Recombinant hybrid protein, Shiga toxin and granulocyte macrophage colony stimulating factor effectively induce apoptosis of colon cancer cells

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MATERIALS AND METHODS

Preparation of purified recombinant chimeric protein (StxA1-GM-CSF)

The catalytic domain of Shiga toxin, StxA1, was fused
Cell culture
HepG2 cell line derived from human hepatoma and LS174T cell line derived from colon carcinoma were provided by Cell Bank of Institute of Development, Aging and Cancer, Tohoku University, Japan. These cell lines were kept in RPMI-1640 medium (Gibco-BRL, Germany) supplemented with 10% fetal bovine serum (Gibco-BRL, Germany) at 37 °C in 50 mL/L CO2 atmosphere.

Cytotoxicity assay
The cytotoxic effect of StxA1-GM-CSF was determined by trypan blue exclusion assay and MTT assay. For trypan blue dye exclusion assay, 2.5 × 104 cells were seeded in one well of 96-well plates and different concentration (10-160 ng) of StxA1-GM-CSF was added to each well. After incubation for 24, 48, and 72 h, the number of viable cells was determined.

For MTT assay, HepG2 and LS174T cell lines were cultured and StxA1-GM-CSF was added as described above. After 24, 48 or 72 h incubation, 10 μL of 0.5 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, Japan) was added to each well and further incubated at 37 °C in 50 mL/L CO2 atmosphere for 4 h to allow MTT to be converted to formazon crystals by reacting with metabolically active cells. Reactions were stopped by addition of 10% SDS in 0.01 mol/L HCl and absorbance at 570 nm was determined.

Neutral comet assay
Neutral comet assay was carried out as previously described with some modifications. Briefly, freshly prepared cell suspension (2 × 108 /10 μL) was mixed with 150 μL of 1% low-melting agarose. The mixture was layered on top of the microscopic slide coated by 1% agarose. After low melting agarose was solidified in a refrigerator for 10 min, the slide was gently immersed in a freshly prepared lysing solution (2% SDS, 0.03 mol/L EDTA) for 30 min protected from light. After the slides were washed with TBE buffer, electrophoresis was carried out at 25 V for 25 min. Then, comets were visualized with 1 mmol/L propodium iodide. One hundred and fifty cells per slide were analysed under fluorescent microscope.

Hoechst staining
After administration of StxA1-GM-CSF, cells were fixed with 1% paraformaldehyde for 30 min, washed with PBS and stained with 1mmol/L Hoechst 33342 (Sigma, Japan) for 10 min. Nuclear morphology of at least 400 cells was randomly observed under fluorescent microscope.

Statistical analysis
The results were expressed as mean ± SD and each value represented the mean of three experiments. Differences between groups were compared using the Student’s t test.
application of this method. Accuracy and ease of manipulation, are likely the advantages of PCR technique.

StxA is an established inhibitor of eukaryotic translation and more potent than ricin[9]. No reduction in its enzymatic inhibitory activity has been reported when it is genetically fused with HIV gp120-binding domain of CD4[8]. Similarly in our study, fusion of StxA1 with hGM-CSF caused no reduction in its inhibitory effect. In addition, the enzymatic domain of diphtheria toxin has been fused to hGM-CSF using recombinant DNA techniques and is under clinical trial[10]. Our results suggest that StxA1-GM-CSF is more toxic than DT-GM-CSF, since IC50 is 20 ± 3.5 ng/mL for StxA1-GMCSF and 70 ± 18 ng/mL for DT-GM-CSF[8]. In this study, LS174T showed very obvious cytotoxic effect and changes characteristic of apoptosis were observed. A close correlation between protein synthesis inhibition and apoptosis induction has also been reported for DT-GM-CSF in which LS174T is not sensitive to apoptosis while other cell lines are more sensitive to cytotoxicity and apoptosis[11].

Agarose gel electrophoresis is one of the commonly used techniques for the detection of apoptotic cells. This technique usually involves a DNA isolation procedure from millions of cells and obtained results can not be quantified[12]. TUNEL assay is an established method for the detection of apoptotic cells, but this method is associated with a number of artefacts[13]. Annexin V labelled with fluorescent protein like FITC is used for rapid cytofluorometric analysis of apoptosis, but this method can produce false positive results when membrane is damaged[14]. Comet assay is sensitive, simple and fast. Usually this method is used to detect DNA damage like single or double strand breaks. On the other hand, this method also can detect single apoptotic cells in a large number of cells[15]. A few studies have tried to delineate the mechanism of apoptosis induction by Stx[16-18]. More knowledge about its mechanism can help us prevent shigellosis and enable us to tackle the problem of resistance to cancer therapy. Induction of apoptosis by hybrid protein indicates that catalytic domain of Shiga toxin in hybrid protein can not activate the cellular apoptosis machinery directly since the toxin inactivates ribosomes that inhibit protein synthesis.

In conclusion, more investigations are required to clarify how StxA1-GM-CSF induces apoptosis. Our results reveal that the hybrid protein is toxic to the colon cancer cell line and can be considered as a therapeutic agent.

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Figure 4 Apoptosis percentage in the presence of 40 ng/mL StxA1-GM-CSF at different time intervals (A). 

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