Neural Cell Adhesion Molecule Mediates Contact-dependent Inhibition of Growth of Near-diploid Mouse Fibroblast Cell Line m5S/1M

Junken Aoki,* Masato Umeda,* Koji Takio,‡ Koiti Titani,‡ Hiroshi Utsumi,§ Masao Sasaki,§ and Keizo Inoue*

*Department of Health Chemistry, Faculty of Pharmaceutical Science, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan; ‡Laboratory for Aging Process Research, Frontier Research Program, RIKEN, 2-1, Hirosawa, Wako, Saitama 351-01, Japan; and §Radiation Biology Center, Kyoto University, Yosida-Konoecho, Sakyou-ku, Kyoto 606, Japan

Abstract. A near-diploid mouse fibroblast cell line m5S/1M used in this study shows a high sensitivity to contact-dependent inhibition of growth, and the addition of EGF causes both morphological change and loss of contact-dependent inhibition of growth. The m5S/1M cell and its transformants obtained by x-ray irradiation have been used to search for the cell surface glycoproteins that are responsible for the growth regulation via cell–cell interactions. Lectin blotting analyses showed that the expression of the cell surface glycoprotein of 140 kD (140KGP) is highly sensitive to the transformation induced either by x-ray irradiation or by the EGF stimulation. We purified the 140KGP and found that it was composed of two glycoproteins. The major component of 140KGP was identified as neural cell adhesion molecule (NCAM) by amino acid sequence analyses of the peptide fragments and by the cross-reactivity with anti-NCAM mAb, clone H28.1.2.3. Monoclonal antibody against 140KGP (clone LN-10) recognizes all three isoforms of NCAM expressed on m5S/1M cell and showed that the expression of NCAM was highly sensitive to the transformation. Furthermore, the immobilized LN-10 strongly inhibited the growth of actively proliferating m5S/1M cells and the LN-10 in a soluble form showed a significant growth-stimulating effect on the confluent quiescent cultures of m5S/1M cells. The results show that NCAM plays a major role in the contact-dependent inhibition of growth of m5S/1M, and that NCAM might be involved in the regulation of cell growth during embryogenesis and formation of nervous systems.

ADHESIVE interactions between cells play a central role in the primary cellular processes operating in the formation of tissues such as growth, motility, morphology, and differentiation. The growth of normal cells in vitro is also highly controlled, leading to the cessation of growth when the cells reach a certain cell density (contact-dependent inhibition of growth). Although a large amount of evidence has indicated that cell surface glycoproteins play a crucial role in the adhesive interactions and in the regulation of cell growth, the molecules which are responsible for the contact-dependent inhibition of growth are not fully characterized.

Concerning the molecules which are involved in the growth regulation via cell–cell contacts, several reports have shown that plasma membranes purified from the various cells (Whittenberger and Glaser, 1977; Raben et al., 1981; Nakamura et al., 1984; Heimark and Schwarz, 1985), or immobilized plasma membrane glycoproteins (Wieser et al., 1985; Wieser and Oesch, 1986, 1987) could mimic the inhibitory effect of cell–cell contacts on the cell growth. Recently, Wieser et al. (1990) isolated the cell surface glycoprotein with an apparent molecular mass of 60–70 kD which is involved in the contact-dependent inhibition of growth of human diploid fibroblasts.

We have used a mouse near-diploid fibroblast cell line m5S/1M to search for the cell surface glycoproteins that are involved in the contact-dependent inhibition of growth. Since many of the cell lines which have been used for these studies such as 3T3 cells are hypotetraploid or hypertetraploid, with most of the genes present in three or four copies, the genetic as well as biochemical phenotypes of the cells are unstable. m5S/1M is unique in retaining a highly stable near-diploid chromosome constitution and in showing high sensitivity to contact-dependent inhibition of growth (Sasaki and Kodama, 1987). This cell line exhibited a unique response to the stimulation of EGF (Watanabe et al., 1990); the addition of EGF caused both morphological change and loss of contact-dependent inhibition of growth of m5S/1M cell, and the analysis of glycosphingolipids of the EGF-stimulated cell revealed that globotriaosylceramide, which is known to be a Burkitt lymphoma–associated antigen, is specifically expressed in the EGF-stimulated cells. Many

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transformants obtained by x-ray irradiation have provided a useful tool for studying the genomic changes during transformation (Kodama and Sasaki, 1987). Since changes of the expression of particular cell surface glycoproteins in transformed cells may reveal the connection between the glycoproteins and the regulation of cell growth, first we searched for the cell surface glycoproteins that show the distinct expression profiles among m5S/1M and its transformants.

In the course of this study, we found that neural cell adhesion molecule (NCAM)[1] is abundantly expressed on m5S/1M cells and the expression is highly sensitive to the transformation. Although a large body of information on the tissue distributions of NCAM during embryonic development and on the adhesive interactions between NCAM molecules are available (Edelman, 1987; Cole et al., 1986; Hoffman and Edelman, 1983), we know very little about the cellular signals followed by the interaction between NCAM molecules. Here we demonstrate that the immobilized monoclonal antibody against NCAM strongly inhibited the growth of m5S/1M cells and suggest the NCAM plays the major role in the establishment of contact-dependent inhibition of growth of m5S/1M cells. These findings raised the possibility that NCAM is involved in the regulation of cell growth during embryogenesis and formation of nervous systems.

Materials and Methods

Cell Culture

m5S/1M cells (Sasaki and Kodama, 1987) and x-ray–induced transformant of m5S/1M cells (Kodama and Sasaki, 1987) were maintained in alpha-modified minimum essential medium (α-MEM/FCS) supplemented with 5% heat-inactivated FCS (Gibco Laboratories, Grand Island, NY), 100 u/ml penicillin (Gibco Laboratories), and 100 μg/ml streptomycin (Flow Laboratories, Inc., McLean, VA) under humidified atmosphere of 5% CO_2-95% air at 37°C (Sasaki and Kodama, 1987). In some experiments, the cells were grown in the presence of the following concentration of ligands by plating 3×10^5 cells in 100-mm culture dishes (Corning Glass Works, Corning, NY) containing 20 ml of α-MEM/FCS; EGF 8 ng/ml (Takara Shuzo Co. Ltd., Kyoto, Japan), PMA (100 nM) (Sigma Chemical Co., St. Louis, MO), bradykinin (1 μM) (Sigma Chemical Co.). The cells, which had become confluent after 5 d of culturing, were washed with PBS (137 mM NaCl, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4) and harvested with a rubber policeman.

Lectin Blot Analysis of Glycoproteins

Membrane glycoproteins were solubilized by incubating the harvested cells with cell lysis buffer (0.5% NP-40, 10 mM Tris, 25% sucrose, 1 mM EDTA, 1 mM PMSF, 5 mM *N*-ethylmaleimide, 1 μM pepstatin A, pH 7.4) at 4°C for 60 min. Cell lysate was then centrifuged at 9,000 *g* at 4°C for 20 min and supernatant was used as glycoprotein fraction. Glycoproteins were separated by SDS-PAGE using 7.5% or 10% acrylamide gel (Laemmli, 1970) and then transferred to nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA) at 50v for 150 min in 25 mM Tris, 192 mM glycine at 4°C using the protein transfer system (Bio-Rad Laboratories) (Towbin et al., 1979). The nitrocellulose membrane with blotted protein was blocked by incubation with PBS containing 30 mg/ml BSA at room temperature for 2 h with gentle shaking. The nitrocellulose membrane was then incubated with PBS 0.05% Tween 20 (PBS–Tween 20) containing biotinylated lectins (Umeda et al., 1986), for 2 h at room temperature with gentle shaking. The concentrations of the lectins used were 20 μg/ml for ricinus communis agglutinin (RCA) and 5 μg/ml for Con A. The nitrocellulose membrane was incubated with HRP-streptavidin (Zymed Labs. Inc., South San Francisco, CA) 1:1,000 diluted with PBS–Tween 20 for 1 h at room temperature with gentle shaking. The binding lectins were detected by color development using 4-chloro-1-naphtol as the substrate (Murakami et al., 1988). Nitrocellulose membrane was washed with PBS–Tween 20 four times after each incubation.

Isolation and Amino Acid Sequence Analysis of 140KGP

The glycoprotein fraction was isolated by applying the total lysate (using cell lysis buffer) of m5S/1M (2×10^9 cells) to RCA-agarose and eluting with 200 mM lactose solution. The eluate was further applied to wheat germ agglutinin (WGA)–agarose and eluted with 200 mM *N*-acetylglucosamine solution. The eluted material was then subjected to preparative SDS-PAGE and 140KGP was electrophoretically eluted by the methods of Hashizume et al. (1987). The purified glycoprotein was subjected to cyanogen bromide fragmentation after reduction and S-carboxymethylation of the cystein/cystine residues. The resulting peptide fragments were subjected to molecular exclusion chromatography in 6 M guanidine hydrochloride/10 mM phosphate, pH 6.0, using TSK G2000SWXL HPLC column. The peptides in each fraction separated by molecular size were then fractionated on a Vydac C4 reverse-phase HPLC column by using a gradient of 0–64% of acetonitrile in 0.1% trifluoroacetic acid. The isolated peptides were sequenced by automated Edman degradation using a protein sequencer (model 477A, Applied Biosystems, Inc., Foster City, CA) connected on-line to a model 120A PTH Analyzer.

mAb against 140KGP(NCAM)

mAb against 140KGP (NCAM of m5S/1M) (LN-10; rat IgM) was raised as follows. Crude glycoprotein fraction was prepared from m5S/1M cell lysate by RCA-I and WGA affinity column chromatography. 8-wk-old female Wistar rats were immunized with the crude glycoprotein fraction of m5S/1M by intra splenic injection (Murakami et al., 1988; Umeda et al., 1989). Spleen cells (5×10^8) of the immunized rat were fused with 5×10^7 P3X63-Ag8.653 myeloma cells (Kearney et al., 1979). Antibody secreting hybridoma cell was screened by ELISA and immunoblot. On immunoblot analysis, LN-10 recognized a triplet of glycoproteins of 180, 130, and 115 kD under nonreduced condition of SDS-PAGE, and weakly bound to a triplet of glycoproteins of 180, 140, and 120 kD under reduced condition. LN-10 mAb was purified from culture supernatant of LN-10 secreting hybridoma by ammonium sulfate precipitation and by gel filtration HPLC column (TSK G4000 SWXL). Rat mAb against mouse embryo brain NCAM (H28.123; IgG2a) was purchased from Immunotech S.A. (Marseille, France). Purity of all mAbs used in this study was checked by SDS-PAGE followed by silver staining (Merril et al., 1981).

Immunoblot Analysis

Purified LN-10 was iodinated (IODO-BEADS, Pierce Chemical Co., Rockford, IL) (Markwell, 1982). 0.5 mCi of $Na^{125}I$ and one bead was incubated with 200 μg of LN-10 in 500 μl of PBS for 15 min at room temperature yielding a specific activity of 2.5×10^6 cpm/μg protein. The labeled LN-10 was separated from free $Na^{125}I$ using PD-10 gel filtration column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). Cell lysate was separated by SDS-PAGE and transferred to nitrocellulose membrane under nonreduced condition as described above. The nitrocellulose membrane with blotted protein was then incubated with 3% BSA–PBS containing ^{125}I labeled LN-10. After extensive wash with PBS–Tween 20, the nitrocellulose membrane was exposed to film (XAR, Eastman Kodak Co., Rochester, NY), and NCAM bands were visualized by autoradiography.

Immunofluorescence Microscopy

Cells were fixed with 3.7% paraformaldehyde in PBS for 30 min at 4°C followed by fixation with ice cold MeOH for 30 min at −20°C and washed with PBS. Then the cells were stained for 60 min at 4°C with FITC-conjugated LN-10 (20 μg/ml), (Harlow and Lane, 1988) in PBS containing 10 mg/ml BSA. After extensive wash with PBS the specimens were viewed in a Labophot microscope (Nikon Inc., Melville, NY).

Cell Proliferation Assays

Growth suppression or stimulation effect of anti-NCAM mAb (LN-10) was investigated as follows. Purified LN-10 was coupled to Eupergit 1CZ beads (average diameter 0.6-14 μm, Röhm Pharma, Weiterstat, Germany) accord-

1. *Abbreviations used in this paper*: NCAM, neural cell adhesion molecule; PHA-E4, phaseolus vulgaris erythroagglutinin; PHA-L4, phaseolus vulgaris leukoagglutinin; RCA-1, ricinus communis agglutinin-1; WGA, wheat germ agglutinin.

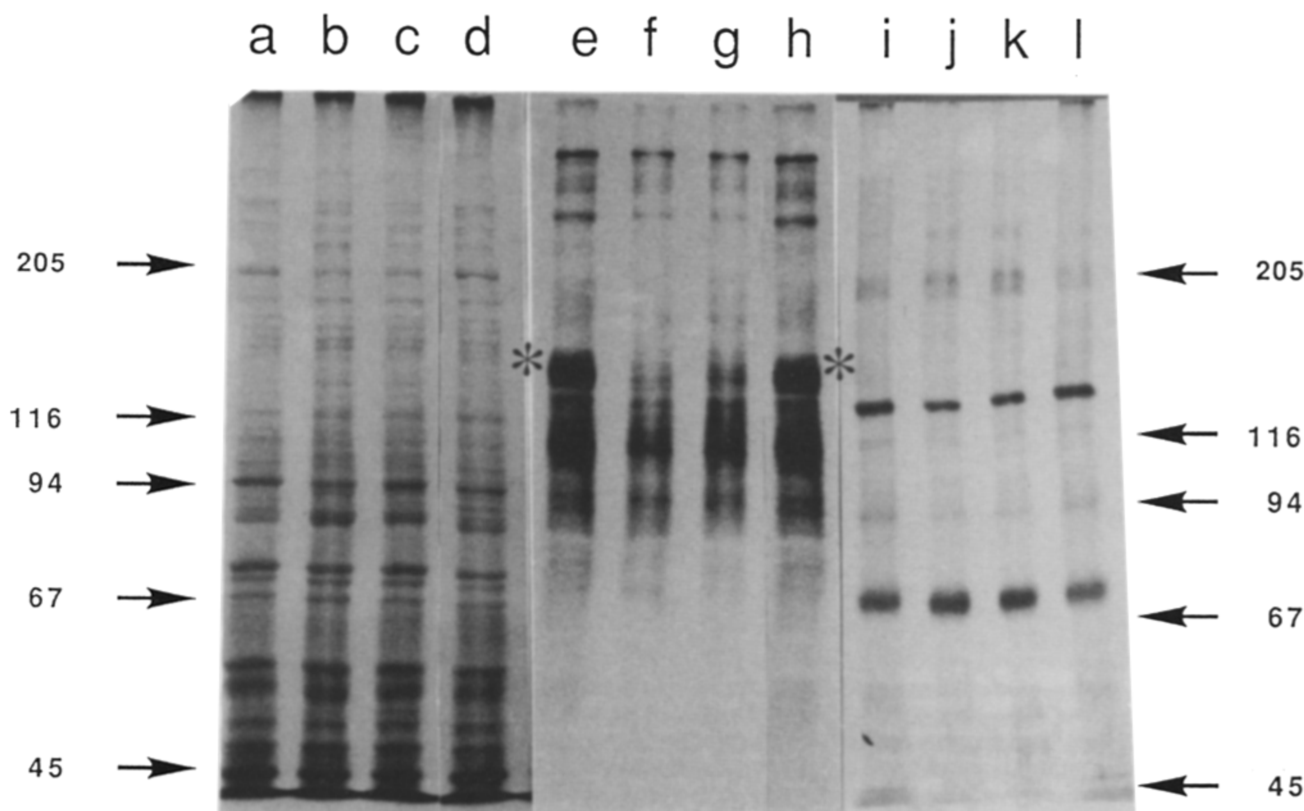


Figure 1. Effect of EGF, PMA, and bradykinin on glycoprotein composition of m5S/1M cell. m5S/1M cells, which had become confluent in the presence or absence of EGF (PMA or bradykinin) in the culture media, were washed with PBS and membrane glycoproteins were solubilized as described in Materials and Methods. 20-μg samples of lysate of nontreated cells (lanes *a*, *e* and *i*), EGF-treated cells (lanes *b*, *f*, and *j*), PMA-treated cells (lanes *c*, *g*, and *k*), and bradykinin-treated cells (lanes *d*, *h*, and *l*) were subjected to SDS-PAGE on 7.5% acrylamide gels under reducing conditions. The proteins were either stained by Coomassie brilliant blue (lanes *a–d*) or transferred to nitrocellulose membrane followed by the lectin blot analysis as described in Materials and Methods. The results obtained with 20 μg/ml RCA-I (lanes *e–h*) and 5 μg/ml Con A (lanes *i–l*) were presented. The molecular masses given are 205 (myosin), 116 (β-galactosidase), 94 (phosphorylase b), 67 (BSA) and 45 kD (ovalbumin). *, 140KGP.

ing to the manufacturer's protocol with the protein concentration of 2 mg/ml of wet beads (immobilized LN-10). The immobilized LN-10 was washed first with 70% ethanol, and then three times with α-MEM with 5% FCS and used. m5S/1M cells were distributed into a 96-well culture plate at 1,500 cells per well in 100 μl of medium.

Measurement of DNA Synthesis. After 3 d (sparse culture) or 5 d (confluent culture), various amounts of LN-10 either coupled to the beads or in the soluble form were added to each well. Controls received rat serum IgG either coupled to the beads or in the soluble form. After 24 h in the case of immobilized LN-10 or 48 h in the case of soluble LN-10, incorporation of ^{3}H-TdR (Kato et al., 1988) was measured. Briefly 4 h before completion of incubation, 0.5 μCi of radioactive thymidine was added and radioactivity of the acid-insoluble fractions of cells was counted.

Determination of Growth Index. After 24 or 48 h of incubation with LN-10, cells were fixed with ice cold MeOH and were stained with 0.2% of crystal violet (Merck, Braco S.p.A., Milan E Merck, Darmstadt, Germany) (Ruff and Gifford, 1980). After solubilization of cell proteins with 1% SDS, absorbance of each well at 550 nm was measured.

The growth index was calculated as follows: growth index = absorbance at 550 nm (after the incubation)/absorbance at 550 nm (before the incubation). Each experiment was carried out as triplicates, and results are shown as mean growth index ± SD of at least three experiments.

Results

140KGP As an EGF-sensitive Cell Surface Glycoprotein

We have previously shown that the m5S/1M cells exhibited a high sensitivity to postconfluence inhibition of cell division and formed a uniform monolayer after they had become confluent (Sasaki and Kodama, 1987). The addition of EGF resulted in loss of contact-dependent inhibition of growth and caused a massive piling up of a multilayered array of cells after they had become confluent (Watanabe et al., 1990). Bradykinin had no significant effect on the growth of m5S/1M cells, while it induced a rapid formation of inositol phosphates and the mobilization of cytosolic free calcium. EGF had no effect either on the formation of inositol phosphates or on the calcium mobilization.

We first examined the effect of EGF and bradykinin on the glycoprotein compositions of m5S/1M cells by the lectin-blotting analysis. Crude membrane fractions from confluent cultures of m5S/1M cells cultured either in the presence or absence of EGF (bradykinin) were solubilized and the electrophoretically separated proteins were transferred to nitrocellulose membrane. Glycoproteins were detected by incubating the blotted membrane with RCA-I, and Con A (Fig. 1). More than 20 bands were detected by each lectin. Major bands of 140 (140KGP) and 110 kD (110KGP) were observed when the glycoproteins from unstimulated m5S/1M cells were stained with RCA-I. A marked decrease in the staining of 140KGP was observed with EGF-treated m5S/1M cells, and a slight but appreciable decrease in the staining of 110KGP

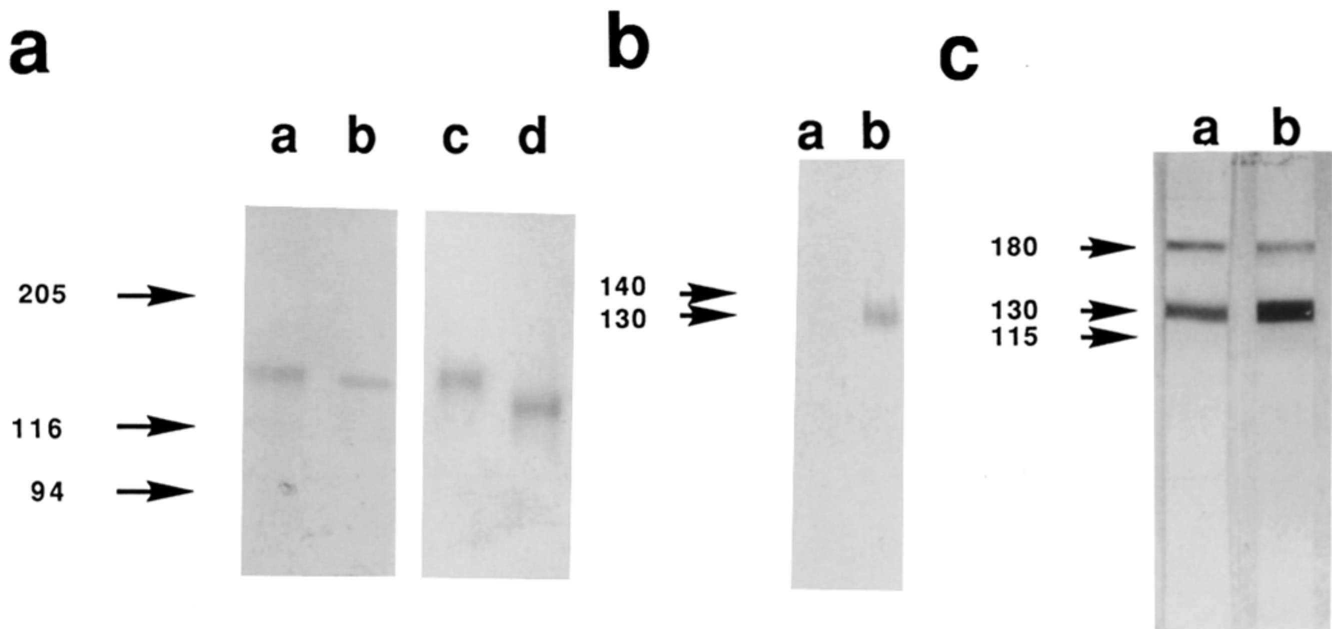


Figure 2. Identification of 140KGP as NCAM. (*a*) SDS-PAGE analysis of 130- and 140-kD glycoprotein. Both 130- and 140-kD glycoprotein were separated by SDS-PAGE under nonreduced condition, and were electroeluted from SDS gel. Each glycoprotein eluted from the gel was then separated by SDS-PAGE (lanes *a* and *c*, 140-kD glycoprotein, and lanes *b* and *d*, 130-kD glycoprotein; lanes *a* and *b*, reduced condition, and lanes *c* and *d*, nonreduced condition). (*b*) Immunoblot analysis of 130- and 140-kD glycoprotein using anti-NCAM monoclonal antibodies (H28.123). 140- (lane *a*) and 130-kD (lane *b*) glycoprotein were electrophoresed under nonreduced condition. After transfer onto nitrocellulose membrane, they were reacted sequentially with anti-NCAM mAbs (H28.123 [10 μg/ml]) and peroxidase-conjugated anti-rat antibodies (1:1,000). (*c*) Immunoblot analysis of m5S/1M crude membrane fraction using anti-NCAM monoclonal antibodies. Crude membrane fraction of m5S/1M was electrophoresed under nonreduced condition. After transfer onto nitrocellulose membrane, they were reacted sequentially with anti-NCAM mAbs (lane *a*, LN-10 [20 μg/ml]; lane *b*, H28.123 [10 μg/ml]), biotin-labeled anti-rat antibodies (1:1,000), peroxidase-conjugated streptavidin (1:1,000), and 4-chloro-1-naphthol.

was also observed. The staining of 140KGP and 110KGP by WGA was also decreased with EGF-stimulated m5S/1M cells (data not shown). Similar reduced binding of the lectins to the glycoproteins from the PMA-stimulated cells were observed, while no significant change in the Con A staining was observed with EGF-stimulated cells. No appreciable change of the glycoprotein profiles was observed when the bradykinin-stimulated cells were examined with this lectin blotting analysis (Fig. 1, lanes *e–h*).

140KGP was also stained with phaseolus vulgaris erythroagglutinin (PHA-E_4), phaseolus vulgaris leukoagglutinin (PHA-L_4) and allomyrina dichotoma agglutinin, and its expression examined with these lectins was also decreased in EGF-stimulated cells (data not shown). Although RCA, WGA, PHA-E_4, PHA-L_4, and allomyrina dichotoma agglutinin could bind to asparagine-linked carbohydrates, the precise recognition sites of these lectins are all different (Merkle and Cummings, 1987). Since the binding of all of these lectins to 140KGP was decreased with EGF-stimulated m5S/1M cells, it is likely that the decrease is not because of the change of carbohydrate structure but rather to the diminution of the glycoprotein.

When cell surface glycoproteins were labeled with ^{125}I by the lactoperoxidase-glucoseoxidase method and the labeled glycoproteins were precipitated using RCA-I coupled to agarose followed by SDS-PAGE, three major bands of 140, 110, and 80 kD were observed with the autoradiography, suggesting that 140KGP resides on the cell surface (data not shown). Another observation showed that 140KGP was highly sensitive to the mild trypsin treatment of the intact cells. These observations indicate that 140KGP is a cell surface glycoprotein and its expression is highly sensitive to the transformation induced by EGF or PMA.

Identification of 140KGP as NCAM

We have undertaken two approaches to study the role of 140KGP in the contact-dependent inhibition of growth; one is to identify the glycoprotein and the other is to make mAb against 140KGP. 140KGP was purified by sequential column chromatography on RCA-I–agarose and WGA-agarose, and by the electroelution of the electrophoretically separated 140KGP. SDS-PAGE analysis showed that it migrated to a single band under the reduced condition, while under the nonreducing condition 140KGP was found to contain a minor component. The major band corresponded to 130 kD and the minor band 140 kD. Each glycoprotein was separated by SDS-PAGE under nonreducing condition and was electroeluted from SDS–polyacrylamide gels and rerun under the reduced condition. Under the reduced condition both the major component and the minor component showed the same molecular mass 140 kD (Fig. 2 *a*). Each glycoprotein was reduced and S-carboxymethylated, and was fragmented by CNBr treatment in 70% formic acid. The resulting peptides were separated by size-exclusion HPLC column and isolated by reverse-phase HPLC column (data not shown). Edman degradation of a peptide from the major 10-kD glycoprotein resulted in a single sequence of amino acids (EEG-KAAFSKDESKEPIVEVRTE) which was revealed to be

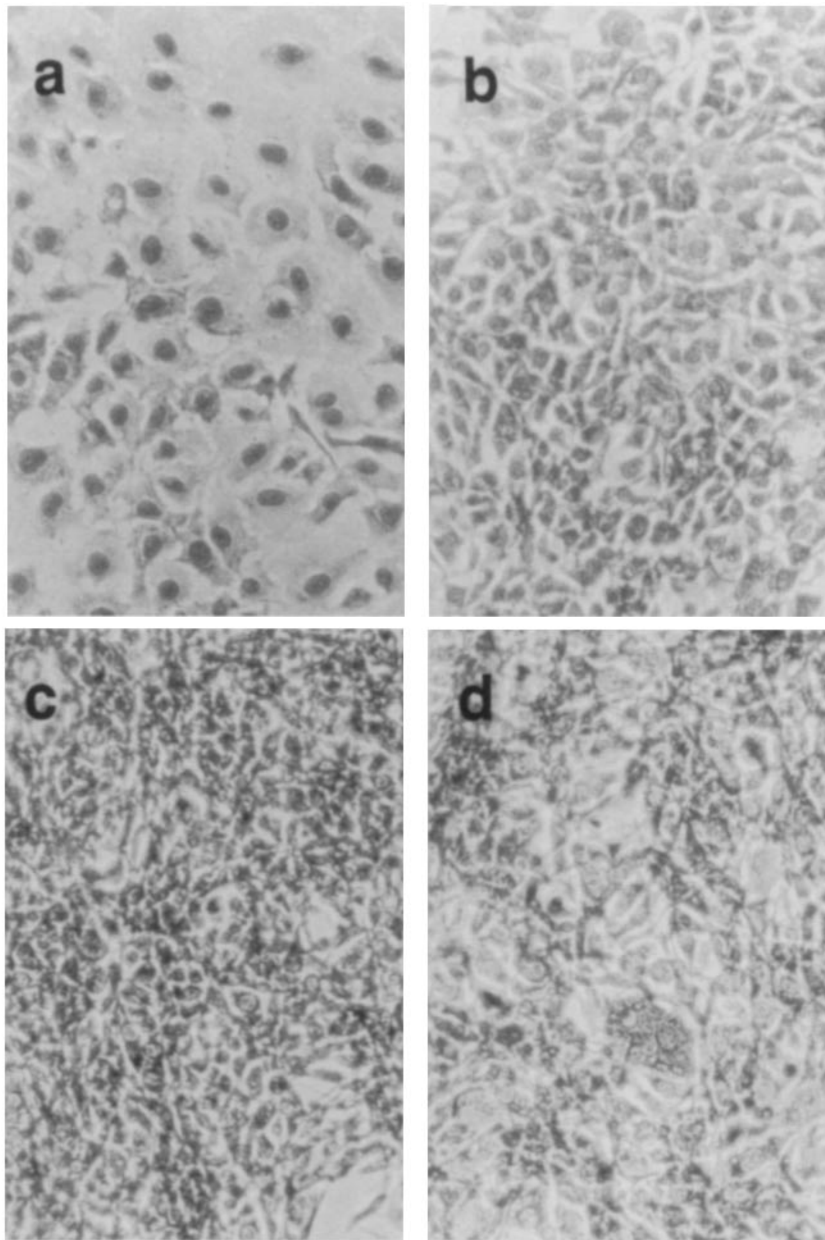


Figure 3. Morphology of the x-ray-induced transformants of m5S/1M cells. X-ray-induced transformants of m5S/1M cells were classified into four groups (*a*–*d*) according to their cell shape, sensitivity to postconfluence inhibition of growth, growth rate, and tumorigenicity. Morphology of the typical clones from each group: (*a*) m5S/1M; (*b*) m5S cl.4102; (*c*) m5S cl.6520; and (*d*) m5S cl.2520 cells were presented. The cells were cultured to confluence and nuclei were stained with Giemsa.

identical to a part of the amino acid sequence (from 744 to 765 amino acid residue) of the mouse brain NCAM (Santoni et al., 1987). The amino acid sequence of a peptide from the minor component showed no significant homology with previously reported proteins searched through the European Molecular Biology Laboratory protein data base (SWISS-PROT; Heidelberg, Germany) (release 15.0, Aug. 1990) and Protein Identification Resource (release 26.0, Sept. 1990) (data not shown).

To further confirm the identification of the 130-kD glycoprotein with NCAM, we established a rat mAb, clone LN-10, against the glycoprotein by the intrasplenic immunization method as previously described (Murakami et al., 1988; Umeda et al., 1989). The immunoblot analysis using LN-10 showed that the mAb bound only to the 130 kD glycoprotein (Fig. 2 *b*) but not to the 140-kD glycoprotein. Three distinct bands corresponding to 180, 130, and 115 kD were observed when a crude membrane fraction of m5S/1M cells was used for the experiments (Fig. 2 *c*). Anti-NCAM mAb, clone H28.123, showed a significant binding to the 130-kD but not the 140-kD glycoprotein (Fig. 2 *b*). H28.123 showed a binding profile identical to those observed with LN-10 when crude membrane fraction of m5S/1M cells was examined by the immunoblot analysis; three major bands of 180, 130, and 115 kD were observed, and each band corresponds to the three isoforms of NCAM: NCAM-180, NCAM-140, and NCAM-120, respectively (Hirn et al., 1981) (data not shown). In the immunoblot analysis, H28.123 also showed an intense binding to the glycoproteins purified by the sequential column chromatography on RCA-I–agarose and LN-10–coupled Sepharose 4B (data not shown). From these observations we identified the 130-kD glycoprotein as NCAM, and m5S/1M cells were shown to express three isoforms of NCAM.

Table I. Characterization of the X-Ray-Induced Transformants of m5S/1M Cells

	Sensitivity to postconfluence growth inhibition	Cell density after cells become confluent	Growth rate	Tumorigenicity	Clones
Type A	+	low	low	–	m5S/1M m5S cl.2540 m5S cl.4502
Type B	+	high	high	–	m5S cl.4102 m5S cl.4103 m5S cl.6010
Type C	–	high	high	–	m5S TR600 m5S cl.6520
Type D	–	high	high	+	m5S cl.2520 m5S cl.2531

Effect of X-Ray-induced Transformation and EGF Stimulation on the Expression of NCAM

We have established a series of transformants by x-ray irradiation (Kodama and Sasaki, 1987). They had different chromosome constitutions and were also different in their morphology, sensitivity to postconfluence inhibition of growth, saturation density after they had reached confluency, and tumorigenicity in nude mice. We selected nine clones and classified them into four groups according to their cell shape, sensitivity to postconfluence inhibition of growth, growth rate, and tumorigenicity (Fig. 3). Distinctive features of each cell lines are summarized in Table I. Cells in type A group exhibited a phenotype similar to that of unstimulated m5S/1M cells and showed a round cell shape, high sensitivity to postconfluence inhibition of growth, and low saturation density after they had reached confluence. Type B cells also showed sensitivity to postconfluence inhibition of growth, but were discriminated from type A cells in that they had a flattened cell shape and high saturation density. Type C cells lost sensitivity to postconfluence inhibition of growth and showed a high growth rate and high saturation density which were the same as those of EGF-stimulated m5S/1M cells. Type D cells had a significant tumorigenicity in nude mice in addition to the nature of type C cells.

Expression of NCAM on m5S/1M cell and its x-ray-induced transformants was studied by the quantitative immunoblot assay using ^{125}I-labeled anti-NCAM mAb LN-10. As low as 0.2 ng of NCAM can be quantitatively determined by this quantitative immunoblot assay (data not shown). Autoradiography obtained with the immunoblot assay is shown in Fig. 4 and results were summarized in Fig. 5. The expression of NCAM on the EGF- or PMA-stimulated m5S/1M cells was significantly decreased and the expression of NCAM on type A, B, C, and D cells was 100–120%, 70–100%, 10–20%, and <5%, respectively, of that of untreated m5S/1M cells. The reduced expression of NCAM in EGF- or PMA-treated m5S/1M cells observed by the immunoblotting analysis is consistent with those observed by the lectin blotting analysis. The lectin blotting analysis of the m5S/1M transformants also showed that the expression of 140KGP in type C cells was markedly decreased, and no appreciable expression was observed with type D cells (data not shown).

The expression of NCAM on cell surface was examined by the indirect immunofluorescent visualization using the FITC-labeled mAb LN-10. The intensity of the staining was significantly decreased in the transformed cells, showing a nice correlation with the results obtained by the immunoblotting assay. The staining profiles of m5S/1M cells shows that NCAM was distributed on the cell surface and was mainly localized at the sites of cell–cell contacts (data not shown). These results show that the expression of NCAM was highly sensitive to the transformation and the reduction in NCAM expression corresponds highly to the degree of decrease in the sensitivity to the contact-dependent inhibition of growth.

Effect of Anti-NCAM mAb LN-10 on the Growth of m5S/1M Cells

We first examined the effect of anti-NCAM mAb LN-10 on the growth of m5S/1M cells by measuring either the DNA synthesis or the increase in cell number of actively proliferating m5S/1M cells. Various amounts of LN-10 (2 mg LN-10/ml of wet beads) were added to the actively proliferating cells at the density of 2.5×10^4 cells/cm^2 and DNA synthesis was measured as the rate of [^{3}H]thymidine incorporation 24 h after the addition of the mAb. The addition of LN-10 immobilized on the beads significantly inhibited the [^{3}H]thymidine incorporation, while no change in the incorporation was observed with the beads coated with control rat immunoglobulins (Fig. 6 *a*). The addition of 0.3 μg/ml of LN-10 on beads exhibited 50% inhibition of the [^{3}H]thymidine incorporation, and 1.0 μg/ml of immobilized LN-10 completely inhibited the DNA synthesis. The effect of LN-10 on the proliferation of m5S/1M cells was also determined by measuring cell numbers after a 24-h incubation of the cells either in the presence or absence of immobilized LN-10. The increase in the cell number was indicated as a growth index where the cell numbers after the 24-h incubation were divided by the cell numbers before the incubation. The results were consistent with those obtained with the [^{3}H]thymidine incorporation analysis; 0.3 μg/ml of LN-10 showed 50% inhibition and 1.0 μg/ml of LN-10 almost completely inhibited the cell proliferation, while control rat immunoglobulin had no effect (Fig. 6 *b*). In contrast, LN-10 in soluble form had no effect either on the [^{3}H]thymidine incorporation or on the increase in the growth index (data not shown).

A

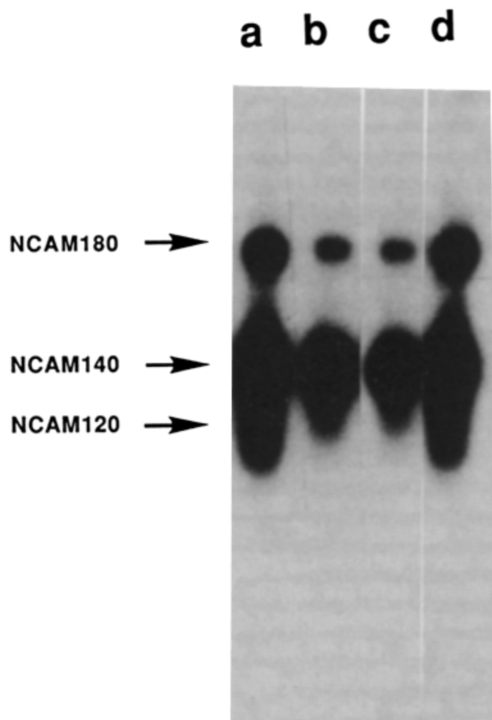
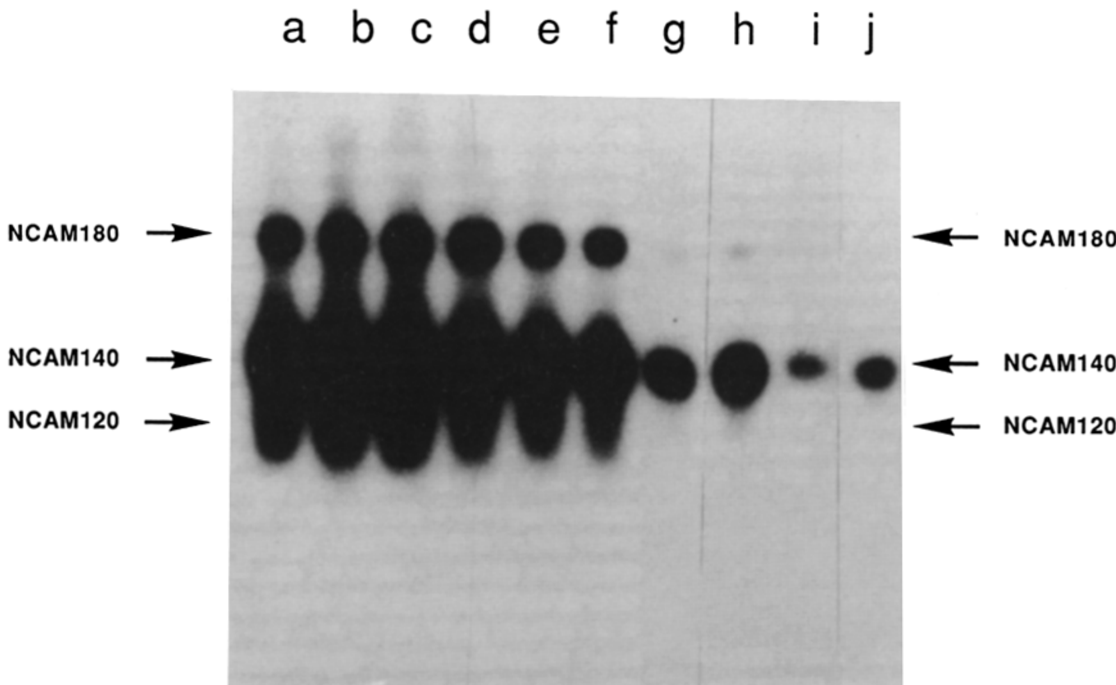


Figure 4. Change in NCAM expression in m5S/1M cells and its x-ray–induced transformants. (*a*) Effect of EGF, PMA, and bradykinin on NCAM expression of m5S/1M cells. Crude membrane fraction of nonstimulated m5S/1M (lane *a*), EGF-treated m5S/1M (lane *b*), PMA-treated m5S/1M (lane *c*), and bradykinin-treated m5S/1M cells (lane *d*) were electrophoresed under nonreduced condition. After transfer onto nitrocellulose membrane, they were reacted with ^{125}I-labeled anti-NCAM mAb, LN-10, and the NCAM bands were detected by autoradiography. (*b*) Effect of x-ray–induced transformation on NCAM expression. Crude membrane fraction of nonstimulated m5S/1M x-ray–induced transformants were electrophoresed under nonreduced condition. After transfer onto nitrocellulose membrane, they were reacted with ^{125}I-labeled anti-NCAM mAb, LN-10, and the NCAM bands were detected by autoradiography. lanes *a*, m5S/1M; *b*, m5S cl.2540; *c*, m5S cl.4502; *d*, m5S cl.4102; *e*, m5S cl.4103; *f*, m5S cl.6010; *g*, m5S TR600; *h*, m5S cl.6520; *i*, m5S cl.2520; and *j*, m5S cl.2531 cells.

B



In the next series of experiments, we examined the effect of soluble anti-NCAM mAb LN-10 on the growth of the confluent quiescent culture of m5S/1M cells. Cell growth was examined both by [^{3}H]thymidine incorporation and by the growth index as described above. When the immobilized LN-10 was added to the culture, we could not observe any change either in the [^{3}H]thymidine incorporation or in the increase in the growth index. However, when high concentrations of LN-10 (100 μg/ml) in a soluble form was added to the m5S/1M cells, both morphological change and loss of contact-dependent inhibition of growth of m5S/1M cells were observed. Simultaneously, the resumption of the [^{3}H]-thymidine incorporation and the increase in the growth index were observed. Namely, the cells started to grow and were released from the contact-dependent inhibition of growth (Fig. 7, *a* and *b*).

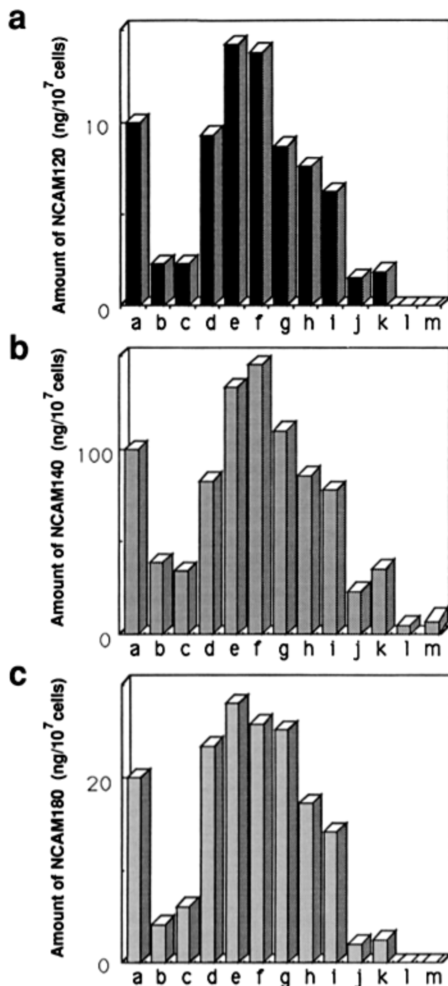


Figure 5. Quantitative analysis of NCAM expression on m5S/1M cells and its x-ray–induced transformants. The nitrocellulose membrane corresponding to each isoform of NCAM in Fig. 5 *b* was cut and the radioactivity was measured by γ-counter. The standard calibration curve was obtained by the experiments where the known amounts of each isoform of NCAM were separated by SDS-PAGE followed by the immunoblot analysis using ^{125}I-labeled anti-NCAM mAb, LN-10. (*a*) NCAM120, (*b*) NCAM140, and (*c*) NCAM180. lanes *a*, m5S/1M; *b*, EGF-treated m5S/1M; *c*, PMA-treated m5S/1M; *d*, bradykinin-treated m5S/1M; *e*, m5S cl.2540; *f*, m5S cl.4502; *g*, m5S cl.4102; *h*, m5S cl.4103; *i*, m5S cl.6010; *j*, m5S TR600; *k*, m5S cl.6520; *l*, m5S cl.2520; and *m*, m5S cl.2531 cells.

Discussion

One striking feature of the transformed cells is their reduced adhesive interactions either between cells, or between cell and extracellular matrix, which may lead to the impaired contact inhibition of growth and the uncontrolled migration of the cells. Previous immunohistochemical localization and biochemical studies have shown that the levels of both extracellular matrices such as fibronectin (Hynes, 1986), collagen and laminin (Yamada, 1983), and its receptor integrins (Plantefaber and Hynes, 1989) on transformed cells are significantly lower than those on normal cells. The alterations of the cell surface molecules which may regulate the growth by cell–cell contacts have also been reported (Wieser et al., 1990).

In the present study, we have found that NCAM is abundantly expressed in the mouse near-diploid fibroblast cell line m5S/1M and that the transformed cells expressed significantly reduced amounts of NCAM. A major finding was that the immobilized monoclonal antibody against NCAM strongly inhibited the growth of actively proliferating m5S/1M cells, and inversely, the monoclonal antibody in a soluble form showed a significant growth-stimulating effect on the confluent quiescent cultures of m5S/1M cells. These findings suggest that NCAM plays an important role in the regulation of cell growth by the cell–cell contact mechanisms.

NCAM as a Transformation-sensitive Cell Surface Glycoprotein

Although NCAM is expressed during embryonic development in a variety of nonnervous tissues on derivatives of all three germ layers (Edelman, 1987), the expression was usually diminished when the cells were transferred into cultures, and most fibroblastic cells are usually NCAM negative. The present amino acid sequence analysis and the immunochemical analyses using the anti-NCAM mAbs clearly demonstrated that NCAM is abundantly expressed on the m5S/1M cells which had been established from embryonic skin fibroblasts of ICR mouse. Our quantitative immunoassay using ^{125}I-labeled anti-NCAM mAb LN-10 showed that m5S/1M cells expressed 5–10-fold higher amounts of NCAM than other cell lines such as Swiss 3T3 and BALB 3T3 (our unpublished observations). The expression of NCAM on m5S/1M cell is highly sensitive to the transformation, and the NCAM expression on the x-ray–induced transformants exhibited a negative correlation with the decrease in the sensitivity to the contact-dependent inhibition of growth. EGF and PMA, both of which induced the morphological transformation and the proliferation of m5S/1M cells, suppressed the NCAM expression, whereas bradykinin, which was shown to induce both the mobilization of cytosolic free calcium and the formation of inositol phosphates but not the transformation of m5S/1M cells (Watanabe et al., 1990), had no effect on the NCAM expression. Our preliminary observations showed that, in the actively proliferating cells, the expression of NCAM is also suppressed and even the molar ratio of each NCAM component expressed in the actively proliferating cells is different from that of the confluent quiescent culture of m5S/1M cells; the expression of the 140-kD component dominates when the cells had become confluent, while an almost equal amount of each component is expressed in the actively proliferating cells (our unpublished observations). These observations suggest that the decreased expression of NCAM is likely to be the secondary consequences of the growth-stimulating signals which may be continuously occurring in the transformed cells.

Several factors such as nerve growth factor (Prentice et al., 1987) and transforming growth factor-beta (Roubin et al., 1990) have been reported to affect the expression of NCAM on nervous cells. Roubin et al. reported that the expression of NCAM in NIH 3T3 cells was highly enhanced by transforming growth factor-beta but was not affected by EGF (Roubin et al., 1990). Brackenbury et al. (1984)

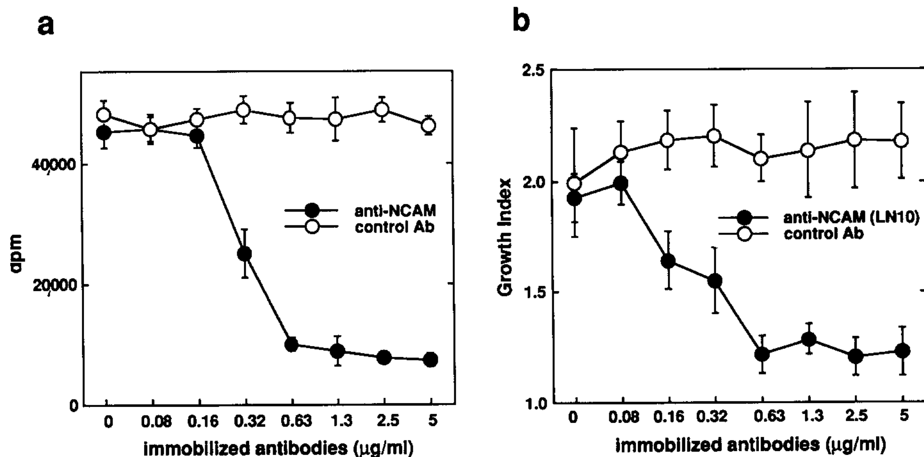


Figure 6. Effect of immobilized anti-NCAM mAb (LN-10) on thymidine uptake and growth of m5S/1M cells. m5S/1M cells were distributed into a 96-well culture plate at 1,500 cells/well, cultured for 3 d. Various amounts of immobilized LN-10 or immobilized control rat IgG (2 mg/ml of wet beads) were added to each well, and after 24 h both thymidine uptake (*a*) and the growth index (*b*) were measured as described in Materials and Methods.

reported that the expression of NCAM was significantly decreased in the chicken retinal cells transformed by Rous sarcoma virus. They suggested that the effect of the transforming gene product of Rous sarcoma virus, pp60src, on the expression of NCAM was most likely indirect. Further studies with a variety of cells transformed by various agents have been required to understand the relationship between the transformation and the expression of NCAM. Our results showed that other transformation-inducing agents such as EGF and x-ray irradiation also affected the NCAM expression of the nonneural cells, again suggesting the relationship between the reduced expression of NCAM and the transformation.

One of the most consistently observed alterations with transformed cells is an expression of large asparagine-linked carbohydrates (Yamashita et al., 1984). The change in size of asparagine-linked carbohydrate has been attributed to an increase in sialic acid (Passaniti and Hart, 1988; Hunt and Wright, 1985) and the addition of beta-1-6-linked lactosamine antennae at the trimannosyl core (Yamashita et al., 1984). Since the carbohydrates on NCAM were reported to affect the adhesive interactions between NCAM (Hoffman and Edelman, 1983), we were curious to know the effect of transformation on the carbohydrate structures of NCAM. NCAM from both the normal and transformed cells, however, showed a similar lectin-binding profile, suggesting that no significant difference in the carbohydrate structures of NCAM was induced by the transformation (our unpublished observation).

Modulation of Cell Growth by anti-NCAM mAb LN-10

Since the lectins such as RCA, WGA, and PHA could bind to NCAM, we first examined the effect of immobilized lectins on the growth of m5S/1M cells. The immobilized lectins, however, had no effect on the cell growth (our unpublished observations). Only the immobilized anti-NCAM mAb LN-10 could inhibit the growth of actively proliferating m5S/1M cells, and even higher concentrations of soluble LN-10 had no effect on the growth. These observations indicate that the inhibition is not a consequence of the nonspecific binding of the beads to the cells, and that NCAM is responsible for the contact-dependent inhibition of growth of m5S/1M cell. The immobilized mAb on the surface of beads may mimic the intercellular interactions of NCAM and the cross-linking of the NCAM may mediate some signal which leads to the cessation of growth. The immobilized LN-10 failed to inhibit the growth of other fibroblastic cell lines such as BALB 3T3 cells where fivefold less amount of NCAM was expressed. We are now investigating the effect of the immobilized LN-10 on the

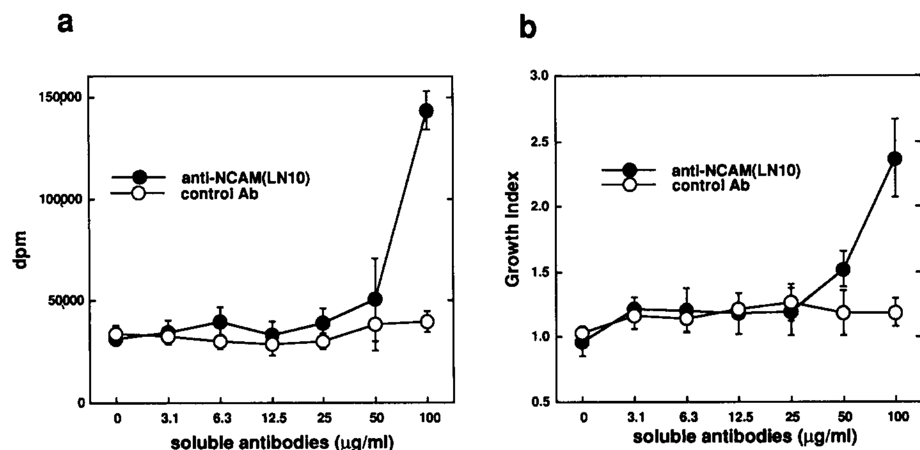


Figure 7. Effect of soluble anti-NCAM mAb (LN-10) on thymidine uptake and growth of m5S/1M cells. m5S/1M cells were distributed into a 96-well culture plate at 1,500 cells/well, cultured for 5 d (confluent culture). Various amounts of soluble LN-10 or control rat IgG were added to each well, and after 48 h both thymidine uptake (*A*) and the growth index (*B*) were measured.

growth of other cell lines which abundantly express NCAM such as astroglial and myoblast cell lines.

The cytoplasmic domain of the 180-kD component of NCAM was reported to be associated with the cytoskeletal protein, spectrin, suggesting the possibility that the 180-kD component transfers the recognition signal to the intracellular components, leading to the initiation of various physiological processes (Pollerberg et al., 1987). The phosphatidylinositol-anchored component of NCAM was also suggested to mediate the cell contacts (Knudsen et al., 1989). We could not, however, define at present which molecule is responsible for the inhibition of cell growth in this system.

The LN-10 in the soluble form releases the confluent quiescent cultures of m5S/1M cells from the contact-dependent inhibition of growth, leading to the resumption of cell growth, though a very high concentration of LN-10 was required. In contrast, the immobilized LN-10 had no effect on the cell growth, when the mAb was added to the confluent cultures of m5S/1M cells. These observations suggest that the mAb in the soluble form could competitively inhibit the interaction between NCAM and further support the idea that NCAM plays the dominant role in the contact-dependent inhibition of growth of m5S/1M cells.

Although many cell-cell adhesion molecules such as NCAM and other members of the immunoglobulin super family (Cunningham et al., 1987), cadherin family (Takeichi, 1988), and integrins (Hynes, 1987) have been shown to be involved in the adhesive interactions of neural cells, little is known about the cellular signals that follow the adhesive interactions. The density-dependent inhibition of cell growth mediated by NCAM is reminiscent of the regulation of cell growth by cell-cell contacts in nervous tissues. Although the growth of nervous cells may be mainly regulated by soluble factors (Prentice et al., 1987), several reports have suggested that cell-cell contacts affect the growth and phenotypic differentiation of neural cells (Hatten, 1985; Ratner et al., 1985). Hatten reported that the proliferation of astroglial cells was inhibited either by fixed neurons or by a cell membrane fraction of granule neurons (Hatten, 1987). Since NCAM, together with other adhesion molecules such as L1, is involved in the neuron-neuron or neuron-glia cell interactions (Kadmon et al., 1990), and in the adhesive interactions between the cells during embryogenesis (Edelman, 1987), the present findings suggest a new hypothesis that NCAM plays a role in growth regulation after stabilization of the cell-cell contacts during embryogenesis and the formation of nervous systems.

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References

Brackenbury, R., M. E. Greenberg, and G. M. Edelman. 1984. Phenotypic changes and loss of N-CAM-mediated adhesion in transformed embryonic chicken retinal cells. *J. Cell Biol.* 99:1944-1954.

Cole, G. J., A. Loewy, and L. Glaser. 1986. Neuronal cell-cell adhesion depends on interactions of N-CAM with heparin-like molecules. *Nature (Lond.).* 320:445-447.

Cunningham, B. A., J. J. Hemperly, B. A. Murray, E. A. Prediger, R. Brackenbury, and G. M. Edelman. 1987. Neural cell adhesion molecule: structure, immunoglobin-like domains, cell surface modulation, and alternative RNA splicing. *Science (Wash. DC).* 236:799-806.

Edelman, G. M. 1987. CAMs and Igs: cell adhesion and the evolutionary origins of immunity. *Immunol. Rev.* 100:11-45.

Harlow, E., and D. Lane. 1988. Antibodies: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 354 pp.

Hashizume S., T. Honjo, K. Kuroda, and M. Komichi. 1987. A method for eluting proteins from SDS-Gel. *Seikagaku.* 59:1155-1160.

Hatten, M. E. 1985. Neuronal regulation of astroglial morphology and proliferation in vitro. *J. Cell Biol.* 100:384-396.

Hatten, M. E. 1987. Neuronal inhibition of astroglial cell proliferation is membrane mediated. *J. Cell Biol.* 104:1353-1360.

Heimark, R. L., and S. M. Schwarz. 1985. The role of membrane-membrane interactions in the regulation of endothelial cell growth. *J. Cell Biol.* 100:1934-1939.

Hirn, M., M. Pierres, H. Deagostini-Bazin, M. Hirsch, and C. Goridis. 1981. Monoclonal antibody against cell surface glycoprotein of neurons. *Brain Res.* 214:433-439.

Hoffman, S., and G. M. Edelman. 1983. Kinetics of homophilic binding by embryonic and adult forms of the neural cell adhesion molecule. *Proc. Natl. Acad. Sci. USA.* 80:5762-5766.

Hunt, L., and S. E. Wright. 1985. Both acidic-type and neutral-type asparaginyl-oligosaccharides of host cell glycoproteins are altered in Rous sarcoma virus-transformed chick embryo fibroblast. *Biochem. J.* 229: 441-451.

Hynes, R. O. 1986. Fibronectins. *Sci. Am.* 254:42-51.

Hynes, R. O. 1987. A family of cell surface receptors. *Cell.* 48:549-554.

Kadmon, G., A. Kowitz, P. Altevogt, and M. Schachner. 1990. The neural cell adhesion molecule N-CAM enhances L1-dependent cell-cell interactions. *J. Cell Biol.* 110:193-208.

Kato, T., I. Kudo, H. Hayashi, K. Onozaki, and K. Inoue. 1988. Augmentation of DNA synthesis in guinea pig bone marrow cells by platelet-activating factor (PAF). *Biochem. Biophys. Res. Commun.* 157:563-568.

Kearney, J. F., A. Radbruch, B. Liesegang, and K. Rajewsky. 1979. A new myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. *J. Immunol.* 123:1548-1550.

Knudsen, K. A., L. Smith, and S. McElwee. 1989. Involvement of cell surface phosphatidylinositol-anchored glycoproteins in cell-cell adhesion of chick embryo myoblasts. *J. Cell Biol.* 109:1779-1786.

Kodama, S., and M. S. Sasaki. 1987. The involvement of chromosome-13 in the x-ray-induced in vitro transformation of mouse m5S cells. *Jpn. J. Cancer Res.* 78:372-381.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680-685.

Markwell, M. A. K. 1982. A new solid-state reagent to iodinate proteins. I. Condition for the efficient labeling of antiserum. *Anal. Biochem.* 125: 427-432.

Merkle, R. K., and R. D. Cummings. 1987. Lectin affinity chromatography of glycopeptides. *Methods Enzymol.* 138:232-259.

Merril, C. R., D. Goldman, S. A. Sedman, and M. H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science (Wash. DC).* 211:1437-1438.

Murakami, M., T. Kobayashi, M. Umeda, I. Kudo, and K. Inoue. 1988. Monoclonal antibodies against rat platelet phospholipase A_2. *J. Biochem. (Tokyo).* 104:884-888.

Nakamura, T., Y. Nakamura, and A. Ichihara. 1984. Reciprocal modulation of growth and liver functions of mature rat hepatocytes in primary culture by an extract of hepatic plasma membranes. *J. Biol. Chem.* 259:8056-8058.

Passaniti, A., and G. W. Hart. 1988. Cell surface sialylation and tumor metastasis. *J. Biol. Chem.* 263:7591-7603.

Plantefaber, L. C., and R. O. Hynes. 1989. Change in integrin receptors on oncogenically transformed cells. *Cell.* 56:281-290.

Pollerberg, G. E., K. Burridge, K. E. Krebs, S. R. Goodman, and M. Schachner. 1987. The 180-kD component of the neural cells adhesion molecule N-CAM is involved in cell-cell contacts and cytoskeleton-membrane interactions. *Cell Tissue Res.* 250:227-236.

Prentice, H. M., S. E. Moore, J. G. Dickson, P. Doherty, and F. S. Walsh. 1987. Nerve growth factor-induced changes in neural cell adhesion molecule (N-CAM) in PC12 cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1-10.

Raben, D., M. Lieberman, and L. Glaser. 1981. Growth inhibitory protein(s) in 3T3 cell plasma membrane. Partial purification and dissociation of growth inhibitory events from inhibition of amino acid transport. *J. Cell Physiol.* 108:35-45.

Ratner, N., R. P. Bunge, and L. A. Glaser. 1985. A neuronal cell surface heparan sulfate proteoglycan is required for dorsal root ganglion neuron stimulation of Schwann cell proliferation. *J. Cell Biol.* 101:744-754.

Roubin, R., H. Deagostini-Bazin, M-R. Hirsch, and C. Goridis. 1990. Modulation of NCAM expression by transforming growth factor-beta, serum, and autocrine factors. *J. Cell Biol.* 111:673-684.

Ruff, M. R., and G. E. Gifford. 1980. Purification and physio-chemical characterization of rabbit tumor necrosis factor. *J. Immunol.* 125:1671-1677.

Santoni M-J., D. Barthels, J. A. Barbas, M-R. Hirsch, M. Steinmetz, C. Gordis, and W. Wille. 1987. Analysis of cDNA clones that code for the transmembrane forms of the mouse neural cell adhesion molecule (NCAM) and are generated by alternative RNA splicing. *Nucleic Acids Res.* 15:8621-8641.

Sasaki, M., and S. Kodama. 1987. Establishment and some mutational characteristics of 3T3-like near-diploid mouse cell line. *J. Cell. Physiol.* 131: 114-122.

Takeichi, M. 1988. The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development (Camb.).* 102:639-655.

Towbin, H., T. Staehlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.

Umeda, M., I. Diego, E. D. Ball, and D. M. Marcus. 1986. Idiotypic determinants of monoclonal antibodies that bind to 3-fucosyllactosamine. *J. Immunol.* 136:2562-2567.

Umeda, M., K. Igarashi, K. S. Nam, and K. Inoue. 1989. Effective production of monoclonal antibodies against phosphatidylserine: stereo-specific recognition of phosphatidylserine by monoclonal antibody. *J. Immunol.* 143: 2273-2279.

Watanabe, S., M. Umeda, T. Nomura, T. Kobayashi, T. Nakano, H. Arita, H. Utumi, M. Sasaki, and K. Inoue. 1990. Mitogenic response of near-diploid mouse cell line m5S/1M induced by epidermal growth factor. *J. Cell. Physiol.* 142:99-107.

Whittenberger, B., and L. A. Glaser. 1977. Inhibition of DNA synthesis in cultures of 3T3 cells by isolated surface molecules. *Proc. Natl. Acad. Sci. USA.* 74:2251-2255.

Wieser, R. J., and F. Oesch. 1986. Contact inhibition of growth of human diploid fibroblasts by immobilized plasma membrane glycoproteins. *J. Cell Biol.* 103:361-367.

Wieser, R. J., and F. Oesch. 1987. Plasma membrane glycoproteins covalently bound to silica beads as a model for molecular studies of cell-cell interactions in culture. *J. Biochem. Biophys Methods.* 15:13-22.

Wieser, R. J., R. Heck, and F. Oesch. 1985. Involvement of plasma membrane glycoproteins in the contact-dependent inhibition of growth of human fibroblast. *Exp. Cell Res.* 158:493-499.

Wieser, R. J., S. Schutz, G. Tschank, H. Thomas, H-P. Dienes, and F. Oesch. 1990. Isolation and characterization of a 60-70-kD plasma membrane glycoprotein involved in the contact-dependent inhibition of growth. *J. Cell Biol.* 111:2681-2692.

Yamada, K. M. 1983. Cell surface interactions with extracellular materials. *Annu. Rev. Biochem.* 52:761-799.

Yamashita, K., T. Ohkura, Y. Tachibana, S. Takasaki, and A. Kobata. 1984. Comparative study of the oligosaccharides released from baby hamster kidney cells and their polyoma transformant by hydrazinolysis. *J. Biol. Chem.* 259:10834-10840.