The Suppression of MUC1 Expression in Cultured Cholangiocarcinoma Cells Using Antisense MUC1 Ribozyme Strategy

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Abstract : **Ribozyme strategy has been employed to suppress the expression of target genes with a better efficacy than that of a regular antisense RNA strategy due to its favorable stoichiometry of the hybridization. A plasmid capable of controlling the expression of a portion of antisense RNA whose sequence is complementary to the 5' region of the coding sequence of MUC1 was constructed to include the requisite sequence of a hammerhead ribozyme. This plasmid is intended to suppress MUC1 expression. This novel plasmid was examined to see whether it functioned inside the harboring cell. Cultured cholangiocarcinoma cells prepared from the sediment of intrahepatic biliary fluid were employed as the transfection target since these cells have been confirmed to have high levels of MUC1 expression. The suppression of MUC1 expression in these cells after stimulation of the plasmid function for 16 h was demonstrated using flow cytometric analysis.**

เรื่องย่อ : **การยับยั้งการแสดงออกของ MUC1 ที่ผลิตจากเซลล์เพาะเลี้ยง Cholangiocarcinoma โดยอาศัยรหัสพันธุกรรมที่สามารถยึดจับและตัดสาย mRNA ของ MUC1 ได้**

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การใช้รหัส ribozyme เพื่อยับยั้งการแสดงออกของรหัสพันธุกรรมเป้าหมายมีประสิทธิภาพสูงกว่าการใช้ antisense RNA ทั่วไปซึ่งมีรหัสเข้าคู่กับ mRNA ของรหัสพันธุกรรมเป้าหมาย ทั้งนี้เนื่องจากแต่ละโมเลกุลของ

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ribozyme สามารถยึดจับกับ mRNA เป้าหมายได้มากกว่า 1 โมเลกุล รหัสพันธุกรรมชนิดวงที่สามารถผลิต RNA ที่มีรหัสยึดจับ mRNA ของ MUC1 บริเวณต้นสายเมื่ออยู่ภายในเซลล์ได้ถูกผลิตขึ้นมาพร้อมกับได้บรรจุรหัสของ hammerhead ribozyme ทั้งนี้เพื่อหวังผลยับยั้งการผลิต MUC1 ที่มีประสิทธิภาพสูงขึ้น จึงมีการทดสอบประสิทธิภาพการทำงานของรหัสพันธุกรรมนี้ที่บรรจุภายในเซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีที่เตรียมจากตะกอนน้ำดีภายในตับของผู้ป่วย โดยที่เซลล์เพาะเลี้ยงมะเร็งท่อน้ำดีนี้ได้ผ่านการทดสอบมาล่วงหน้าแล้วว่าสามารถผลิต MUC1 ได้ในปริมาณสูงในภาวะปกติ การทดสอบโดยอาศัย flow cytometry ได้ยืนยันว่ามีการลดการแสดงออกของ MUC1 ได้ชัดเจนแม้ภายใน 16 ชั่วโมงหลังจากการกระตุ้นการทำงานของรหัสพันธุกรรมนี้

INTRODUCTION

Ribozymes have been employed as tools to suppress the expression of a target gene to which its mRNA can be annealed[1]. The stoichiometry of the reaction between the ribozyme to its target mRNA is not limited to a 1:1 ratio as experienced in a regular antisense RNA strategy[2]. After the target cleavage, the ribozyme can spontaneously detach from the cleaved products due to the fact that the new dissociation temperatures between the ribozyme and the 2 cleaved products is lower than the physiological 37°C. The released ribozyme can then hybridize with another molecule of target mRNA and repeat the cycle of cleavage (Figure 1). One of the most popular ribozyme used is the hammerhead ribozyme due to its simple structure. The target gene in which we intended to suppress its expression using a ribozyme strategy is the gene encoding MUC1.

Mucin-1 (MUC1) is a highly glycosylated membrane protein with a single hydrophobic transmembrane domain. The polypeptide core consists of 30-100 repeats of the 20-amino acid sequence (GVTSAPDTRPAPGSTAPPAH)[3]. The molecular structure of MUC1 is rigid due to its rich glycosylation along the length of the proline-rich polypeptide core. A high level of expression of MUC1 is associated with a poor prognosis and rapid cancer progression in a variety of epithelial cancer types (e.g., breast[4,5], pancreas[6], and colon[7]). Primary breast cancer in MUC1-null mice had a lower growth rate and metastatic potential. Cancer-associated MUC1 contains truncated carbohydrate chains (1- 6 sugar residues) and therefore exposes internal sugar residues and cryptic core peptide sequences.

It has been proposed that MUC1exerts paradoxical role in tumor immunogenicity[3]. The MUC1 molecule of may be a target for cytotoxic T lymphocytes (CTL) directed against the tumor. Since MUC1 itself is a tumor antigen, the soluble form of MUC1 secreted to the surrounding environment could serve as a decoy for the CTLs and induce T cell anergy. The large, rigid and dense glycosylation of MUC1 may serve as a protective shield to restrict the accessibility of CTLs to HLA or other tumor epitopes. The degree of inhibition of T-cell proliferation is directly proportional to the number of tandem repeats present on MUC1 synthetic peptides. The signal transduction through the phosphorylation of the cytoplasmic domain can down-regulate the activities of catenins, cadherins or integrins, thus facilitating invasion and metastatic potential[8]. However, the carbohydrate moieties of MUC1 serve as ligands for selectin-like molecules on endothelial cells and for intercellular adhesion molecule 1 (ICAM1). Immunotherapy using MUC1 as a target for immune-mediated cytolysis has been ongoing using a MUC1 peptide vaccine[9] or either MUC1-expressing tumor cells[10] or MUC1-pulsed dendritic cells[11] as antigen presenting cells. Multiple cytolytic mechanisms are required for the rejection of the MUC1-expressing mouse melanoma tumor line[12].

Cholangiocarcinoma is an attractive model for the study of MUC1 suppression. The tumor is endemic in the northeastern part of Thailand where there is an association with liver fluke (*Opisthorchis viverrini*) infestation and nitrosamine ingestion. Human cell lines of cholangiocarcinoma have been developed and characterized[13]. We recently isolated

cholangiocarcinoma from bile obtained at endoscopic examination. Our preliminary results indicate that every primary cultured cell line of cholangiocarcinoma is positive for MUC1 staining. This research asks whether the novel construct can functionally suppress the expression of MUC1.

MATERIALS AND METHODS

Chemicals

Dulbecco's modified Eagle medium (DMEM), sodium pyruvate, transferrin, sodium selenite, non-essential amino acid solution, and *l*-glutamine were of tissue culture grade (GibcoBRL, USA). Fetal bovine serum were purchased either from GibcoBRL or from Hyclone. Penicillin and streptomycin were of pharmaceutical grade and were prepared as 100 × stock (50,000 unit/mL and 50,000 µg/mL respectively).

Primary culture of cholangiocarcinoma cells from biliary fluid

Cultured cells of human cholangiocarcinoma were prepared from biliary fluid routinely aspirated at endoscopic retrograde cholangiopancreatography (ERCP) for palliative treatment in subjects with cholangiocarcinoma and clinical evidence of biliary obstruction. The operations were performed in the endoscopy unit of Siriraj Hospital without disturbing regular treatment. The collected fluid was centrifuged at 300 × g to remove the supernatant. The remaining pellet was washed 4 times by centrifugation at 300 × g with 10 mL serum-free DMEM. The cell pellet was dispersed and seeded at a density of 6,000-10,000 cells/cm^2 in 25-cm^2 tissue culture flasks. Cultured cells were propagated in growth medium (Dulbecco's modified Eagle medium (DMEM), 15% fetal bovine serum, 1 mM sodium pyruvate, 1 mg/mL insulin, 0.66 mg/mL transferrin, 0.67 µg/mL sodium selenite, 0.1 mM non-essential amino acid solution, 2 mM *l*-glutamine, 50 unit/mL penicillin, and 50µg/mL streptomycin) at 37°C with 5% CO_2. The cultured cells were morphologically homogeneous under light microscopy after 4-week's incubation. The epithelial origin of the cultured cells was confirmed by their intense staining with primary antibody against low molecular weight cytokeratin.

Immunohistochemistry

The cultured samples were grown on either chambered slides or cover slips until reaching subconfluent density. The attached cells were air-dried overnight and fixed by dipping into acetone-containing jar for 5 min. They were immunohistochemically stained with the monoclonal mouse anti-human Cytokeratin (Pan) antibody (clone AE1/AE3; Zymed, CA, USA). The rabbit secondary antibody was conjugated with biotin and directed against the Fc fragment of the mouse primary antibody (Dako, Japan). The streptavidin conjugated with horse radish peroxidase (Dako, Japan) would be attracted to the biotin group of the secondary antibodies. The conjugated peroxidase activity would catalyze the appearance of brown color upon exposure to DAB substrate (Sigma, USA).

The construction of the pAnti-MUC1-ribozyme

The coding sequence of MUC1 mRNA at the invariable 5' region (nt5-34) was designed as the target for antisense RNA hybridization. Two oligonucleotide strands were synthesized (5'-GC CUCGAG GGGCACCCAGU-3' and 5' CCAGCTAGC GCAG GAAGAAAGGA CTGATGAGTCCGCGAGGA CGAA ACTGGGTGCCCCTCGAGGC 3', Figure 2). Both strands were amplified using PCR. The full length sequence contains the requisite sequence for a hammerhead ribozyme[14] flanked by the target sequence with *Xho* I or *Nhe* I restriction sites at each appropriate end that can generate the antisense orientation after ligation with the vector. The expression vector was derived from pAnti-IGF-I[15] that had been engineered to contain *Nhe* I at the 5' end and *Xho* I at the 3' end of the coding sequence respectively. The vector was maintained in a DH5α strain of *E. coli*. Both the insert and the vector were digested with *Xho* I and *Nhe* I to generate "sticky" ends that will generate the antisense orientation of MUC1 upon ligation. The structure of the novel episome (Figure 3) was confirmed for inclusion of the insert using the restriction map obtained after cleavage with *Kpn* I and *Xho* I or *Nhe* I and *Xho* I.

Transfection of cultured cholangiocarcinoma with pAnti-MUC1-ribozyme

Cells were transfected with pAnti-MUC1-

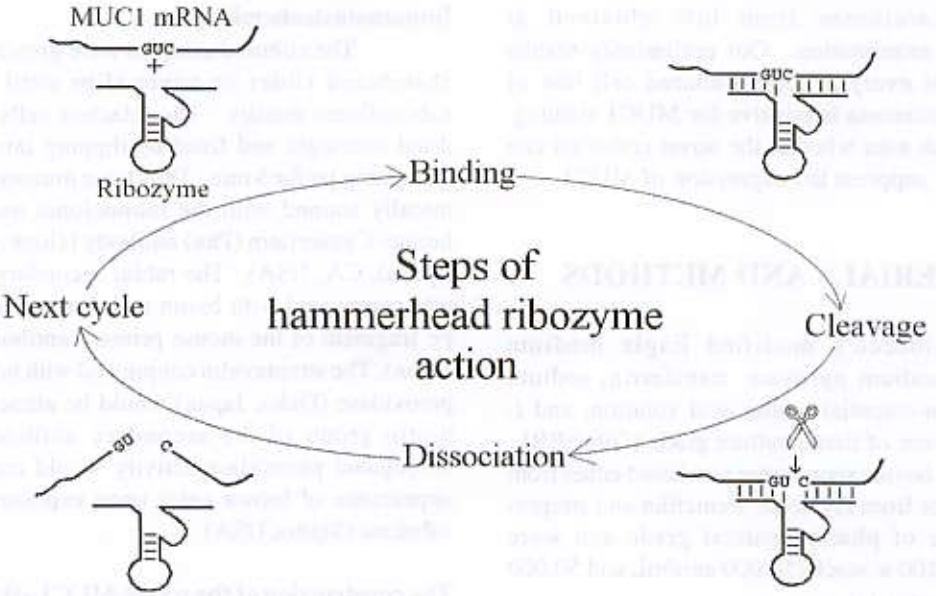


Figure 1. A scheme presenting the cycle of the interaction between the hammerhead ribozyme and the targeted MUC1 mRNA inside the cell.

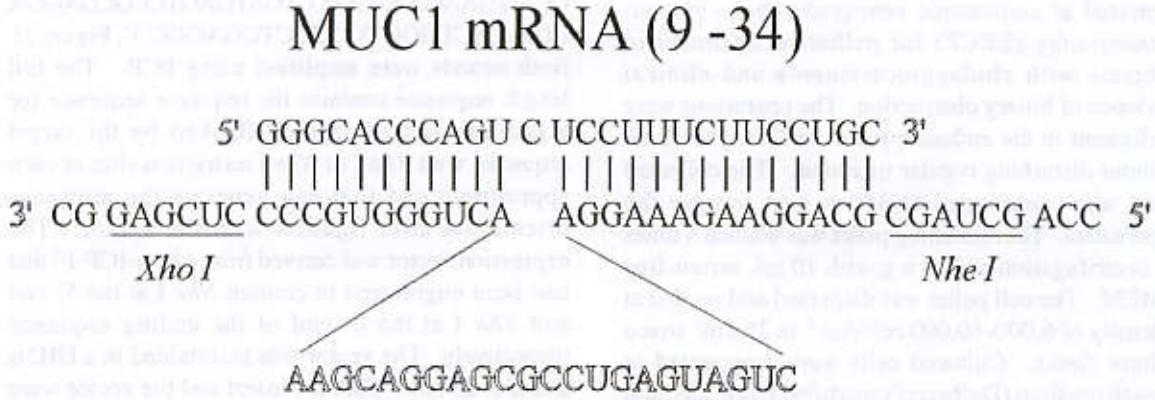


Figure 2. The strategy for the construction of pAnti-MUC1-ribozyme that targets the 9th – 34th bp of the coding sequence of MUC1 mRNA.

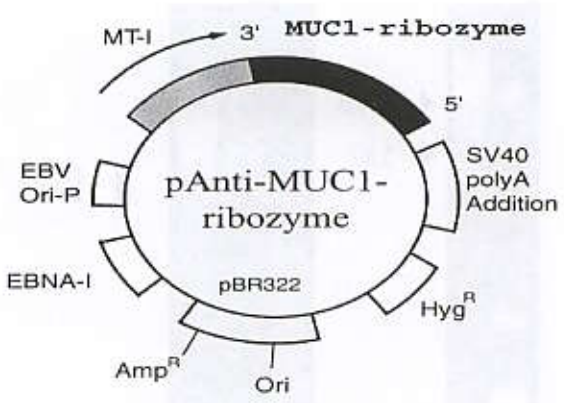


Figure 3. The structure of pAnti-MUC1-ribozyme. The transcription of the antisense MUC1 RNA-ribozyme followed by a polyA tail is driven by a MT-I promoter that is activated upon exposure to $ZnSO_4$. The ampicillin resistant gene (Amp^R) and hygromycin resistant gene (Hyg^R) provide selective survival advantage in bacteria and mammalian cells respectively that contain this construct. This construct can be replicated inside mammalian cells using EBV origin of replication (EBV Ori-P) and EBV nuclear antigen-1 (EBNA-1)

ribozyme using a lipofectamine reagent kit (Gibco BRL) according to the manufacturer's instructions. The transfected cells were enriched to produce a large number of copied episomes by adding hygromycin starting at 0.5 µg/mL for 1-2 weeks. The hygromycin concentration was gradually raised by 0.5 - 1 µg/mL every 1 - 2 weeks until a minimum concentration of 2 µg/mL was reached. The activation of MT-I promoter that drove the expression of antisense MUC1-ribozyme was achieved by cellular incubation with 100 µM $ZnSO_4$ in serum-free growth medium for 16 h. The suppression of MUC1 expression was determined by immunohistochemical staining using mouse primary monoclonal antibodies against human MUC1. Goat anti mouse IgG secondary antibody was conjugated with FITC (PharMingen).

Fluorescence-activated cell sorting analysis of MUC1 expression in cultured cholangiocarcinoma cells

Cell suspensions (1×10^5 cells/100 µL) collected from a brief trypsin-digestion of attached cultured cells were aliquoted to 15-mL conical polypropylene tubes. The detached cells were fixed with 4% (w/v) paraformaldehyde/PBS pH 7.4 for 10 min. The fixed cells were washed once, resuspended in 1 mM Na citrate buffer pH 6, and heated in waterbath at 100°C for 20 min. The cell suspension was washed and incubated with 500-µL FACS Permeabilization solution (Becton Dickinson) at room temperature for 10 min, washed by centrifugation at 500 × g with 1.6-mL wash buffer (PBS, 5% FBS). The cell pellet was incubated with 2 µL mouse anti-human MUC1 monoclonal antibody (HMPV, PharMingen) for 45 min at 4°C, incubated with 3-mL wash buffer for an additional 45 min before washing by centrifugation for 3 min at 500 × g at 4°C, and resuspended in 50-mL wash buffer. The washed cells were incubated with 4-µL of secondary antibody (goat anti-mouse IgG) conjugated with FITC for 45 min, added to 3 mL PBS and incubated for an additional 45 min, washed by centrifugation for 3 min at 1,500 × *g*, 4°C, and finally resuspended in residual solution prior to counting with a flow cytometer.

RESULTS

Primary culture of cholangiocarcinoma

The cultured cells (HubCCA1) were confirmed as cholangiocarcinoma cells according to the following characteristics: (1) they were collected from intrahepatic bile fluid upstream from the tumor blockade; (2) the cultured cells had epithelial-like morphology (Figure 4A); (3) they stained positive for low molecular weight cytokeratin (Figure 4B). The doubling time of this cell line ranges between 2-4 days.



Figure 4. The adherent cultured cholangiocarcinoma cells (HubCCA1) derived from the sediment of biliary fluid were propagated in growth medium (20 × objective lens, A). The cultured cells were transferred to chambered slide and immunohistochemical stained using the mouse primary antibody raised against human low molecular weight cytokeratin (B, courtesy of Dr. Mongkol Uiprasertkul).

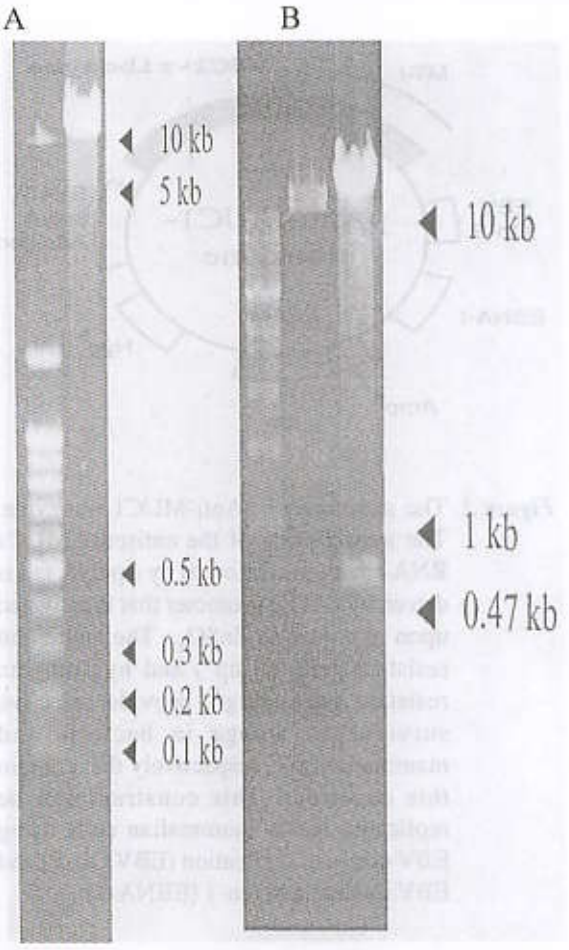


Figure 5. The restriction endonuclease analysis of the pAnti-MUC1-ribozyme. The plasmid was digested either by (A) *Nhe* I and *Xho* I; or (B) *Kpn* I and *Xho* I. The right lane of both (A) and (B) are the digested plasmids while other lanes contain standard-size markers.

The construction of pAnti-MUC1-ribozyme

The structure of the novel pAnti-MUC1-ribozyme inclusion sequence was confirmed using *Nhe* I and *Xho* I digestion. The resulting products consisted of a 54-bp-insert and a 11.4 kbp vector (Figure 5A). The alternative digestion with *Kpn* I and *Xho* I yielded fragments of 724 bp and 10.7 kbp (Figure 5B).

The suppression of MUC1 expression in the transfected cultured cells

The expression of MUC1 in HubCCA1 was determined using flow cytometric analysis (Figure 6, 7). HubCCA1 (Figure 6) and other cultured cholangiocarcinoma cells (data not shown) abundantly expressed MUC1. The addition of $ZnSO_4$ to HubCCA1 did not alter the expression level of MUC1. The pAnti-MUC1-transfected HubCCA1 already had a detectable degree of reduced MUC1 expression. The addition of 100 μM $ZnSO_4$ for 16 h resulted in a larger suppressive action (Figure 7). Inoculation with non-transfected cells did not alter the expression pattern of MUC1.

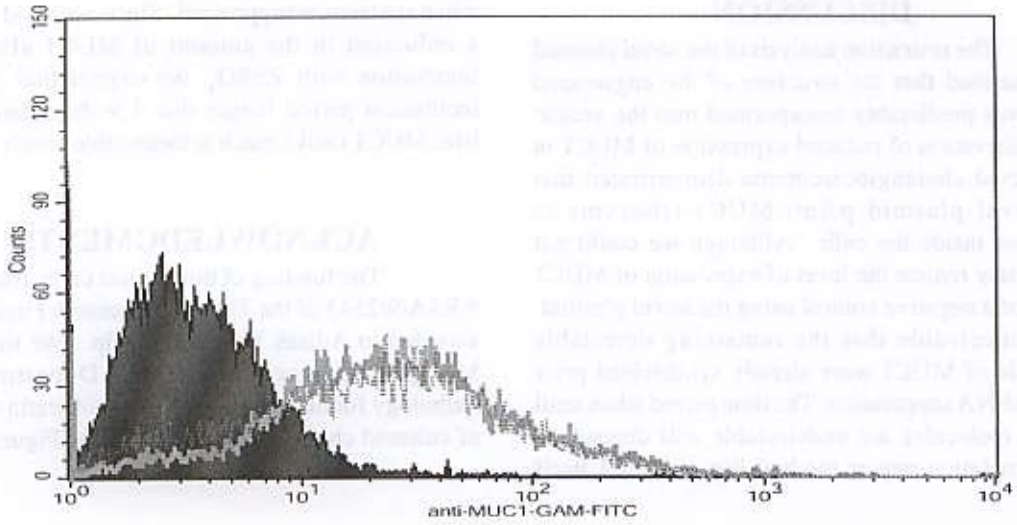


Figure 6. Flow cytometric analysis for the expression of MUC1 in non-transfected cholangiocarcinoma cells. Cultured cholangiocarcinoma cells (HubCCA1) were detached from the tissue culture flasks using trypsin digestion. The cell suspensions were stained with monoclonal antibody against MUC1 (light-grey line), followed by staining with goat anti-mouse antibody conjugated with FITC. The presence of $ZnSO_4$ did not alter the expression of MUC1 (dashed dark-grey line).

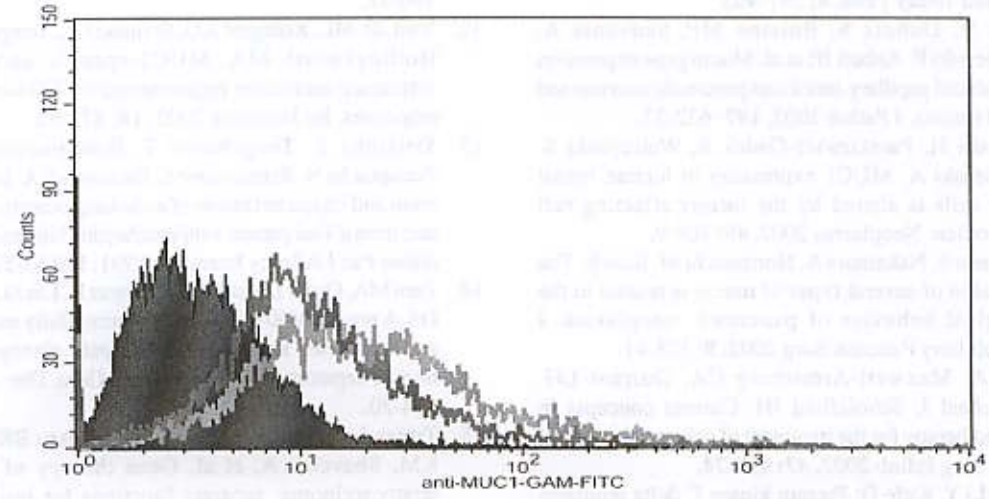


Figure 7. Flow cytometric analysis for the expression of MUC1 in transfected cholangiocarcinoma cells. The transfected cells were treated in the same fashion as in Fig. 5. The transfected cells already have a lower basal expression level (light-grey line) than that of the non-transfected counterpart. The presence of $ZnSO_4$, although did not completely abrogate the MUC1 expression, significantly lowered the expression of MUC1 beyond the baseline level (dark-grey line).

DISCUSSION

The restriction analysis of the novel plasmid demonstrated that the structure of the engineered insert was predictably incorporated into the vector. The observation of reduced expression of MUC1 in transfected cholangiocarcinoma demonstrated that the novel plasmid pAnti-MUC1-ribozyme is functions inside the cells. Although we could not completely reduce the level of expression of MUC1 to that of a negative control using the novel plasmid, it is conceivable that the remaining detectable molecule of MUC1 were already synthesized prior to the mRNA suppression. The time period taken until MUC1 molecules are undetectable will depend on the degradation rate or the half-life of MUC1 itself when synthetis is suppressed. Since we could observe a reduction in the amount of MUC1 after 16-h incubation with $ZnSO_4$, we expect that with an incubation period longer that 4 × degradation half life, MUC1 could reach undetectable levels.

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