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以胃癌動物模式研究胃癌細胞表現黏液素在細胞癌化過程之作用

研究成果報告(精簡版)

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Introduction

The first discovery of secretory mucin gene is mucin 2. It constitutes tandem repeat sequences rich in threonine. MUC2 expressed by the goblet cells of colon and small intestinal and involved in the suppression of tumor formation. In MUC2-deficient mice, the intestinal goblet cells apparently absent and develop colitis. Thus, MUC2 play an important role for intestinal carcinogenesis.

The most common in populations at high risk is the “intestinal” type of gastric carcinomas. The preceded by a precancerous stage follow a series steps: superficial gastritis, atrophic gastritis, intestinal metaplasia, and dysplasia. The intestinal MUC2 did not detected in normal gastric mucosa, but expressed in intestinal metaplasia. The previous reports suggested that MUC2 is a tumor marker of malignant transformation of gastric mucosa. Moreover, the relationships between a worst survival in patients with a high MUC2 expression were analyzed. Therefore, a curious phenomenon of MUC2 expression in intestinal metaplasia of gastric mucosa implicated that MUC2 has a beneficial effect of malignant cell progression.

MUC2 functions involved decreased proliferation, increased apoptosis, and decreased migration of intestinal epithelial cells. However, the effect of MUC2 expression on metastatic potential was associated with an increase in liver colonization. On the other hand, mucins secreted by colon cancer cells induce COX-2 in monocytes/macrophages, resulting in PGE2 production. Then, these findings suggested that PGE2 secreted from macrophages binds to surface receptor on cancer cells, and activated cancer cells and macrophages produce VEGF. These results suggested that MUC2 expression have a distinctively effect in mucinous carcinomas of tissue origin.

This study focuses on the MUC2 expression of murine gastric cancer cell lines whether enhance tumorigenesis, metastasis and suppress immunity. We established a unique MUC2 positive tumor cell lines and designed MUC2 siRNA. The silences of MUC2 expression of tumor cell lines affect tumor cell growth, migration, invasion, apoptosis and host immunity. This animal model is the first to provided that the effect of MUC2 expression of gastric mucosa for tumorigenicity, metastasis and local immunity.

Materials and methods

Establishment of murine gastric cancer cell lines (MGCC)

Female ICR mice (8 weeks old) were treated with 200 μl of corn oil or 3 mg of benz[a]pyrene (B[a]P) in 200 μl corn oil by p.o. gavage twice a week for 4 weeks (Watterberg 1977). The mice were then sacrificed at week 40 and 50 after the first administration of B[a]P. Fresh forestomach tumor tissues from the stomach were

washed with Hank's balanced salt solution (HBSS), cut into small pieces with sterile scissors, treated with 0.05% Trypsin/EDTA for 30–60 min at 37℃ in a water bath, and after vigorous pipetting were allowed to settle. Supernatant fluid containing cell clumps was collected after centrifugation at 1500 rpm, 5 min. Cell pellets were washed and resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin, and then incubated on plastic dishes in a humidified 5% CO2 incubator at 37℃. Any remaining fibroblasts were removed by mechanical scraping and a differential attachment selection method with trypsin- EDTA, after which pure epithelial cell cultures were obtained. Cell lines have now been cultured for more than half years without apparent phenotypic change.

***In vitro* cell growth assay**

To examine the growth rate of murine gastric cancer cell lines *in vitro*, the cells were plated at 2.5 $\times 10^4$ cells in a 60 mm-dish were at first starved in 0.1% FCS/DMEM for 24 h, and then re-grew in a regular 10% FCS/DMEM. Cells were harvested at intervals to determine the number of viable cells by Trypan blue exclusion method.

Chemotaxis

Cell migration was evaluated in modified Boyden chambers for 6 h, as previously described (Sozzani 1997). A modified Boyden chamber (NeuroProbe, Inc., Gaithersburg, MD) was used to assay the cell migration ability through filter. To evaluate the migration potential, cancer cell lines from monolayer cultures were treated with 0.05% trypsin/EDTA, washed twice with serum-free media and resuspended in serum-free media. 5×10^5 of cells per ml were suspended in serum-free medium. Then, 2.5×10^4 of cells were added into the upper chamber. Polycarbonate filter with 8-μm pore size was used to separate the Boyden chamber into upper and bottom chamber. Either 10% FCS or serum-free medium was added to the bottom chamber as the chemo-attractant. After 6-h incubation, cells on the top of filter were scraped and the cells on the bottom side of filter were fixed and stained. Use a microscope set at 100× magnification to count the number of stained cells in 3 to 5 fields.

Semi-quantitative and real-time PCR

Total RNA was extracted from 5×10^5 cells and purified using the RNeasy Kit according to the manufacturer's instructions (Qiagen) and converted to cDNA by Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase with oligo(dT) primer in the presence of RNAsin (Promega). Semi-quantitative PCR for MUC2, MUC5AC, TGF-β, C-myc, cyclin E, MMP-9, VEGF, COX-2 and β-actin was

performed as described previously (Chen 2002; 2003; Okamoto 2004; Matsuura 2005). The cDNA generated was subjected to 30-35 cycles of PCR amplification on a DNA Thermal Cycler. The β-Actin served as a quantitative control for PCR. PCR products were fractionated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light.

Assay for tumorigenesis in mice

To examine tumorigenicity of cancer cell lines in ICR and NOD/SCID mice, growing cells were harvested with 0.05% trypsin-EDTA, washed with PBS, and 5×10^6 cells in 0.5 ml HBSS were injected subcutaneously into the left abdominal flanks of 8-week-old male ICR or NOD/SCID mice maintained under specific-pathogen-free (SPF) conditions. Tumor size was measured with a caliper every week. ICR or NOD/SCID mice bearing subcutaneous tumor were autopsied 4 weeks after injection; then lymph nodes were removed and analyzed cell phenotypes. The numbers of macroscopic lung metastases were determined by counting visible and were confirmed by histological examination with H&E stained specimens.

siRNA sequences and constructs

Using GenBankTM sequence NM 009263 for murine MUC2 cDNA , we selected two candidate sequences in the MUC2 cDNA sequence for RNAi. These 21-nt sequences showed no homology with other known mouse genes. Synthetic, annealed, siRNA oligonucleotides were synthesized chemically and gel-electrophoresis purified and used during transient transfection experiments. Murine mismatch or scrambled siRNA sequences possessing limited homology to mouse genes served as a negative control. For stable RNAi we designed a hairpin siRNA sequence that contains both sense and antisense siRNA sequences against MUC2 target 2 and flanking BamH1 and HindIII sites. This sequence was chemically synthesized and PAGE-purified (Sigma-Genosys, The Woodlands, TX) and cloned into pSilencer neoTM, an expression vector containing an H1 RNA polymerase III promoter and a neomycin antibiotic resistance gene. A pSilencer neoTM vector that expresses mismatch hairpin siRNA with limited homology to mouse genes served as our negative control. Plasmids were amplified and purified using QIAfilterTM Plasmid Maxi Kit (QIAGEN, Valencia, CA).

Transfection of siRNA oligonucleotides and stable siRNA plasmids

Harvesting of cancer cells using Trypsin was done 24 h prior to transfection, and plated at a density of 5×10^5 cells/well in 6-well plates (Costar, Corning Inc., NY) in DMEM/10% FBS without antibiotics. Reconstituted, annealed siRNA against target 1 (sR1) and siRNA against target 2 (sR2) were diluted in OPTIMEM I (Invitrogen) to a final concentration of 50 nM and transiently transfected into cancer cells using Lipofectamine 2000 (Invitrogen). The medium was replaced with DMEM/10% FBS after 4 h. Cancer wild-type cells (WT), cells incubated with Lipofectamine 2000 alone

in the absence of siRNA and cells incubated with Lipofectamine 2000 and mismatch siRNA (sR-) were used as controls. Cells were harvested 48 h after transfection and MUC2 protein levels were quantified by western blot-analysis in triplicate assays. In separate experiments, 2 mg of purified pSilencer neoTM expression vectors containing either the MUC2 siRNA insert (pS-MUC2) or the negative-control mismatchsequence (pS-MM) were transfected into cancer cells as described above. Antibiotic selection using Geneticin (Invitrogen) was initiated 24 h after transfection to generate stable clones. Western blotting for MUC2 was performed, 72 h after transfection, to assess the selectivity of MUC2 knockdown amongst WT, MUC2tpS-OPN and MUC2tpS-MM cell-lines. Clonal cell lines that stably express MUC2-siRNA and mismatch-siRNA were selected and cultured for up to 4 months.

Histological examination and immunohistochemistry.

Tissues were surgically obtained and immersed in buffered 10% formalin solution for at least 24 h. Sections 4 μm thick were dehydrated, embedded, and stained with hematoxylin and eosin. The tissues for immunohistochemical staining were embedded in OCT compound, frozen in liquid nitrogen, and stored at −20°C. Five-μm thick cryosections were placed on poly-L-lysine-coated glass slides and fixed with 3.7% formaldehyde in either PBS for 15 min plus acetone for 3 min or 3.7% paraformaldehyde for 5 min plus acetone for 1 min. The endogenous peroxidase activity was depleted by incubation in PBS containing 3% H_2O_2 for 3 min. The primary antibody was diluted with antibody diluent (DAKO), and included rat anti-NK mAb (DX5), rat anti-CD4 mAb (H129.19), rat anti-CD8 mAb (53-6.7), rat anti-Ly-6G (RB6-8C5) mAb, or rat anti-F4/80 mAb. The secondary antibodies were sheep anti-rat IgG peroxidase conjugated. Peroxidase stain of reddish-brown color was developed with an AEC substrate kit. Sections were counterstained with hematoxylin and mounted with glycerol gelatin.

Western blot-analysis

Total cell lysates were prepared and analyzed by SDS-PAGE as previously described (Chen 2002). Briefly, 35 μg of protein/lane was resolved on 4-20% polyacrylamide gels (Gradipore Inc., Hawthorne, NY) and transferred to polyvinylidene (PVDF) membranes (Amersham Biosciences, Piscataway, NJ).

The antibodies used are listed below (Ootani 2003). A rat polyclonal antibody cathepsin E (Wako Pure Chemicals, Osaka, Japan) was used to detect the gastric surface mucous cells.A mouse monoclonal antibody 45M1 (Novocastra, Newcastle, UK) was used to detect the gastric mucin MUC5AC. A mouse monoclonal antibody HIK1083 (M-GGMC-1, Kanto Kagaku, Tokyo, Japan) was used to detect the gastric mucous neck cells. Mouse monoclonal antibodies MUC2 and CD10 (Novocastra, Newcastle, UK) were used to identify the intestinal goblet cells and absorptive cells,

respectively. Mouse monoclonal anti-cytokeratin was from Nichirei (Tokyo, Japan), and mouse monoclonal anti-vimentin was from Dako (Kyoto, Japan). Immunodetection was performed using HRP-based SuperSignal Chemiluminescent Substrate (Pierce, Rockford, IL). For quantification, the bands were scanned into AlphaImager 3400 (Alpha Innotech, San Leandro, CA) and normalized by dividing the measured density of protein bands by the density of GAPDH control bands from corresponding cell lysates.

Results and discussion

Morphology of cultured cells

In vitro, murine gastric cancer cell lines (MGCC) 3I and 5J appeared homogeneous by phase-contrast microscopy. Two cell lines, MGCC3I and 5J, derived from stomach cancer and grew adherent in monolayer. MGCC3I cell was polygonal-shaped epitheloid. MGCC5J was round and form typical epithelial monolayers.

C-myc, cyclin E, TGF-β1, COX-2, VEGF, MMP-9 and MIP-2 mRNA expression in murine gastric cancer cell lines

Phenotypic marker expression was determining by examining the expression of gastric mucin. *In vitro*, MGCC3I and 5J expressed MUC2 and MUC5AC and indicated goblet-specific. Moreover, MGCC3H and 5I expressed cytokeratin and indicated epithelial nature. Morphologically, the MGCC3H and 5I cell lines are epithelial cell-like and produce MUC2 mucins as do goblet cells. Moreover, high expression of C-myc, cyclin E, TGF-β1, COX-2, VEGF, MMP-9 and MIP-2 were analyzed in two cell lines.

Murine gastric cancer cells migration in response to 10% FCS

The ability of MGCC3I and 5J cell cells to migrate in response to 10% FCS was investigated using a micromultiwell chemotaxis chamber assay. The chemotactic response of MGCC3I and 5J cells to 10% FCS. MGCC3I and 5J cell exhibited significant chemotactic responses to 10% FCS.

Tumorigenicity potentials in ICR mice

Those two cell lines appeared to be tumorigenic in sygeneic mice and development subcutaneous tumors. To analyzed the mechanism whereby MUC2 positive expression by tumor cells results in increased tumor progression *in vivo*, we assessed the level of neutrophil, CD4 and CD8 cell infiltration in the excised tumors. MUC2 expression by cancer cells results in increased neutrophil infiltration of tumor nodules. Therefore, this study is first established cultured cell lines and derived from mouse forestomach cancer; the newly derived two cell lines exhibit markedly tumorigenesis.

Conclusions: Establishment and characterization of two novel mouse gastric cancer

cell lines with differentiated intestinal phenotype derived from forestomach cancer. A useful animal models for understand the mechanism of immune regulation and development of new molecular targeting therapy for gastric cancer especially mucins.