combined with lack of a transmembrane potential, contribute to the protection of erythrocytes from the peptides' attack (5).

Recently, it was reported that pancreatic cancer growth is inhibited by endogenous peptide YY (PYY), secreted predominantly by endocrine cells or L-cells of the ileum, colon and rectum in response to food (11,12). The growth-inhibitory effect of PYY is believed to involve a hormonal effect, at the p-nM range of concentration in cell culture system (12). Interestingly, other authors have reported that PYY has potent antibiotic activity with its amphiphilic cationic α-helical structure (13,14), at the n- μM range of concentration, which can be physiologically attained at least, in brain tissues. Thus, PYY may have a additional effect against pancreatic cancer cells, as shown here with apoE 133-162, by forming ion-permeable "pores" through membranes followed by membrane-potential dissipation which results in cell lysis.

The use of an endogenous peptide like apoE 133-162 for cancer therapy may have the advantages of low antigenicity and probably low side-effects. The stability and the activity of the peptide can be improved through modification, following the example of other peptides with amphiphilic cationic α-helical structure (15).

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