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Antisense GLUT1 RNA Suppresses the Transforming Phenotypes of NIH 3T3 Cells Transformed by N-*Ras*

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An antisense approach was attempted to investigate the role of antisense GLUT1 RNA in suppressing tumor cell phenotypes using N-ras-transformed NIH 3T3 cells. The established cell line transformed by ras showed typical biological characteristics of cancer cells, such as increased glucose transport, GLUT1 mRNA contents, and the ability to form colonies on the soft agar.

In this system, the plasmids (pMAM-GLUT1(rev)) which can transcribe the antisense GLUT1 RNA were transfected and the accompanying changes in the phenotypes of the ras-transformed cells were observed. The expression of antisense GLUT1 RNA by induction with dexamethasone reduced the glucose transport by 30% (1.97±0.13 nmoles) after 4 min incubation when compared to the non-induction group of transformed cell (2.85±0.19 nmoles). Also, the number of colonies sized over 50 μm on the soft agar was reduced significantly in the antisense RNA expressing group compared to non-induction group. These results suggest that the expression of antisense GLUT1 RNA reduced the glucose transport and transforming potential in soft agar possibly by hybridization with GLUT1 mRNA in N-ras-transformed NIH 3T3 cells.

Key Words: GLUT1 expression, antisense RNA, *ras*

Recently, antisense approaches have been attempted to suppress the tumor phenotypes *in vitro*. Introduction of complementary antisense RNA into a cell or tissue is one of the effective way to inhibit a specific gene expression. Of these, applications of antisense technology to the treatment of cancer is just emerging; the suppression of the growth of carcinoma cell lines by expressing E6/E7 antisense RNA (Steele *et al.*, 1992), and EGF receptor antisense RNA (Moroni *et al.*, 1992).

Warburg (1956) established that most tumor cells exhibit elevated rates of glucose uptake and metabolism compared to non-transformed cells. This phenomenon is one of the most distinctive features of tumor cells and specific to transformation process (Hatanaka *et al.*, 1970).

Recent advances in molecular biology enabled us to uncover the potential mechanism of increased glucose transport in tumor cells. The transformation of NIH 3T3 cells with the activated cellular oncogenes (*src*, *ras*, and *fps*) increased glucose transport by 4-fold whereas nuclear oncogene (*myc*) did not show any ef-

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Abbreviation: GLUT1 (erythrocyte-type glucose transporter), DMEM (Dulbecco's minimum essential media), PCR (Polymerase chain reaction), MMTV-LTR (Mouse mammary tumor virus-long terminal repeat), BME; basal media Eagle

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fect (Birnbaum *et al.*, 1987; Flier *et al.*, 1989). Currently, these effects are thought to be mediated by the elevation in GLUT1 mRNA and GLUT1 protein (Merrall *et al.*, 1993). Therefore, GLUT1 may play an important role in the maintenance of tumor cell phenotypes. To test this hypothesis, we tried to suppress tumor cell growth by transfecting with a plasmid which can express antisense GLUT1 RNA. In this study, we have transformed NIH 3T3 cells with activated *ras* and investigated the effect of antisense GLUT1 RNA on the transformed phenotypes of tumor cells.

MATERIALS AND METHODS

Cell culture

NIH 3T3 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, U.S.A). The cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (GIBCO, BRL. MD, U.S.A.), 100 mg of streptomycin, 100 units of penicillin G, and 0.25 mg amphotericin B per milliliter of media at 37℃ under standard condition.

Transformation of NIH 3T3 cells by pBN-*ras*

pBN-*ras* was purchased from ATCC. It has a known transforming activity in NIH 3T3 cells (Murray *et al.*, 1983). Transfections were performed by calcium phosphate coprecipitation of pBN-*ras*, and pRSV-neo which is used for neomycin selection (Chen and Okayama, 1987). Transformed cells were removed from the foci by treating with trypsin and the stable cell line was established by selection with neomycin at a concentration of 700 mg/ml media.

Detection of N-*ras* in the chromosome of transformed NIH 3T3 cell

To detect the N-*ras* in the chromosome of tranformed NIH 3T3, polymerase chain reaction (PCR) was performed. Chromosomal DNA was prepared from the transformed NIH 3T3 cells (Sambrook *et al.*, 1989) and the exon 2 region of N-*ras* was amplified by oligonucleotide primers (sense; pCCCCCGAATTCTTACAGA AAA, antisense; pCTGGATCCTGTAGAGGTT-AATATCCGC). The first thermal cycle of PCR consisted of 6 mins' denaturation at 94℃, annealing for 1.5 min at 58℃ and polymerization at 72℃ for 2 min. Between the first and the last cycle, DNA was denatured for 1 min at 94℃, annealed for 1.5 min at 58℃ and polymerized at 72℃ for 2 min. During the last cycle of PCR, the DNA was denatured at 94℃ for 1 min and annealed at 58℃ for 1.5 min and polymerized for 10 min at 72℃. The stable cell line of transformed NIH 3T3 cells were chosen by confirming the presence of exon 2 region (193 bp) using PCR (data not shown).

Glucose transport activity

The transport of 2-deoxy-D-glucose was determined in NIH 3T3 cells, N-*ras*-transformed NIH 3T3 cells, and NIH 3T3 cells transfected with antisense GLUT1 construct (*ras*/pMAM-GLUT(rev)) according to the method of Hasegawa *et al.* (1990). The 2.5×10^5 cells were seeded in 12 well culture plates. Cells were further cultured for 2~3 days with or without 1 μM dexamethasone until they reached confluency. The cells were rinsed three times with phosphate buffered saline (pH 7.4) and incubated for designated time in Krebs-Henseleit Hepes buffer (pH 7.4) with 0.5 μM(2 mCi) 2-deoxy-D-[1-^{3}H] glucose (specific activity, 17.4 mCi/mmole). The isotopes remaining in the media were washed three times with phosphate buffered saline after the designated incubation times. The cells were solubilized in 1 ml of 2% SDS and 0.8 ml lysates were taken to measure the amount of transported glucose in liquid scintillation counter. Transport assays were done in triplicate and protein concentration was measured by Bradford method (1976). The specific uptake of 2-deoxy-D-[1-^{3}H] glucose was expressed in nmoles/mg protein.

Construction of antisense GLUT1 expression vector and transfection

Human GLUT1 cDNA was subcloned into *Sal* I site of pGEM4Z and named pGEM-

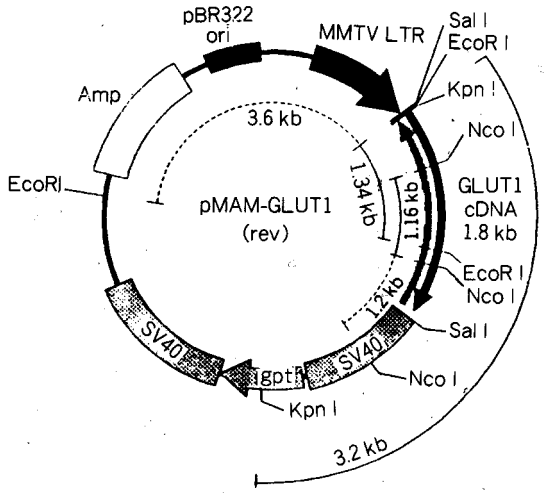

Fig. 1. *Construction of plasmid expressing antisense GLUT1 RNA. The Sal I fragment of GLUT1 cDNA in pGEM-GLUT1 was excised out and subcloned into eukarytoic expression vector, pMAMneo. The recombinant plasmid containing cDNA in reverse orientation, relative to MMTV LTR, was selected by restriction enzyme analysis and named pMAM-GLUT1 (rev).*

GLUT1. 1.8 kb GLUT1 cDNA encoding the entire open reading frame was excised out using *Sal* I restriction enzyme and the fragment was isolated using GeneClean II kit (Bio101, La Jolla, CA). The cDNA was subcloned into *Sal* I site of dexamethasone inducible eukaryotic expression vector, pMAMneo. The orientation of GLUT1 cDNA in the pMAMneo was confirmed by restriction enzyme analysis. The plasmid that encode antisense GLUT1 RNA was named pMAM-GLUT1(rev) (Fig. 1). This DNA was used to transfect *ras*-transformed NIH 3T3 cells using Lipofectin (GIBCO BRL, USA) according to the manufacturer's instruction. After 20 hrs, the growth media was replaced with DMEM containing 10% fetal calf serum and the cells were used for the measurement of glucose transport or for soft agar assay.

RNA preparation and northern blotting

mRNA was isolated from cells using Quick Prep micro mRNA purification kit (Pharmacia Biotech, NJ), and was stored at −70℃. 5 μg of mRNA was run in formaldehyde agarose gel electrophoresis, then transferred to the nitrocellulose membrane. [^{32}P]-labeled RNA probes of sense and antisense transcripts were prepared using T7 polymerase and SP6 RNA polymerase using in vitro transcription system (Promega, WI, USA) and were used to detect antisense and sense GLUT1 RNA, respectively.

Anchorage independence

Assay for anchorage independence was performed according to the method of Velu *et al.* (1989). 3×10^4 cells were seeded in 0.33% agarose in BME supplemented with 10% FBS and antibiotics on the top of 0.5% agarose basal layer. Focus formation was assessed at 21st days by scoring the number of colonies that were larger than 50 μm under inverted microscope.

Inducing agents. The expression of GLUT1 antisense RNA was induced by the addition of dexamethasone at the concentration of 1 μM when the cells were seeded for glucose transport or soft agar assay.

Statistics

Transfections of pMAM-GLUT1 (rev) or glucose transport studies were carried out in three independent experiments, where triplicate dishes were used for each group. Data are expressed as mean±S.D. and compared by the Student's t-test.

RESULTS AND DISCUSSION

Comparison of phenotypes between control NIH-3T3 and N-*ras* transformed NIH 3T3 cells

To test whether the cells transformed by N-*ras* have increased glucose transport, glucose transport between control NIH 3T3 and N-*ras* transformed NIH 3T3 cells was compared (Fig. 2). The transport of glucose began to show marked difference after 4 min of incubation in the presence of 2-deoxy-D-[1-^{3}H]

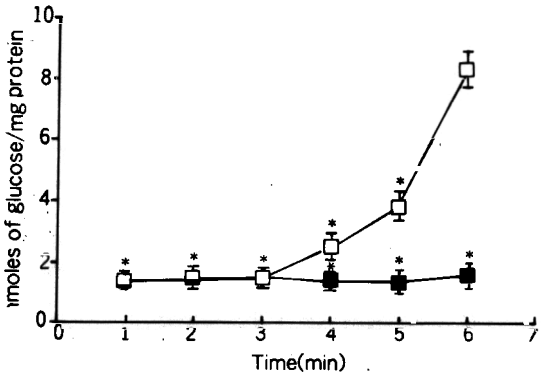

*Fig. 2. Time course of glucose transport observed in NIH 3T3 cells and N-ras-transformed NIH 3T3 cells. The transport of 2-deoxy-D-[1-^{3}H]-glucose in control and N-ras-transformed NIH 3T3 cells was determined by the method of Hasegawa et al. (1990). Detailed methods are described in "Materials and Methods". Closed square: control NIH 3T3 cells, open square: N-ras-transformed NIH 3T3 cells *: Mean±S.D.*

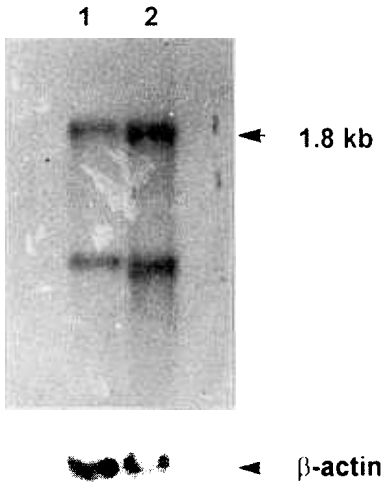

Fig. 3. Northern blot of GLUT1 mRNA isolated from control and N-ras-transformed NIH 3T3 cells. mRNA (5 μg) was isolated from control and N-ras-transformed NIH 3T3 cells and subjected to gel electrophoresis. The separated mRNAs were transferred to nitrocellulose membrane and stained with methylene blue or hybridized with ^{32}P-labeled GLUT1 antisense probe. The amount of mRNA in each lane were confirmed to be the same by hybridization with a probe to detect β-actin mRNA. Autoradiography was performed at −70℃ overnight. Lane 1: control NIH 3T3 cells, Lane 2: N-ras-transformed NIH 3T3 cells.

glucose. The amounts of transported glucose increased 2.5 , 4.5, and 6.0 fold at 4min, 5min and 6min, respectively, after the addition of 2-deoxy-D-[1-^{3}H]-glucose when compared to the control group. The northern blot showed marked increase in GLUT1 mRNA sized 1.8 kb in transformed cells (Fig. 3). These data clearly support the current belief that the increased glucose transport is due to increased GLUT1 transcription at least in *ras*-transformed tumor cells (Birnbaum *et al.* 1987). In this experiment, small sized RNA(~0.8 kb) were hybridized in both group. They may be degradation products of GLUT1 mRNA. However, we don't know why the degradation products appeared as discrete bands.

The ability to form colonies in soft agar showed marked difference. The N-*ras*-transformed cells readily formed colonies after 14 days of incubation. Judging from these data, N-*ras*-transformed NIH 3T3 cells have characteristic finding of cancer cells and may serve as an excellent cancer cell model.

Expression of antisense RNA

Attempts to inhibit the growth of tumor cells using antisense RNA has been expanded rapidly (Walder, 1988; Helene and Toulme, 1990). The increased transcription of GLUT1 mRNA in many types of tumor cells makes the GLUT1 mRNA one of the attractive targets for antisense inhibition. We have constructed a vector which can express full length antisense GLUT1 RNA within cells and transfected the DNA into a tumor cell line (NIH 3T3) which was established by transfecting with N-*ras*. The transcription of antisense GLUT1 RNA was induced by the addition of dexamethasone (1 μM). Northern blot revealed that dexamethasone induced the transcription of antisense GLUT1 RNA sized 2.4 kb (Fig. 4). Although the size of GLUT1 cDNA was 1.8 kb, the subcloning in the oppo-

site direction in pMAMneo vector resulted in longer transcript (2.4 kb, Fig. 1) because the poly A signal lies about 600 bp downstream of the GLUT1 cDNA insert.

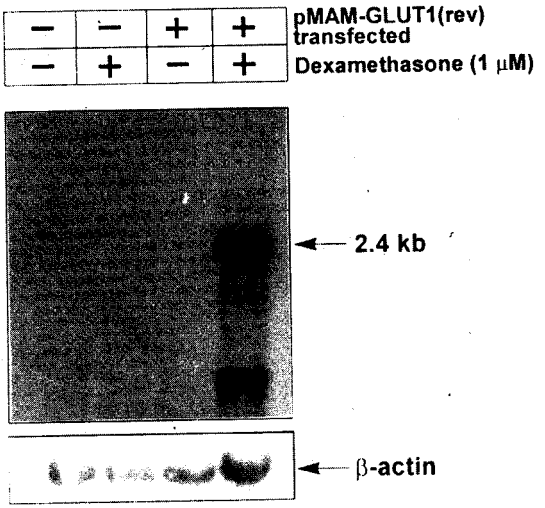

Fig. 4. *Induction of antisense GLUT1 transcripts by dexamethasone. Cells (1×10^5) were seeded and grew in the presence or absence of dexamethasone (1 μM) for 24 hrs. Cells were harvested and mRNAs were fractionated as described in the "Materials and Methods". Antisense GLUT1 RNA transcripts were detected using [^{32}P]-labeled riboprobe of sense strand of GLUT1 cDNA.*

Inhibition of glucose transport by GLUT1 antisense RNA

In examining the effect of GLUT1 antisense RNA on the glucose transport of transformed cell, the effect of antisense RNA inducing agent, dexamethasone was tested. Of the concentrations of tested, 1 μM dexamethasone was used because the glucose transport was not affected upto this concentration (Table 1, $P > 0.05$). Table 1 shows the inhibition of glucose uptake by antisense GLUT1 RNA. Although a slight increase in the glucose transport rate was observed (3.36 ± 0.54 nmoles /mg protein) when compared to the control groups (pMAM-GLUT1(rev) untransfected groups), this was not statistically significant ($p > 0.05$). This result suggests that the rate of glucose transport was not affected when the antisense GLUT1 RNA was not induced to express inside the transformed cells. However, the induction of antisense RNA by dexamethasone caused significant decrease (30%) in the glucose transport ($p < 0.05$). This result suggests that the antisense GLUT1 RNA inhibited the glucose transport possibly by hybridizing with GLUT1 mRNA.

Anchorage independence

N-*ras*-transformed cells transfected with

Table 1. Effect of antisense GLUT1 RNA expression in the glucose transport in N-*ras*-transformed NIH 3T3 cells. 2.5×10^5 cells were seeded in 12 well culture plates and cultured for another 2~3 days until they reached confluency. For glucose transport measurements, cells were incubated for 4 minutes in the presence of 2-deoxy-D-[1-^{3}H]-glucose. The glucose transports into the various test groups were determined as described in the "Materials and Methods". Data represent mean±S.D. for three independent determinations.

Dexamethasone (1 μM)	−	+	−	+
pMAM-GLUT1(rev) transfected	No	No	Yes	Yes
Glucose transported (nmoles/mg protein)	2.85±0.19	2.99±0.12	3.36±0.54	1.97±0.13*

* Significantly different from other groups by Student's *t*-test ($p < 0.05$)

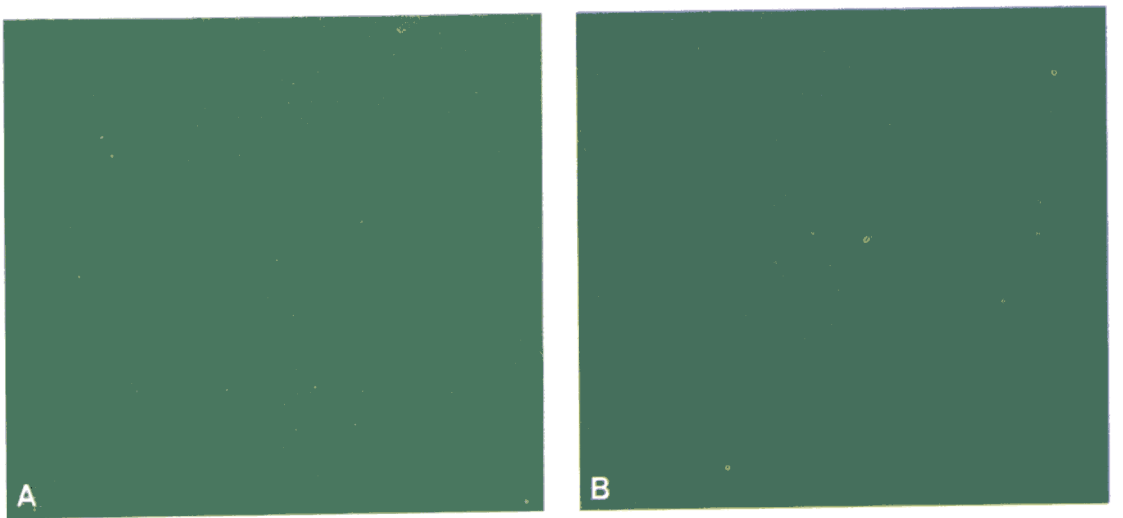

Fig. 5. Effect of antisense GLUT1 in the suppression of the tumor cell growth in soft agar. Cells were transfected with pMAM-GLUT1 (rev) and grew for another 20 hrs. The transfected cells were seeded (3×10^4) in 0.33% agarose in basal media Eagle (BME) supplemented with 10% FBS and appropriate amounts of antibiotics as described in the "Materials and Methods" and poured on the top of 0.5% agarose basal layer. 1 μM dexamethasone was added in order to induce the transcription of antisense GLUT1 RNA. (A) Non-induction group, (B) Induction group.

pMAM-GLUT1 (rev) readily produced anchorage independent foci in soft agar (Fig. 5A) because the expression of antisense GLUT1 RNA was not induced by dexamethasone. However, dexamethasone treatment to this group decreased the numbers of colonies as well as the size of foci (Fig. 5B). The number of colonies on the soft agar plate was decreased significantly ($P<0.05$) in antisense GLUT1 RNA expressing cells by 42% (20 ± 3.4 foci/60 mm plate) when compared to the uninduced control group (34 ± 2.8 foci/60 mm plate). Also, colonies produced by the antisense expressing group grew as very small colonies. The size of the colonies did not further increase even if they kept in culture for more than a month.

The mechanism of these phenotypic changes by antisense GLUT1 RNA is not known yet. But it is quite possible that the antisense GLUT1 RNA transcribed transiently in the tumor cell line may hybridize with overproduced GLUT1 mRNA leading to the blockage of translation of GLUT1 mRNA or other inhibitory functions elicited by antisense GLUT1 RNA (Helene and Toulme, 1990). Taken together, it is tempting to speculate that the inhibition of GLUT1 expression by antisense GLUT1 RNA resulted from the decrease in GLUT1 in cancer cells. The reduced ability to form colonies in soft agar might be caused by the decrease in glucose which is an essential nutrient for tumor cell growth. Finally, the inhibition of some tumor cell phenotypes by antisense GLUT1 RNA raises the possibility that GLUT1 mRNA could be a target for cancer therapy, especially for cancer cells overexpressing GLUT1.

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