Cancer Letters, 69 (1993) 27–32
Elsevier Scientific Publishers Ireland Ltd.

Partial reversion of transformed phenotype of B104 cancer cells by antisense nucleic acids

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(Received 17 September 1992)
(Revision received 7 December 1992)
(Accepted 8 December 1992)

Summary

We used antisense RNA to inhibit the expression of oncogene neu and investigated the effects of diminished neu expression on the phenotypes of B104 cells containing activated oncogene neu. Antisense MT-neu and pSV-neo plasmids were cotransfected into neuroblastoma B104 cells. Southern analysis showed the integration of anti-neu DNA into B104 cells. The expression of neu was inhibited up to 90% as quantitated by immunoprecipitation. The growth rate and the potential to differentiate in these transfectants were not affected as compared to the parental cell lines. The ability to grow in soft agar was inhibited more than 90% in these transfectants. Our results indicated that antisense-RNA against a specific oncogene can decrease the tumorigenicity of tumor cells but may not be able to revert it to normal cells completely.

Keywords: *neu*; antisense; oncogene; neuroblastoma

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Introduction

One effective way to modify or turn off the activity of a particular gene selectively is to create antisense molecules that bind specifically to the target gene RNA messages, thereby interrupting the translation of that gene [1–3]. Such an approach has been used to generate artificial mutants in studying the functions of oncogenes and other genes in vitro and in vivo. Antisense RNA or DNA techniques also hold promise as therapeutics in treating diseases or cancer by inhibiting the production of a specific virus-encoded protein or the expression of cancer-associated genes [4–11]. Cancer cells containing oncogenes activated by point mutation, such as *ras* or *neu*, would be the preferred choices to perform the antisense experiments, because the expression of proto-oncogene will be unaffected when selective oligonucleotides against the mutated sequences are employed [8]. However, no experimental evidences has been presented so far that anti-neu nucleic acid can revert the transformed phenotypes of cancer cells.

The activated oncogene *neu* is carried in the rat neuroblastoma B104 cells, which were derived from neoplasms that were induced transplacentally with nitrosourea during the 15th day of gestation [12]. The *neu* oncogene was detected by transfection of mouse NIH3T3 cells with genomic

0304-3835/93/$06.00

DNA from B104 cells, and isolated from foci of transformed cells [13] The oncogene *neu* encodes a tumor-associated antigen p185 bearing similarities to the EGP receptor [14]. The activation of *neu* results from a change of valine to glutamic acid in the transmembrane domain of the p185 [15]. The human *neu* (HER-2) has been found to be amplified in a significant proportion of human breast cancer and other carcinoma, and this amplification appears to correlate with a more malignant phenotype [16–20]. Therefore, it is important to study whether the antisense-RNA can revert the *neu*-associated cancer cells to normal cells or cells with less transformed phenotype. In this study, we inhibited the expression of *neu* by introducing the anti-*neu* plasmid into B104 cells. The transfectants grow normally but with little ability to grow in soft agar. The results suggest that anti-*neu* message can partially revert the transformed phenotype to normal cells.

Materials and Methods

Cell culture

B104 cells and the antisense transfectants were cultured in Dulbecco's modified Eagle's medium with 10% calf serum (Boehringer Mannheim), penicillin (100 U/ml), and streptomycin (10^{-4} g/ml). Selection for the expression of the *neu* gene [21] was carried out in medium containing 4×10^{-4} g/ml G418. Induction of the metallothionein (MT) promoter was performed in medium containing 5×10^{-5}M $ZnCl_2$. The MT promoter was a kind gift from Dr. Kazuo Maruyama.

Transfection and selection of transfectants

The antisense MT-*neu* plasmids and pSV-neo plasmid were cotransfected into half-confluent B104 cells at the ratio of 10:1 by the calcium phosphate method [22]. Cells were subcultured and subjected to selection in medium containing G418. G418 resistant colonies were isolated 2 weeks later using cloning cylinders.

DNA isolation and Southern blotting

Genomic DNA was isolated by standard method [23]. Ten micrograms of DNA was digested with *Eco*R I and separated in 0.8% agarose gel. Blotting of DNA to nitrocellulose was done as described by Southern [24]. Probes with high specific activity was prepared by random-primed DNA labeling kit (Boehringer Mannheim). The probe for hybridization is 0.7 kb *Pst*I-*Sma* I fragment of *neu* cDNA [14].

Immunoprecipitation

Cells were grown to 80% confluent in 10-cm dishes in DMEM 10% calf serum, and 5×10^{-5} M $ZnCl_2$. Cells were then starved in methionine-free medium for 1 h, and labeled with [^{35}S]methionine for 6 h. Cells were lysed in lysis buffer containing 50 mM Tris, 1% NP-40, 0.1% sodium deoxycholate, 0.15 M NaCl, 1 m MPMSF, 4 mM EDTA, 2 mM vandate, and 10 mM sodium fluoride. Lysates were centrifuged at $30\,000 \times g$ for 30 min. The anti-*neu* antibody (Ab3 from Oncogene Science) was added to the supernatant for incubation of 2 h. The immunocomplexes were collected using protein A-sepharose, and resuspended in electrophoresis sample buffer for separation on 7% polyacrylamide gel.

Soft agar assay

The assay was performed as described by Rizzino [25]. The base layer is 0.6% agar (agar noble from Difco) in culture medium containing 10% calf serum. Cells were trypsinized and plated 2×10^3 cells/10-cm dish in 5 ml culture medium containing 10% calf serum and 0.3% agar on the top of solidified base layer. Both layers contain 5×10^{-5} M $ZnCl_2$. Once the top layer has been added and solidified, the dishes are incubated at 37°C in a 5% CO_2 atmosphere.

Results and Discussion

The full length *neu* 4.7 kb cDNA was inserted in the opposite orientation, under the control of metallothionein promoter as shown in Fig. 1 [26]. Downstream of *neu* gene are the simian virus 40 small t antigen splice and polyadenylation sites. The backbone of this vector is derived from pUC18. The anti-*neu* plasmid generates a 9-kb linear fragment when digested with *Eco*R I.

Thirteen G418-resistant colonies were isolated when B104 cells were transfected with both

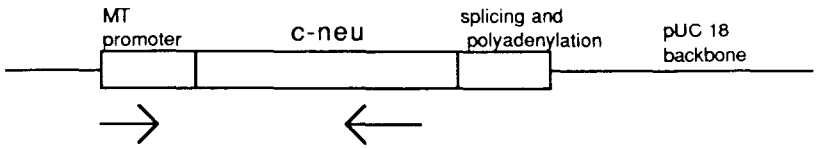

Fig. 1. The map of anti-*neu* plasmid DNA. The arrows indicates that the *neu* cDNA is in the opposite orientation relative to the promoter.

anti-*neu* and pSV-neo plasmids, and the presence of integrated anti-*neu* DNA in the transfectants was demonstrated by Southern blot analysis. Seven of the 13 clones, C-1, C-2, C-4, C-8, C-10, C-12 and C-13, contained anti-*neu* plasmid DNA. A representative Southern blotting is shown in Fig. 2. The presence of strong 9 kb band in lane 2 and 3 indicates the integration of multiple copies of anti-neu plasmid in the genomic DNA of C-1 and C-10 transfectants. The 9 kb band was not observed in the parental B104 cells (lane 1, Fig. 2).

The amount of *neu*-encoded protein p185 was determined in the transfectants and B104 cells by immunoprecipitation with monoclonal antibody against p185. As shown in Fig. 3, the level of p185 in C-1, C-10, and C-12 transfectants were markedly reduced comparing with that of B104 cells. This result indicates that the expression of oncogene *neu* was inhibited more than 90% in these transfectants.

Tumorigenicity of the transfectants was determined in vitro by soft agar assay. The ability of

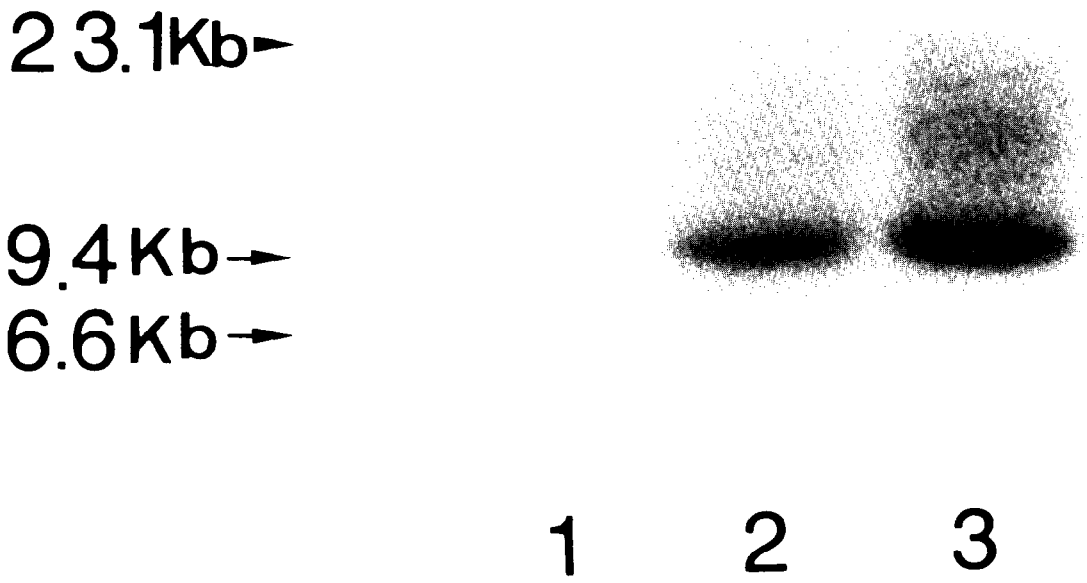

Fig. 2. Southern analysis of the anti-*neu* DNA. *Eco*R I digested genomic DNA from B104, C-1 and C-10 cells were probed with *Pst*I-*Sma* I fragment of *neu* cDNA. Lane 1, B104 cells; lane 2, C-1 transfectants; lane 3, C-10 transfectant. Lambda phage *Hind* II-generated fragments were used as molecular weight markers in kilobases.

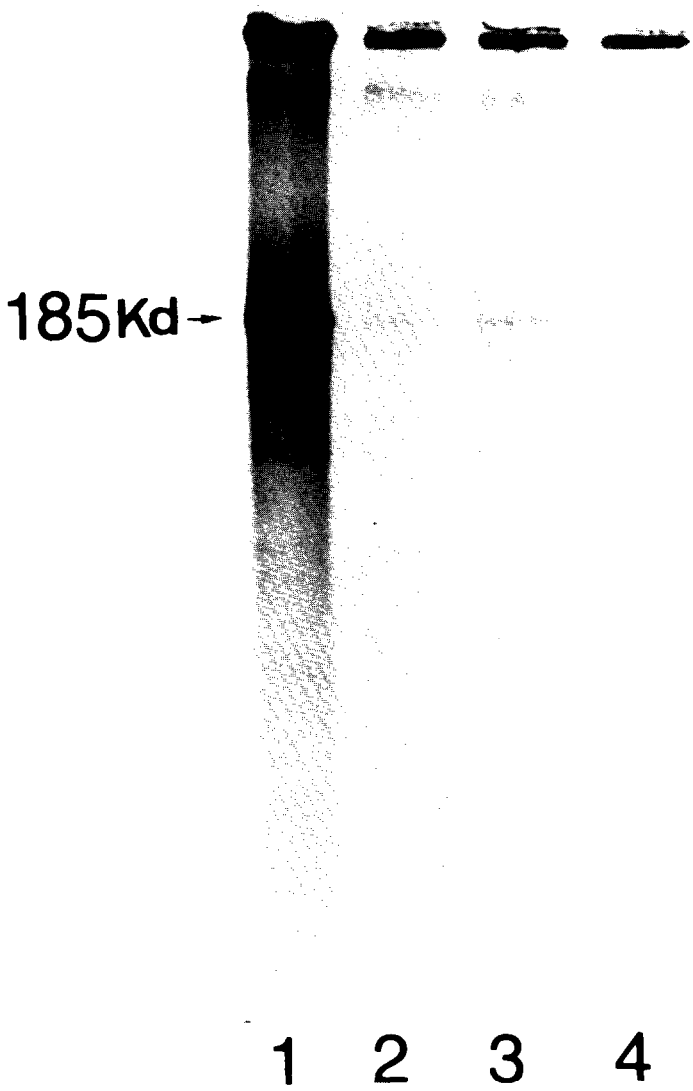

Fig. 3. SDS-PAGE analysis of anti-*neu* immunoprecipitates from metabolically labeled cells. [^{35}S]methionine-labelled cells of B104 and three transfectants were immunoprecipitated with monoclonal anti-neu anibodies as shown. Lane 1, B104 cells; lane 2, C-1 transfectant; lane 3, C-10 transfectant; and lane 4, C-12 transfectant. The precipitation of the 185-kDa polypeptide was indicated by the arrow.

cells to grow in soft agar correlates well with the ability to form tumor in vivo [27]. B104 cells gave rise to many colonies after being seeded in 0.3% soft agar for 2 weeks, while normal NIH3T3 cells did not form any colony. The anti-neu transfectants lost most of the ability to grow in soft agar, and formed few or no colonies (Table I). The growth rates of B104 cell line and other transfectants in 10% calf serum are almost identical (Fig. 4); therefore, decreased colony formation observed here is unlikely to be due to different growth rates. The control transfectants L1 and L2, which only

Table I. Soft agar assay of B104 cells and transfectants.

Cell Lines	No. of colonies[a]
B104	39
NIH3T3	0
L-1	41
L-2	35
C-1	1
C-2	8
C-4	3
C-8	2
C-10	0
C-12	2
C-13	4

[a]The average number of three independent experiments.

pSV-neo integrated, grow in soft agar as well as parental B104 cells. This suggests that loss of growth in soft agar was not due to transfection or selection process. The soft agar results indicated that the anti-neu transfectants has lost one important property of tumor cells, anchorage-independent growth. The inhibition of growth in soft agar was observed in almost all transfectants containing anti-neu, which rules out the possibility that the reversion is due to inactivation of cellular gene by random integration of plasmid DNA.

However, the anti-*neu* transfectants seems to retain a little ability to grow in soft agar as shown in Table I. Therefore, the antisense technique may be an effective way to repress the transformed phenotype of cancer cells but cannot completely revert the cancer cells to normal cells, at least in the B104 cells we examined here.

The proto-oncogenes have been shown to be involved in cellular differentiation in several cell lines, for example, anti-*myc* message has been shown to accelerate differentiation in Friend murine erythroleukemia cells [28]. Therefore, the transfectants were further assayed for the ability to differentiate. Neutrite extension was observed in the transfectants in the presence of 1 mM dibutyryl cAMP or serum starvation (data not shown). The inhibition of neu expression was not able to affect normal differentiation of B104 cells as judging from morphological observation. However, it cannot be excluded that very low amounts of p185 are sufficient for differentiation of B104 cells.

Our current studies have demonstrated that anti-*neu* message can partially though not completely revert the cancer cells to normal cells. Cancer cells from primary tumor are known to contain multiple mutations and many activated oncogenes [29]. Our present study suggests that antisense nucleic acids against one of the activated oncogenes are able to suppress the transformed phenotype remarkably.

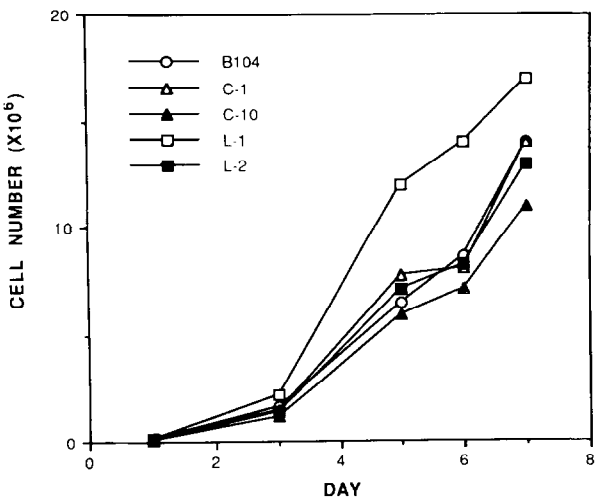

Fig. 4. Growth rates of B104 cells and the transfectants. 1×10^5 cells of B104 cells (circles), C-1 (open triangles), C-10 (closed triangles), L-1 (open squares) and L-2 (closed squares) transfectants were innoculated on the 9-cm plastic dishes. The dishes were cultured in the 10% calf serum with 5×10^{-5} M $ZnCl_2$ and on the indicated days cell numbers were determined in duplate cultures. The average cell number are shown.

Acknowledgements

This work is supported by grant (NSC-78-0412-B-002-163) from National Science Council in Taiwan, Republic of China. I am grateful to Dr. Robert A. Weinberg for providing the B104 cells, and to Dr. Mien-Chie Hung for the gift of *neu* cDNA.

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