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^{99m}Tc-sestamibi to monitor treatment with antisense oligodeoxynucleotide complementary to MRP mRNA in human breast cancer cells

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Objective: Technetium-99m sestamibi (MIBI) has been utilized to evaluate multi-drug resistance (MDR) phenomenon of malignant tumors and to predict chemotherapeutic effects on them. The current investigation examined the possibility of monitoring changes with respect to mRNA expression of multi-drug resistance associated protein (MRP) following antisense oligodeoxynucleotide (AS-ODN) treatment involving ^{99m}Tc-MIBI. ***Methods:*** The human breast cancer MCF-7 cell line and its MDR-induced MCF-7/VP cell line were employed. Cell suspensions of the two cell lines at 1×10^4 cells/m*l* were inoculated in 24-well plates (0.2 m*l*/well) and incubated for one day. Antisense (AS) 20-mer phosphorothioate ODN complementary to the coding region of MRP mRNA and its sense (S) ODN were administered at final concentrations up to 25 μM, followed by a 5-day incubation. ^{99m}Tc-MIBI solution was added to each well and incubated for 30 min. Cellular ^{99m}Tc-MIBI uptake was corrected for protein concentration. MRP mRNA expression levels were analyzed via the reverse transcription polymerase chain reaction (RT-PCR). ***Results:*** Cellular uptake of ^{99m}Tc-MIBI in MCF-7/VP cells was only 15% of that of MCF-7 cells. Following AS-ODN treatment at 25 μM for five days, ^{99m}Tc-MIBI uptake in MCF-7/VP cells increased 2.4-fold in comparison with non-treated control cells. ^{99m}Tc-MIBI uptake in MCF-7 cells was unaffected by AS-ODN administration. Sense ODN did not alter uptake in either cell line. RT-PCR confirmed reduction of MRP mRNA in MCF-7/VP cells following AS-ODN treatment. ***Conclusion:*** Effects of AS-ODN administration on MRP function can be monitored via assessment of cellular uptake of ^{99m}Tc-MIBI.

Key words: ^{99m}Tc-sestamibi, multi-drug resistance, multi-drug resistance associated protein, antisense, oligodeoxynucleotide

INTRODUCTION

MULTI-DRUG RESISTANCE (MDR) phenomenon associated with expression of ATP-binding cassette transporters (ABC), such as P-glycoprotein/MDR1 (Pgp) and multi-drug resistance-associated proteins (MRP), is a major factor contributing to chemotherapeutic failure in malignancies.[1] These cell surface proteins eliminate chemotherapeutic agents from tumor cells, thus neutralizing their cytotoxic effects. Numerous approaches have been evaluated in an attempt to overcome tumoral MDR functions. Chemical modulators of ABC transporters have been intensively investigated with respect to inhibition of pumping function.[2–4] In order to regulate upstream regions of transporter production, translational inhibition of mRNA coding ABC transporters with antisense

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oligodeoxynucleotide (ODN) or hammerhead ribozymes has been considered as well.[4,5]

Technetium-99m-labeled lipophilic, cationic compounds, namely, tetrofosmin (TF) and sestamibi (MIBI), serve as substrates for these transporters; as a result, TF and MIBI may enable characterization of tumors regarding therapeutic response or outcome.[6] Previous reports indicated the potential of these radiotracers in terms of assessment of the effects of modulators on cell membrane transporters.[7–13] However, insufficient data exist as to whether successful inhibition of the translation step of MDR functions induced by antisense ODN or hammerhead ribozymes can be accurately monitored with these tracers.

The current investigation examined ^{99m}Tc-MIBI uptake in MCF7 breast cancer cells, a wild type breast cancer cell line lacking MDR ability, and its MDR-induced subclonal cell line MCF7/VP which expresses high levels of MRP1, a member of the MRP family. Uptake was monitored following treatment with antisense phosphorothioate ODN complementary to the mRNA sequences of MRP1 in order to determine imaging feasibility of this type of therapeutic interference of cellular functions.

MATERIALS AND METHODS

Cell lines

MCF7 human breast cancer cells (American Type Culture Collection, Rockville, MD, USA) and etoposide-resistant MCF7/VP cells were grown in DMEM medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal calf serum. MCF7/VP cells express high levels of MRP1, whereas MCF7 cells do not.[14,15] Neither cell line expresses P-gp/MDR1.[14] Functional status of MCF7/VP cells is relatively unchanged in DMEM medium regarding ^{99m}Tc-MIBI efflux. Growth speed of the two cell lines is comparable. Cells were harvested with 0.1% trypsin and 0.02% ethylenediaminetetraacetate, followed by washing with medium twice.

Oligonucleotides

A 20-mer phosphorothioate antisense ODN (AS-ODN) [5′-TGCTGTTCGTGCCCCCGCCG-3′] complementary to the coding region located at 2107–2126 of MRP mRNA and its corresponding sense sequence (S-ODN) [5′CGGCGGGGGCACGAACAGCA3′] (Grana, Tokyo, Japan) were used according to the previous report showing that AS-ODN of this sequence was the most effective at lowering MRP protein levels.[16] Following solubilization in autoclaved nano-pure water, ODNs were stored at −80°C prior to the experiments.

Treatment of cells with oligonucleotides

Cells (concentration, 1×10^4 cells/m*l*) were inoculated in 24-well culture plates at 0.2 m*l*/well and incubated at 37°C in 95% air and 5% CO_2 for one day. Subsequently, AS-ODN and S-ODN were introduced at final concentrations up to 25 μM in DMEM medium supplemented with 10% fetal calf serum. Due to the time lag between the reduction of MRP quantity on the cell surface and inhibition of mRNA translation induced by AS-ODN, the treatment was applied daily for 4–5 consecutive days by changing the medium. Cell viability was assessed by the trypan blue exclusion test following incubation.

^{99m}Tc-MIBI uptake

^{99m}Tc-MIBI (Daiichi Radioisotope Laboratory, Tokyo, Japan) was prepared with 2 m*l* of ^{99m}Tc-pertechnetate (370 MBq/m*l*). Cells in the logarithmic growth phase were washed with fresh medium in culture plates. ^{99m}Tc-MIBI solution (10 μ*l*), which was diluted to 74 MBq/m*l*, was added to the culture medium; subsequently, cells were incubated at 37°C in 95% air and 5% CO_2. Following a 30-min interval, incubation was terminated by removal of culture medium, and cells were washed twice with chilled PBS. Next, 200 μ*l* of 2 N NaOH solution was introduced to each well for cell lysis. An aliquot of the lysate was collected for radioactive counting in a well-type gamma counter. Wells containing the culture medium without cells were similarly treated to assess non-specific radioactivity absorbed to the plates. Typically, non-specific background radioactivity was 10–20% of cell-associated radioactivity. Upon completion of radioactive counting, protein concentration in each sample was determined via the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Cellular uptake of ^{99m}Tc-MIBI was corrected for protein content. Triplicate samples were measured in each experiment. The results were expressed as ratios of ^{99m}Tc-MIBI uptake in cells treated with ODN to that in non-treated control cells. Statistical analysis was performed with one-way analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD). The level of significance was set at 5% in this analysis.

The influence of ^{99m}Tc-MIBI concentration in wells on its cellular uptake was assessed by applying an increasing amount of ^{99m}Tc-MIBI from 0.74 kBq to 1480 kBq 4 days after inoculation of MCF7 cells. Cell numbers exceeded 1×10^5/well at this time point.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis

MCF-7/VP cells were incubated in the presence of S-ODN or AS-ODN at 5 μM or 25 μM overnight. Total RNA isolated from MCF-7/VP cells with the RNeasy® Mini kit (QIAGEN Sciences, MD, USA) was analyzed utilizing a SuperScript One-Step RT-PCR kit with Platinum Taq (Invitrogen Corp., Carlsbad, CA, USA). Primers for mRNA coding human MRP gene were 5′-CTTGTGCACATTTGCCGTCTAC-3′ representing bases 1879–1900 and 5′-CTCTTCCACTACGGTA

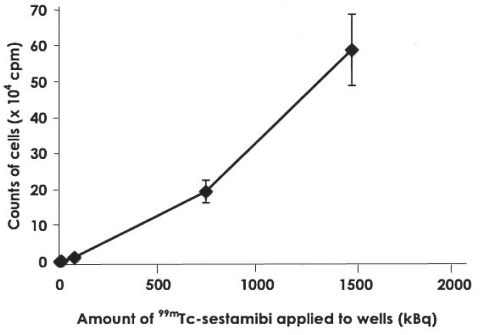

Fig. 1 Effects of the amount of ^{99m}Tc-MIBI applied to wells on uptake in MCF7 cells. Mean ± s.d. of a triplicate assay.

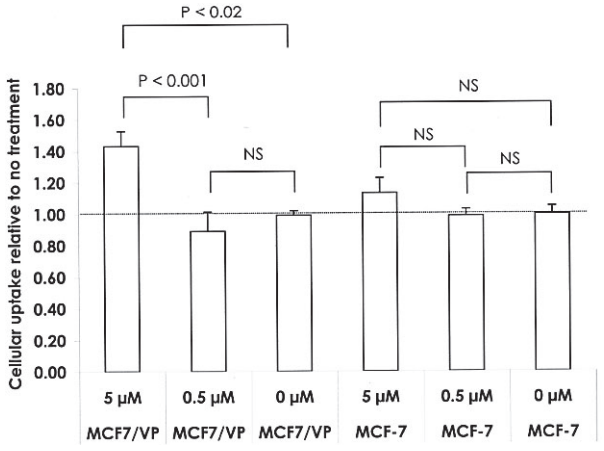

Fig. 2 Effects of treatment with antisense ODN to MRP mRNA on ^{99m}Tc-MIBI uptake in MCF-7/VP and MCF-7 cells. Cells were treated for 4 consecutive days at various concentrations. NS, difference is not statistically significant.

AGTCACAC-3′ representing bases 2173–2196. The amount of RNA template and the cycle number for amplification were selected in quantitative ranges at which reactions proceeded in linear fashion: 150 ng of template and 35 cycles. Temperatures and time periods employed for melting, annealing and extension were 94°C and 30 s, 60°C and 60 s and 72°C and 60 s, respectively. An aliquot of each RT-PCR reaction mixture was electrophoresed on 2% agarose gel and stained with ethidium bromide. β-actin served as an internal reference to ensure equality of RNA loading. Primers for human β-actin mRNA were 5′-CAACCGCGAGAAGATGACCC-3′ representing bases 382–402 and 5′-TCTGCATCCTGTCGGCAATG-3′ representing bases 965–984. The density of MRP mRNA and β-actin mRNA products was measured, corrected by background density, and the ratio of MRP/β-actin was obtained. Results were compared to those of MCF-7 and MCF-7/VP cells not treated with S-ODN or AS-ODN.

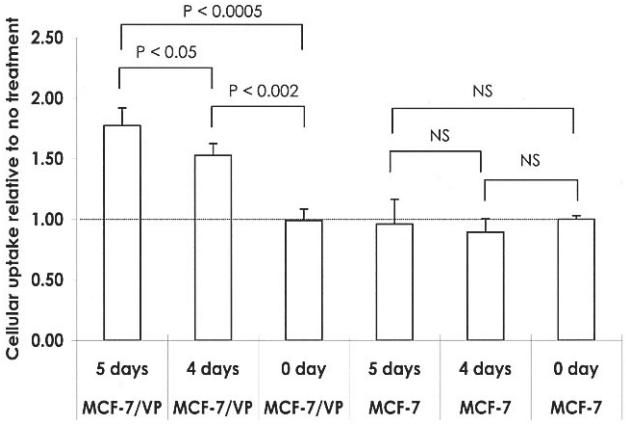

Fig. 3 Effects of treatment with antisense ODN to MRP mRNA on ^{99m}Tc-MIBI uptake in MCF-7/VP and MCF-7 cells. Cells were treated for 4 or 5 consecutive days at 5 μM. NS, difference is not statistically significant.

RESULTS

The trypan blue exclusion test revealed that cell viability of the two cell lines was unaffected by the treatment: >90% viability was detected in all sets of experiments.

As shown in Figure 1, ^{99m}Tc-MIBI uptake was proportional to the applied amount ranging 0.74 kBq to 1480 kBq, indicating that there was no saturation at the concentration used in further experiments, 740 kBq. ^{99m}Tc-MIBI uptake in MCF-7/VP cells under control conditions was only 15% of that in MCF-7 cells: 0.22 ± 0.02%/μg protein and 1.48 ± 0.05%/μg protein, respectively ($p < 0.0001$). This difference was reproducible in all experimental sets, which served as confirmation of MRP expression of these cell lines throughout this study. In the first experiment, cells were incubated in the presence of AS-ODN at 0.5 or 5 μM for 4 days (Fig. 2). Administration of 0.5 μM AS-ODN failed to improve ^{99m}Tc-MIBI uptake in MCF-7/VP cells. However, at 5 μM, uptake increased 43%. Uptake in MCF-7 cells was not appreciably affected at either concentration.

Based on these results, effects of treatment duration were observed in the presence of 5 μM AS-ODN (Fig. 3). Five-day treatment afforded superior enhancement of ^{99m}Tc-MIBI uptake in MCF-7/VP cells in comparison to 4-day treatment. Uptake in MCF-7 cells was unchanged under these conditions.

Five-day treatment with 25 μM AS-ODN provided no significant advantage in terms of ^{99m}Tc-MIBI uptake enhancement in MCF-7/VP cells relative to that detected at 5 μM (Fig. 4). Uptake in MCF-7/VP cells was not altered by S-ODN at any concentration. Neither AS-ODN nor S-ODN affected uptake in MCF-7 cells. These results confirmed the specificity of AS-ODN treatment in MCF-7/VP cells.

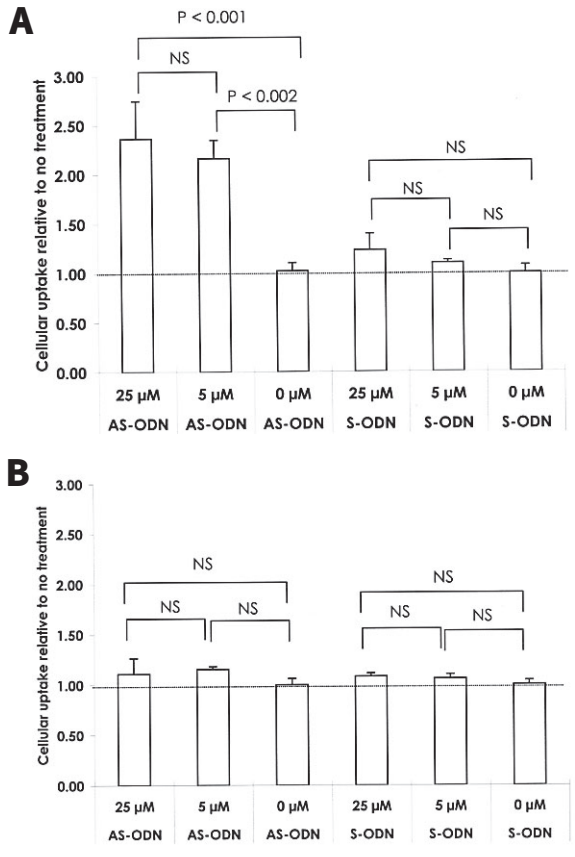

Fig. 4 Effects of treatment with antisense ODN (AS-ODN) and sense ODN (S-ODN) to MRP mRNA on ^{99m}Tc-MIBI uptake in MCF-7/VP (A) and MCF-7 (B) cells. Cells were treated for 5 consecutive days at indicated concentrations. NS, difference is not statistically significant.

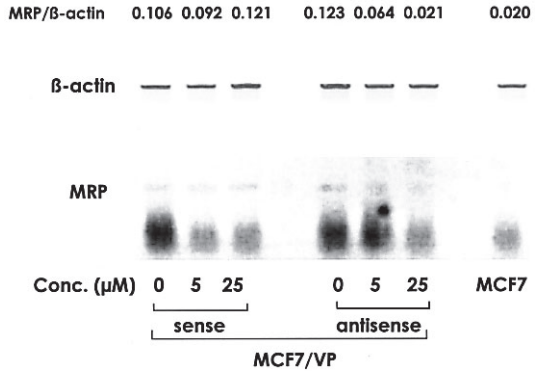

Fig. 5 RT-PCR products derived from MRP mRNA of MCF7/VP treated with antisense ODN (AS-ODN) and sense ODN (S-ODN) to MRP mRNA at indicated concentrations. β-actin served as an internal reference. Results with parental MCF7 cells are also shown. Values above the PCR profiles indicate the density ratios of MRP/β-actin.

RT-PCR analyses demonstrated MRP mRNA expression in MCF-7/VP cells (Fig. 5). In contrast, MRP mRNA was negligible in MCF-7 cells. AS-ODN administration suppressed MRP mRNA expression in MCF-7/VP cells in a dose-dependent fashion; the intensity of the MRP mRNA band decreased partially at 5 μM. On the other hand, the band was faintly recognizable at 25 μM. These findings were supported by MRP/β-actin ratios semi-quantitatively. No marked difference in β-actin mRNA expression level was observed between AS-treated and S-treated MCF-7/VP cells, a finding consistent with the equality of RNA loading in each group.

DISCUSSION

The current investigation demonstrated that effects of AS-ODN treatment on MRP function in cells possessing MDR capability can be monitored via detection of cellular uptake of ^{99m}Tc-MIBI. The most significant result of this study reveals that changes in mRNA levels clearly affect cellular ^{99m}Tc-MIBI uptake. Previously, several reports documented the association between mRNA expression of MRP or Pgp and ^{99m}Tc-MIBI accumulation in a group of patients;[17–19] however, as tracer uptake *in vivo* is a multifactorial event,[20] such *in vivo* findings do not necessarily provide direct evidence regarding the correlation between changes in ^{99m}Tc-MIBI accumulation and functional level of mRNA with respect to MDR. Nakamura et al.[21] described elevated ^{99m}Tc-MIBI uptake in tumor cells following treatment with AS-ODN to *mdr1* mRNA; however, neither mRNA nor protein level was measured in their study. The present investigation is the first to demonstrate a direct correlation between ^{99m}Tc-MIBI uptake and mRNA levels in a particular cell line during AS-ODN treatment.

AS-ODN administration enhanced ^{99m}Tc-MIBI uptake in a dose-dependent fashion; however, reversing effects of this particular AS-ODN appeared to reach a plateau around 5 μM. This type of dose-dependency was shown in a previous report.[21]

Cells were incubated with AS-ODN for 4–5 days based on the 19-h half-life of MRP in a small cell lung cancer line.[22] In the same cell line, MRP mRNA levels returned to 30%, 70% and 100% of those of controls at 24, 48 and 72 h, respectively, following AS-ODN administration, which suppressed MRP mRNA >90% at 4 h.[16] Under identical conditions in MCF-7/VP cells, treatment for five consecutive days may have reduced MRP protein expression to negligible levels. However, 5-day AS-ODN treatment did not reverse completely ^{99m}Tc-MIBI uptake in MCF-7/VP cells in comparison to that in MCF-7 cells; uptake in MCF-7/VP cells under these conditions was approximately 35% of that of MCF-7 cells. In addition, effects of 25 μM AS-ODN did not vary significantly from those of 5 μM AS-ODN in terms of improvement of ^{99m}Tc-MIBI uptake; however, RT-PCR analyses revealed greater inhibition of mRNA expression in the presence of 25 μM AS-ODN. These data suggest that the time course

of cellular activities including transcription, translation and lifetime of MRP in MCF-7/VP cells may occur more rapidly than the aforementioned reported values.

Another possible factor for the discrepant results between the effects on RT-PCR products and those on ^{99m}Tc-MIBI uptake would be related to the ^{99m}Tc-MIBI retention mechanism in cells. Previous reports indicated that the mitochondrion is the major localization site of ^{99m}Tc-MIBI.[23–26] In contrast, ^{99m}Tc-MIBI that is pumped out by MRP would locate in the cytosol other than the mitochondria, meaning that a relatively limited amount of ^{99m}Tc-MIBI is prone to be influenced by AS-ODN modulation. Therefore, sufficient inhibitory effects by AS-ODN on ^{99m}Tc-MIBI clearance would be attained even with incomplete suppression of mRNA expression. In other words, reduction of MRP mRNA achieved by 5 μM of AS-ODN would possibly be sufficient to maximize the inhibition of ^{99m}Tc-MIBI efflux from cells.

Stability of ODN is a key factor with respect to consideration of clinical application. Phosphorothioate ODN, which was utilized in this investigation, generally possesses satisfactory stability profiles;[27] in our previous experience, in excess of 80% of phosphorothioate ODN remained in non-degraded form in serum for 24 h. Therefore, this level of ODN stability, in conjunction with treatment for five consecutive days, was not a serious concern in the current study.

A second critical issue regarding the utility of ODN for interruption of cellular activities relates to insufficient delivery to cells. Receptor-mediated endocytosis or pinocytosis has been postulated as the possible mechanism by which phosphorothioate ODN enters tumor cells.[28] The negative charge of ODN disturbs intracellular entry; consequently, incorporation into carriers is often required for effective targeting of ODN to cells.[16,29] Naked ODN was employed to modulate mRNA function in MCF-7/VP cells, which resulted in the requirement of relatively high concentrations of AS-ODN in order to obtain objective responses. In spite of this difficulty, specific effects of AS-ODN in MCF-7/VP cells are obvious in contrast to the lack of responses in MCF-7/VP to S-ODN and MCF-7 cells to S-ODN and to AS-ODN, respectively.

Evaluation of the reversing effects of chemical modulators, such as verapamil and cyclosporin A, on the MDR phenomenon has been attempted with ^{99m}Tc-MIBI and ^{99m}Tc-TF.[7–13] However, these reversing agents simultaneously affect cationic transporter function on the cell surface,[30–32] on which tracer accumulation is significantly dependent.[23–26] Therefore, ^{99m}Tc-MIBI and ^{99m}Tc-TF would not be ideal for precise assessment of the effects exerted by these types of modulators. In contrast, these tracers may provide suitable estimation of therapeutic effects induced by genetic modulators such as AS-ODN and, probably, hammerhead ribozymes as these modulators do not, in theory, alter ion transporters on cells.

In conclusion, treatment with the 20-mer phosphorothioate AS-ODN specific to the coding portion of MRP mRNA significantly increased ^{99m}Tc-MIBI uptake in human breast cancer cells possessing MDR function through MRP. This result indicates that effects of AS-ODN treatment can be monitored via detection of cellular uptake of ^{99m}Tc-MIBI.

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