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Differential regulation of C-CAM isoforms in epithelial cells

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SUMMARY

C-CAM is a Ca^{2+}-independent cell adhesion molecule (CAM) that mediates intercellular adhesion of isolated rat hepatocytes. It is widely distributed in epithelia, where its presence both at lateral cell borders and on apical cell surfaces suggests that it may have diverse biological functions. Two major isoforms, C-CAM1 and C-CAM2, which differ in the lengths of their cytoplasmic domains, have been identified. The lack of suitable in vitro systems has so far prevented a detailed study of the physiological role of C-CAM in epithelia. We now report on the identification, biochemical characterization and functional analysis of C-CAM isoforms in the established epithelial cell line NBT II, derived from a chemically induced carcinoma of rat bladder. C-CAM in NBT II cells is a 110-115 kDa cell surface glycoprotein located predominantly at sites of cell-cell contact but also present on the apical cell surface. Northern blotting analysis revealed the presence of both C-CAM1 and C-CAM2, with the major transcripts for both isoforms present within the 4.0 kb size range. The dissociation of NBT II cell colonies by anti-C-CAM antibodies indicated that at least one function of C-CAM in these cells is to mediate intercellular adhesion. The maintenance of extensive cell-cell contacts and the expression of C-CAM at the contact sites in cells grown in low Ca^{2+} medium suggested that, like its counterpart in hepatocytes, C-CAM in NBT II cells may be a Ca^{2+}-independent cell-cell adhesion molecule. The co-localization and coordinate reorganization of both C-CAM and actin by anti-C-CAM antibodies indicated that these two proteins were associated and suggested that interactions with the cytoskeleton may be important for the regulation of C-CAM function. The specific upregulation of C-CAM1 in cells induced to undergo epithelial to mesenchymal-like transitions (EMT) by the serum substitute Ultroser G suggested that C-CAM isoforms are important modulators of the adhesive properties of these cells.

Key words: C-CAM, cell adhesion molecule, NBT II cell, bladder cancer

INTRODUCTION

Cell-cell adhesion is required at all stages of development and is of fundamental importance in the establishment and maintenance of the organized structure and function of multicellular organisms. In recent years, a number of specific cell-cell adhesion molecules (CAMs) have been identified that mediate this process (Takeichi, 1990, 1991; Edelman and Crossin, 1991). Within a given tissue, CAMs appear to be expressed in a characteristic spatiotemporal sequence (Edelman, 1988; Takeichi, 1988) and alterations in the normal distribution of CAMs are often associated with changes in cell morphology (Matsuzaki et al., 1990; Takeichi, 1991).

C-CAM (Cell-CAM 105) is a Ca^{2+}-independent CAM that has been shown to mediate the intercellular adhesion of isolated rat hepatocytes (Ocklind and Öbrink, 1982) by a homophilic binding mechanism (Tingström et al., 1990). Sequence comparisons (Lin and Guidotti, 1989; Aurivillius et al., 1990; Margolis et al., 1990) indicate that C-CAM is the rat homologue of biliary glycoprotein (BGP), and together with homologous mouse and human proteins forms the carcinoembryonic antigen (CEA) subgroup of the Ig superfamily (Thomson et al., 1991).

Alternative splicing of the rat C-CAM gene (Najjar et al., 1993) generates two isoforms, C-CAM1, which has a cytoplasmic domain of 71 amino acids, and C-CAM2, whose cytoplasmic domain is only 10 amino acids in length (Lin and Guidotti, 1989; Culic et al., 1992; Edlund et al., 1993). The conservation of this splicing pattern in the homologous human gene (Barnett et al., 1989) suggests that the generation of both long and short cytoplasmic domain isoforms has important functional significance. Using isoform-specific antibodies (Culic et al., 1992) it has been demonstrated that C-CAM1 and C-CAM2 correspond to the 110 kDa and 105 kDa C-CAM proteins, previously identified in rat liver (Odin et al., 1986). Two different sequences have been detected in the extracellular N-terminal Ig domain of C-CAM cloned from different outbred rat stocks, which give rise to the a and b variants of both C-CAM1 and C-CAM2 (Edlund et al., 1993).

Both C-CAM1 and C-CAM2 can be phosphorylated on serine residues (Odin et al., 1986; Culic et al., 1992) and C-CAM1 is, in addition, a substrate for the tyrosine kinase activity of the insulin receptor (Margolis et al., 1990), suggesting a role for differential phosphorylation in the regulation of C-CAM isoform function. That C-CAM may be involved in a variety of complex cellular interactions and signalling events

is further suggested by the binding of the cytoplasmic domains of both C-CAM1 and C-CAM2 to calmodulin (Edlund and Öbrink, 1993). The demonstration that C-CAM1a is identical to a rat liver ecto-ATPase (Lin and Guidotti, 1989) and can act as a bile acid transporter (Sippel et al., 1993) indicates that this protein has diverse isoform-specific functions, whose relationship to its adhesive properties remains to be established.

C-CAM appears late in development (Odin and Öbrink, 1986) primarily in differentiated epithelia (Odin et al., 1988) and its presence at sites of cell-cell contact in many tissues (Odin et al., 1988) is consistent with a role in intercellular adhesion. However, C-CAM has also been localized to the bile canaliculi of mature rat liver (Odin et al., 1988; Mowery and Hixson, 1991) and to the brush borders of intestinal epithelium (Hansson et al., 1989) where it has been suggested that it plays a role in the organization of microvilli. These differences in cellular location, together with the existence of C-CAM isoforms, suggest that C-CAM may have diverse biological functions, but with a shared role in the organization of differentiated epithelia via membrane-membrane interactions.

The rat Nara Bladder Tumour (NBT II) cell line has previously been shown to be a useful model system in which to study epithelial adhesion (Boyer et al., 1989; Tucker et al., 1990). In culture, the cells are extensively linked by desmosomes (Boyer et al., 1989) and express E-cadherin, a Ca^{2+}-dependent CAM of the cadherin family on their surface (Boyer et al., 1992). The ability of NBT II cells to undergo epithelial to mesenchymal like transitions (EMT) in response to soluble factors (Gavrilovic et al., 1990; Valles et al., 1990a,b) or growth on collagen substrata (Tucker et al., 1990) also makes them a useful system in which to study the role of cell surface molecules in this process.

Although highly expressed in rat liver, transplantable hepatocellular carcinomas generally lack or express only low levels of an altered form of C-CAM (Hixson et al., 1985; Hixson and McEntire, 1989) and, to date, no established cell line expressing significant amounts of this protein has been described. We now report on the expression of C-CAM in NBT II cells and on the use of this cell line for the study of the biological functions, cellular interactions and regulation of C-CAM isoforms.

MATERIALS AND METHODS

Cell culture

The NBT II cell line, established by Toyoshima et al. (1971) from a chemically induced carcinoma of rat bladder was obtained from Prof. J. P. Thiery (CNRS, Paris, France). The cells were cultured in DMEM supplemented with 10% heat-inactivated FCS (foetal calf serum), 2 mM L-glutamine, penicillin (100 i.u./ml) and streptomycin (100 mg/ml) (Gibco, Paisley, Scotland) (standard medium) in a 5% CO_2 humidified atmosphere at 37°C.

In some experiments, cells established in standard medium were transferred to S-MEM (Gibco, Paisley, Scotland) supplemented with antibiotics, 10% dialyzed FCS and 5 μM $CaCl_2$ (low Ca^{2+} medium) or to standard medium containing 2% Ultroser G (Gibco, Paisley, Scotland) or 100-200 ng/ml recombinant human acidic-FGF (a-FGF) (a generous gift from Drs Y. Cao and R. Pettersson, Ludwig Institute for Cancer Research, Stockholm, Sweden; see Cao and Pettersson, 1990, for details).

In experiments designed to investigate the role of the actin cytoskeleton in C-CAM organization, cells were incubated in standard medium containing cytochalasin B (1 μg/ml) (Sigma) for 1 hour at 37°C, prior to processing for fluorescence microscopy.

To determine the role of transcription in the expression of C-CAM, NBT II cells were grown in either standard medium or medium containing 2% Ultroser G for 8 hours at 37°C, in the presence of 0.5 μg/ml actinomycin D prior to extraction and immunoblotting.

Fluorescence microscopy

NBT II cells, grown directly on glass coverslips were fixed in medium A (137 mM NaCl, 4.7 mM KCl, 0.6 mM $MgSO_4$, 1.2 mM $CaCl_2$, 10 mM 4-(2-hydroxymethyl)-1-piperazineethanesulphonic acid, pH 7.4) containing 2% paraformaldehyde and 0.05% glutaraldehyde and permeabilized in the same buffer containing 0.1% Triton X-100. Cells were incubated in glycine-containing buffer to quench reactive aldehyde groups and, after extensive washing, were incubated with rabbit affinity-purified polyclonal antibodies against rat liver C-CAM (Ocklind and Öbrink, 1982; Odin et al., 1986), overnight at 4°C. When the mouse monoclonal anti-C-CAM antibody, F7 (Aurivillius, 1990), was used, unfixed cells were incubated with antibody for 1 hour at 4°C.

Primary antibodies were localized by the addition of appropriate FITC- or TRITC-conjugated secondary antibodies (Dakopatts) for 1 hour at room temperature (1 hour at 4°C for F7-treated cells). Filamentous actin was visualized by incubation of fixed/permeabilized cells with TRITC-phalloidin (Sigma) for 20 minutes at room temperature.

Cells were examined with the ×40 standard and ×100 oil immersion objective of a Nikon Labophot phase-epifluorescence microscope and photographed on Tri-X pan film at 400 ASA.

Isolation of RNA and northern blotting analysis

Poly(A)$^+$ RNA was prepared from cell monolayers as described previously (Jin et al., 1991). Briefly, cells were detached from culture dishes into ice-cold STE buffer (0.1 mM NaCl, 20 mM Tris-HCl, pH 7.4, 10 mM EDTA). Proteinase K (400 μg/ml) and SDS (0.5%) were added immediately prior to homogenization. Homogenized samples were incubated for 30 minutes at 37°C and then shaken gently with oligo(dT)-cellulose for 1-2 hours at room temperature. Poly(A)$^+$ RNA was eluted using 1 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.2% SDS and quantified by ultraviolet spectroscopy. Samples of 10 μg poly(A)$^+$ RNA were electrophoresed on 1% agarose, 1.5 M formaldehyde gels and transferred to Hybond-N membranes (Amersham). Membranes were hybridized for 18 hours at 42°C in 5× SSC (1× SSC is: 150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5× Denhardt's solution (100× Denhardt's solution is: 2% Ficoll, 2% BSA, 2% poly(vinylpyrrolidone)), 20 mM sodium phosphate, 100 mg/ml calf thymus DNA, 10% dextran sulphate, 0.5% SDS and 1.0×10^6 cpm/ml of ^{32}P-labelled probe. The filters were washed to a final stringency of 1× SSC, 0.5% SDS at 55°C and exposed to Fuji RXL X-ray film at −70°C. For quantitative analysis, films were scanned using a Shimadzu CS-930 scanner, equipped with an automatic integrator.

Probes for northern blotting analysis

Four isoforms of C-CAM (C-CAM1a,b and C-CAM2a,b) have been cloned from rat liver cDNA libraries. C-CAM1 and C-CAM2 differ in the lengths of their cytoplasmic domains (Lin and Guidotti, 1989; Culic et al., 1992; Edlund et al., 1993) while differences in the sequence of the N-terminal Ig domain give rise to a or b variants of both C-CAM1 and C-CAM2 (Culic et al., 1992; Edlund et al., 1993).

Antisense oligonucleotide probes designed to distinguish these isoforms were synthesized with the following sequences (the corresponding nucleotide sequence numbers of C-CAM cDNA (Edlund et al., 1993) are indicated): Ig2, CACCCTGTCACCTTCTGAGAGGCTTTCACCATTTCTGCTC (nt 532-572); Ig3, AGCTCTTGGGAGGATGTCTGGAGCTTCTCATTGATAAGCC (nt 811-851); Ig4, GGTCTATTCTGAGGGTGCTGTTGTCCTGGGAGAGCGT-

CATCCTGTCT (nt 1096-1143), which recognize sequences common to both C-CAM1 and C-CAM2. Probes F and G, which recognize sequences contained only in the long (C-CAM1) and short (C-CAM2) cytoplasmic domain isoforms of C-CAM, respectively, as well as probes H and I, designed to distinguish the a and b variants of both C-CAM1 and C-CAM2, have been described previously (Edlund et al., 1993). A gene-specific oligonucleotide probe recognizing rat cytoplasmic β-actin and having the following sequence: GACGAC-GAGCGCAGCGATATCGTCATC, was purchased from Clontech. Oligonucleotide probes were 3′-end-labelled using [α-^{32}P]dATP and terminal deoxynucleotidyl transferase.

Extraction of cells and deglycosylation

Rat liver plasma membranes were prepared as described previously (Ocklind and Öbrink, 1982). NBT II cells were rinsed twice with ice-cold PBS, harvested by scraping and pelleted by mild centrifugation. Cell pellets and liver plasma membranes were extracted with TBS (10 mM Tris(hydroxymethyl) aminomethane, 150 mM NaCl, pH 7.4) containing 1% Triton X-100, 1000 ki.u./ml Trasylol (aprotinin) and 1 mM PMSF for 30 minutes at 4°C. Samples were centrifuged at 15,000 ***g*** for 15 minutes and the protein concentration of the supernatant was estimated, after dilution, by a modified micro Lowry Assay (Findlay, 1990) using BSA as a standard. In some experiments, cells were directly extracted with SDS sample buffer (Laemmli, 1970) at 100°C for 5 minutes.

For deglycosylation experiments, cells were extracted as described above in a buffer containing 1% Nonidet P-40, PBS, 10 mM EDTA, 1000 ki.u./ml Trasylol and 1 mM PMSF and treated as described by Barnett et al. (1993) with minor modifications. Briefly, to 100 μl samples of the supernatants were added 10 μl 20% SDS and 10 μl 100 mM DTT, and the samples were incubated at 95°C for 10 minutes. After cooling, 120 μl digestion buffer (40 mM $NaPO_4$ (pH 8.5), 3% Nonidet P-40, 10 mM EDTA, 1000 ki.u./ml Trasylol and 1 mM PMSF) was added together with 5 units *N*-glycosidase F (Boehringer-Mannheim Biochemicals) and samples were incubated overnight at 37°C.

Immunoblotting

Detergent extracts of cells were mixed with an equal volume of 2× SDS sample buffer (Laemmli, 1970) and incubated at 100°C for 5 minutes. Samples were electrophoresed on 7.5% polyacrylamide gels and transferred to nitrocellulose filters (Burnette, 1981). After blocking with 5% defatted milk powder, 0.05% Tween-20, TBS, pH 7.4, membranes were incubated with affinity-purified anti-C-CAM antibodies, overnight at 4°C. Specific antibody binding was detected by use of swine anti-rabbit antibodies coupled to alkaline phosphatase and developed with nitro blue tetrazolium (NBT) and 5-bromo-chloro-indolylphosphate (BCIP) (Blake et al., 1984). For quantitative analyses, the ECL chemiluminescence western blotting kit (Amersham) was used according to the manufacturer's instructions and filters were exposed to Fuji RXL X-ray film at room temperature. Films were analysed by scanning densitometry as described above.

Antibody perturbation

To determine the role of both C-CAM and E-cadherin in cell adhesion, NBT II cells cultured in serum-free standard medium were incubated at 37°C with either rabbit non-immune or anti-C-CAM antibodies at a concentration of 1 mg/ml or with 1/10 volume of non-immune or anti-E-cadherin antiserum (a generous gift from Dr R. Kemler, Max Plank Institute, Freiburg). The effects of antibodies were determined by observing cells by phase-contrast microscopy at various times after antibody addition.

Antibody induced reorganization of C-CAM in NBT II cells

NBT II cells grown on glass coverslips in standard medium were incubated with either non-immune or affinity-purified anti-C-CAM antibodies diluted to 20 μg/ml with medium A, for 1 hour at 37°C prior to fixation and permeabilization. Cells treated with anti-C-CAM antibodies were directly labelled with FITC-conjugated secondary antibodies for 1 hour at room temperature while non-immune treated cells were first incubated with affinity-purified anti-C-CAM antibodies overnight at 4°C. Filamentous actin was localized by subsequent incubation of cells with TRITC-phalloidin for 20 minutes at room temperature and the cells were examined by fluorescence microscopy.

RESULTS

Localization of C-CAM in NBT II cells

Under standard culture conditions, NBT II cells formed monolayers whose 'cobblestone' organization is typical of epithelia (Fig. 1a). Indirect immunofluorescence staining using affinity-purified polyclonal antibodies demonstrated that C-CAM was predominantly localized to lateral cell membranes at sites of cell-cell contact (Fig. 1b), a distribution indistinguishable from that in cultured hepatocytes (Tingström and Öbrink, 1989). In fixed cells, the monoclonal antibody, F7, gave a weak but continuous staining of cell-cell contact sites. A much stronger, but somewhat discontinuous, staining was detected in unfixed cells (Fig. 1c), most likely due to antibody-induced micropatching when the cells were transferred to room temperature during fluorescence microscopy. Staining with anti-C-CAM antibodies was specific as judged by the lack of reaction with non-immune antibodies. A faint staining for C-CAM was detected on the basal cell surface (Fig. 1e) and on the apical cell surface, C-CAM had a strong, punctate staining that appeared to be associated with surface projections, presumably corresponding to microvilli (Fig. 1f). In subconfluent cultures, C-CAM was absent from lateral cell membranes not in contact with an adjacent cell (Fig. 1b, arrows).

To determine the total cellular distribution of C-CAM, cells were permeabilized with Triton X-100 prior to incubation with antibodies. The similarity in the staining patterns of fixed (Fig. 1b) and fixed/permeabilized cells (Fig. 1d) demonstrated that C-CAM is a cell surface protein concentrated at sites of close cell-cell apposition in NBT II cells.

Both C-CAM1 and C-CAM2 are expressed in NBT II cells

To determine the expression pattern of C-CAM isoforms in NBT II cells, we have designed oligonucleotide probes that recognize sequences common to both C-CAM1 and C-CAM2 (probes Ig2, 3 and 4) or sequences unique to C-CAM1 (probe F) or C-CAM2 (probe G).

When used in northern blotting analysis, oligonucleotide probes Ig2, -3 and -4 gave identical results and detected a major 4.0 kb transcript for C-CAM in NBT II cells (Fig. 2, lanes 1, 2 and 3). Probe F, specific for C-CAM1 detected a major 4.0 kb, and minor 6.0 kb and 8.5 kb, transcripts (Fig. 2, lane 4), while probe G, specific for C-CAM2, detected a major 4.0 kb and a minor 3.0 kb transcript (Fig. 2, lane 5). Probes recognizing different sequences in the N-terminal Ig domain designed to distinguish between the a and b variants of C-CAM (Edlund et al., 1992) were poorly reactive, but the results suggested that NBT II cells express the a variant form of C-CAM (not shown).

The results indicate that both the long (C-CAM1) and short (C-CAM2) cytoplasmic domain isoforms of C-CAM are

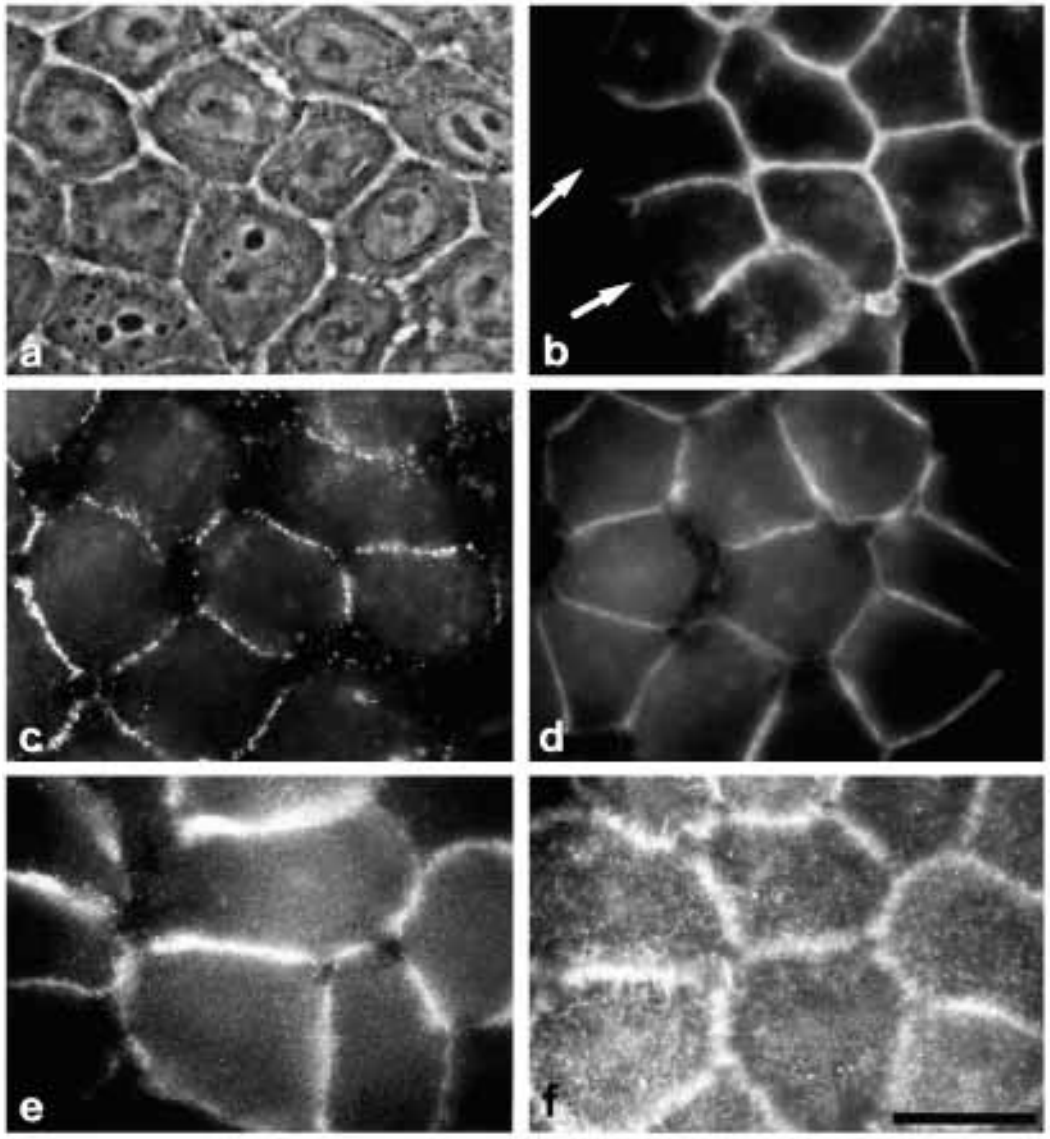


Fig. 1. Immunolocalization of C-CAM in NBT II cells. Phase-contrast micrograph of NBT II cells showing the 'cobblestone' organization typical of epithelia (a). NBT II cells grown in standard medium were fixed (b,e,f) or fixed and permeabilized (d) prior to indirect immunofluorescence staining with anti-C-CAM antibodies. C-CAM was localized predominantly to lateral cell borders at sites of cell-cell contact (b,d) but absent from free cell edges (b, arrows). By focusing at different levels, it was found that C-CAM was also present on both basal (e) and apical (f) cell surfaces where it had a punctate distribution. Staining of unfixed cells with the monoclonal anti-C-CAM antibody F7 also demonstrated that C-CAM was localized to sites of cell-cell contact (c). Bar, 25 μm.

expressed by NBT II cells with the major transcripts for both isoforms present in the 4.0 kb size range. In NBT II cells, the 3.0 kb trancripts are specific for C-CAM2 while the 6.0 kb and 8.5 kb transcripts are specific for C-CAM1. A similar pattern of C-CAM transcripts has been described in rat liver (Edlund et al., 1993).

Both C-CAM1 and C-CAM2 are highly glycosylated

Rat liver C-CAM appeared on western blotting as two bands of apparent molecular mass 110 and 105 kDa (Fig. 3, lane 1), which correspond to C-CAM1 (long cytoplasmic domain isoform) and C-CAM2 (short cytoplasmic domain isoform), respectively (Culic et al., 1992). In contrast, NBT II C-CAM appeared as a broad, diffuse band with an apparent molecular mass of 110-115 kDa (Fig. 3, lane 3).

Rat liver C-CAM is heavily *N*-glycosylated (Odin et al., 1986). Enzymatic removal of N-linked sugars by *N*-glycosidase F gave rise to two closely spaced bands (Fig. 3, lane 2) whose apparent molecular mass of about 52 kDa is close to that of the cloned protein (Lin and Guidotti, 1989). Two bands of the same molecular mass were obtained both after deglycosylation of C-CAM in NBT II cells (Fig. 3, lane 4) and of an 80 kDa form of C-CAM expressed in colon carcinoma cells (unpublished observations). These results suggest that both isoforms of C-CAM are expressed by these cells and that differences in the molecular mass of C-CAM in different cell types may, to a large extent, be due to differences in glycosylation.

C-CAM mediates adhesion in NBT II cells

Disruption of cell-cell adhesion by specific antibodies, a technique devised by Beug et al. (1970), has been a powerful tool in the identification of cell surface CAMs (Edelman, 1988; Takeichi, 1988). In the present study, anti-C-CAM (Fig. 4b) but not non-immune (Fig. 4a) antibodies were found to cause the disruption of cell-cell contacts in NBT II cell cultures, with the dissociated cells having an elongated morphology. The effect was completely reversible, indicating a lack of antibody-induced cytotoxicity. Since the anti-C-CAM antibodies used recognized only C-CAM by immunoblotting, the present results provide strong evidence that C-CAM is involved in cell-cell adhesion in NBT II cells.

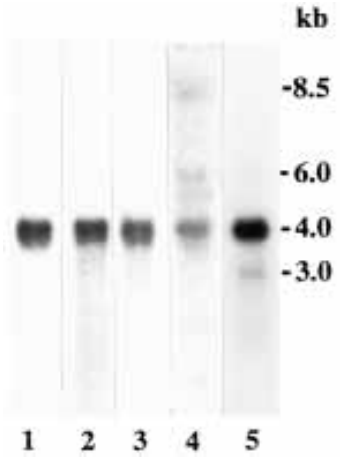


Fig. 2. Northern blot analysis of mRNA from NBT II cells. Poly(A)$^+$RNA (10 μg/lane), isolated from NBT II cells grown in standard medium, was electrophoresed on agarose/formaldehyde gels, transferred to Hybond N membranes and probed with ^{32}P-labelled oligonucleotides recognizing different domains of C-CAM. Probes Ig2 (lane 1), Ig3 (lane 2) and Ig4 (lane 3) recognized sequences present in Ig domains 2, 3 and 4, common to both C-CAM1 and C-CAM2. Probe F (lane 4) recognized sequences present in the cytoplasmic domain of C-CAM1, while probe G (lane 5) recognized sequences present in the cytoplasmic domain of C-CAM2. The migration positions of RNA size markers are shown.

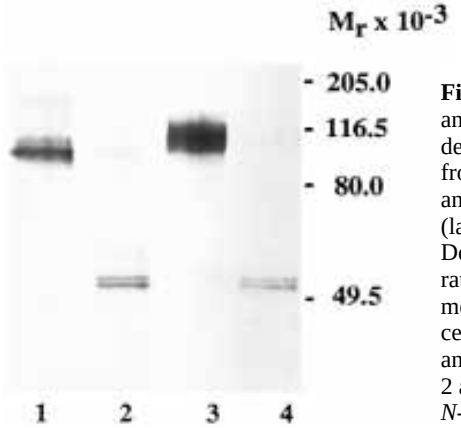


Fig. 3. Immunoblot analysis of deglycosylated C-CAM from rat liver (lanes 1 and 2) and NBT II cells (lanes 3 and 4). Detergent extracts of rat liver plasma membranes and NBT II cells before (lanes 1 and 3) and after (lanes 2 and 4) treatment with *N*-glycosidase F were subjected to SDS-PAGE and immunoblotting with affinity-purified anti-C-CAM antibodies. The migration positions of M_r markers are indicated.

This effect of anti-C-CAM antibodies was, however, somewhat surprising, since NBT II cells have been shown to express functional E-cadherin (Boyer et al., 1992). Indeed, addition of anti-E-cadherin antiserum (Fig. 4c) also caused dissociation of NBT II cells, while non-immune serum had no effect (not shown). These results indicate that both C-CAM and E-cadherin contribute to the overall adhesive properties of NBT II cells and suggest that there may be a functional association between these two adhesion systems.

When transferred to low-Ca^{2+} medium, NBT II cells round up and lose some intercellular contacts. However, even after 24 hours in low-Ca^{2+} conditions, where the functional activity of desmosomes (Mattey and Garrod, 1986) and Ca^{2+}-dependent CAMs (Kartenbeck et al., 1991) are likely to be abrogated, the cells remained extensively associated and continued to express C-CAM at cell-cell contact sites (Fig. 4d). These results, together with the identification of Ca^{2+}-independent adhesion systems on the suface of NBT II cells (Boyer et al., 1992) and the reported Ca^{2+} independence of C-CAM-mediated adhesion (Tingström et al., 1990) are consistent with a role for C-CAM in mediating the Ca^{2+}-independent adhesion of NBT II cells.

C-CAM and actin are associated in NBT II cells

The finding that C-CAM1 and C-CAM2 differ in the lengths of their cytoplasmic domains (Lin and Guidotti, 1989; Culic et al., 1992; Edlund et al., 1993) has suggested that intracellular interactions may be important for their function. For the cadherin family of CAMs, the interaction of their cytoplasmic domain with the actin cytoskeleton has been shown to be neccessary for their adhesive function (Nagafuchi and Takeichi, 1989). In attempting to identify interactions between C-CAM and actin in NBT II cells, we have taken advantage of the ability of anti-C-CAM antibodies to specifically reorganize the surface distribution of C-CAM.

In cells treated with affinity-purified anti-C-CAM antibodies for 1 hour at 37°C, C-CAM was detected only as a punctate outline at cell-cell borders (Fig. 5a), but there was no detectable reorganization of actin (Fig. 5b). Actin is one of the most abundant eukaryotic cell proteins and actin filaments of the subcortical belt are associated with the intercellular adherens junctions as well as with a variety of cell surface proteins (reviewed by Geiger, 1985; Carraway and Carraway, 1989). Thus, the failure to detect a reorganization of actin at cell-cell contacts may be due either to the absence of a physical linkage with C-CAM at these sites or the inability of these methods to detect the small changes in actin distribution

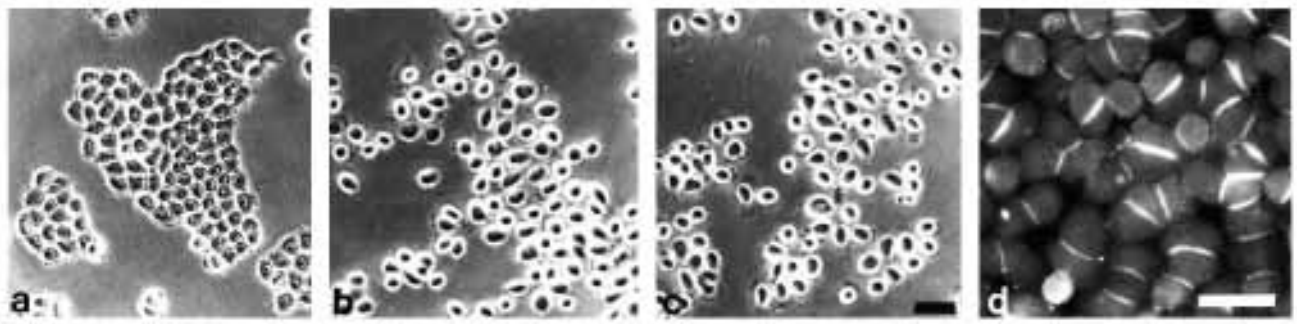


Fig. 4. The role of C-CAM and E-cadherin in the adhesion of NBT II cells. Incubation of subconfluent cultures of NBT II cells with 1 mg/ml non-immune IgG (a) had no effect on NBT II cell colonies while addition of 1 mg/ml anti-C-CAM antibodies (b) or 1/10 volume of anti-E-cadherin antiserum (c) for 3-4 hours at 37°C caused cell dissociation, with the individual cells having an elongated morphology. NBT II cells, established in standard medium, were transferred to low Ca^{2+} containing medium for 24 hours prior to fixation and staining with anti-C-CAM antibodies. The cells were rounded but retained extensive cell-cell contacts, which were positively stained for C-CAM (d). Bars, 50 μm.

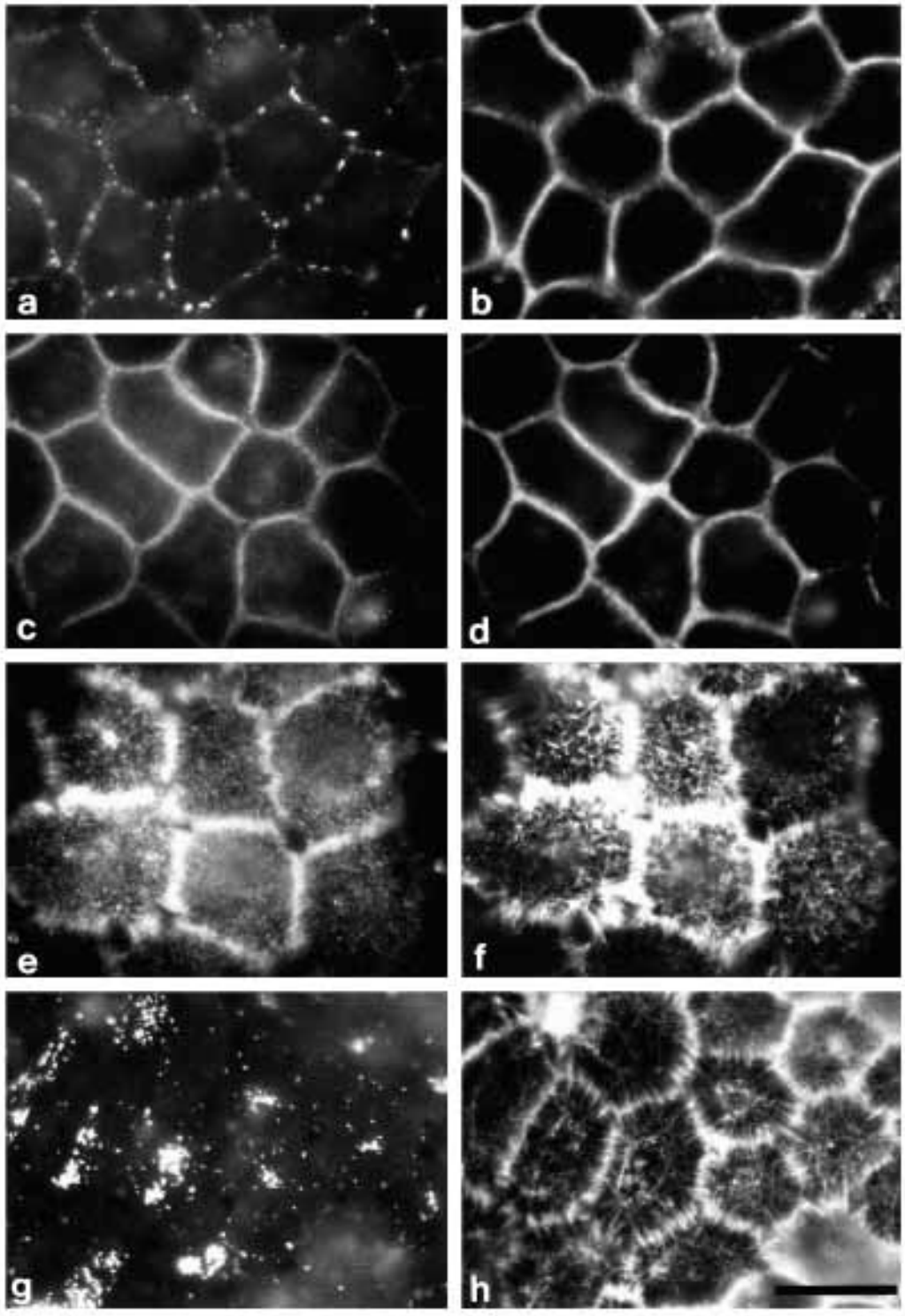


Fig. 5. Localization of C-CAM and actin in NBT II cells - effect of anti-C-CAM antibodies. Cells were incubated with either anti-C-CAM antibodies (a,b,g,h) or non-immune IgG (c,d,e,f) (20 μg/ml) for 1 hour at 37°C prior to fixation, permeabilization and double labelling for C-CAM (a,c,e,g) and actin (b,d,f,h) as described in Materials and Methods. By focusing at the level of cell-cell contact in cells treated with anti-C-CAM antibodies, it was found that C-CAM at the lateral cell membranes was reorganized and detected only as a punctate cellular outline (a) but there was no detectable redistribution of the cortical actin filaments (b). Non-immune IgG had no effect on the distribution of either C-CAM (c) or actin (d) at lateral membranes. By focusing at the apical surface of non-immune IgG-treated cells, C-CAM (e) and actin (f) were found to be evenly distributed and apparently localized in cell surface microvilli. On the apical surface of cells treated with anti-C-CAM antibodies, C-CAM was reorganized into patches (g) and there was a coordinate reorganization of actin (h). Bar, 25 μm.

expected from an interaction with C-CAM. Sodium azide prevented the reorganization of C-CAM (not shown), indicating that this is an energy-dependent, rather than a passive, process and supports the notion that it involves an association with cellular components. In cells treated with non-immune antibodies, there was no detectable reorganization of either C-CAM (Fig. 5c) or actin (Fig. 5d) at cell-cell contacts.

On the apical surface of cells treated with non-immune antibodies, C-CAM had a punctate pattern (Fig. 5e) and actin appeared to be present in surface projections, presumably corresponding to microvilli (Fig. 5f). In cells treated with anti-C-CAM antibodies, C-CAM on the apical surface was redistributed into patches (Fig. 5g) and actin was reorganized into either compact clusters that co-localized with C-CAM or ring-like structures that followed the outline of C-CAM patches (Fig. 5h) and from which filaments appeared to radiate.

When treated with cytochalasin B, large aggregates of cellular material were detected both at lateral cell borders and

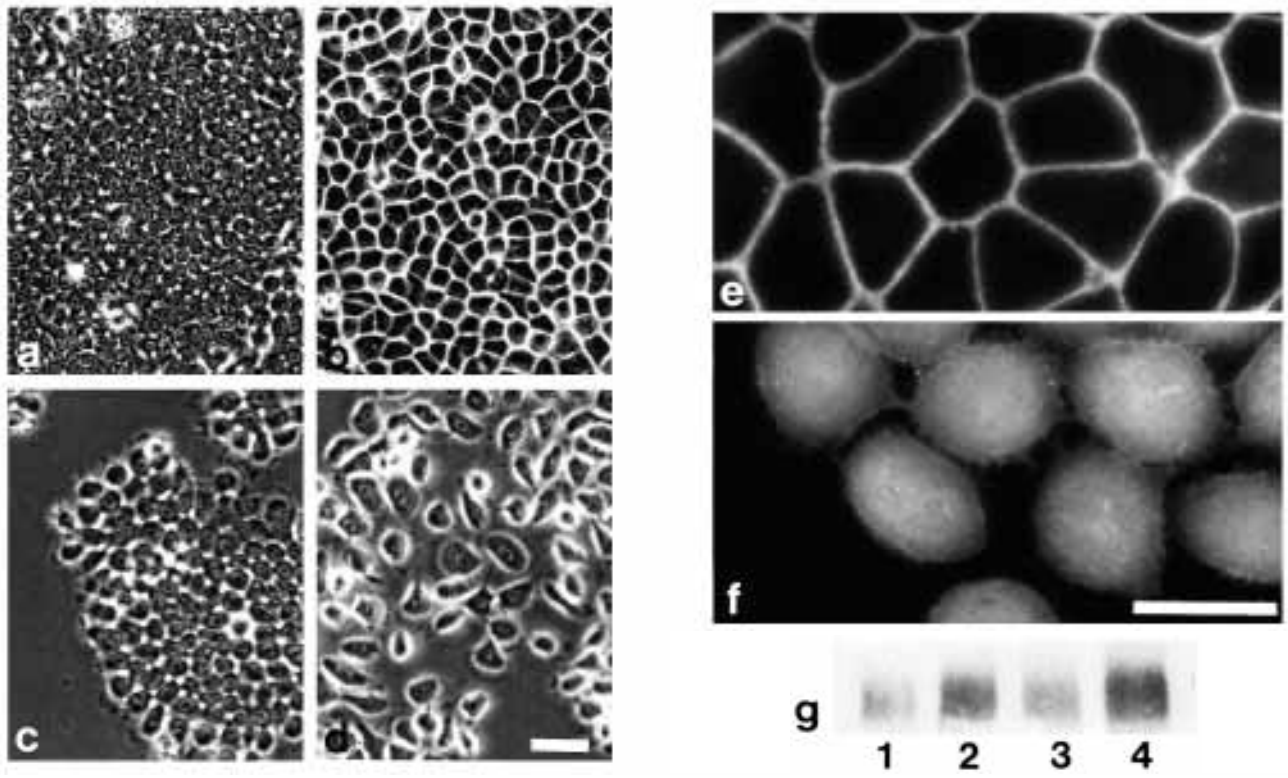


Fig. 6. Modulation of C-CAM in NBT II cells. Confluent (a,b,e) and subconfluent (c,d,f) cultures of NBT II cells established in standard medium were either maintained in standard medium (a,c) or transferred to standard medium containing 2% Ultroser G (b,d,e,f) for 16 hours, prior to phase-contrast microscopy (a-d) or fixation and indirect immunofluorescence staining with anti-C-CAM antibodies (e,f). At confluence, untreated cells formed compact cellular arrays (a), while Ultroser G-treated cells were less tightly packed, with apparently widened intercellular spaces (b). In confluent cultures of Ultroser G-treated cells C-CAM was predominantly localized to lateral cell borders at sites of cell-cell aposition (e). At subconfluent density, cells formed small colonies (c), which were dissociated by Ultroser G treatment (d). The individual cells had an elongated morphology and C-CAM was diffusely distributed over the entire cell surface (f). Bars: (a-d) 50 μm; (e,f) 25 μm. (g) Equal amounts of cellular protein from Triton X-100 extracts of subconfluent (lanes 1 and 2) and confluent (lanes 3 and 4) cultures of NBT II cells prior to (lanes 1 and 3) and after (lanes 2 and 4) Ultroser G treatment were electrophoresed on 7.5% polyacrylamide gels under non-reducing conditions and analysed by immunoblotting with anti-C-CAM antibodies. Blots were developed using the chemiluminescence ECL procedure and quantified by densitometry.

on the apical cell surface (not shown). Actin was no longer detected in cell surface microvilli but could still be detected in the subcortical belt. Double immunofluorescence microscopy demonstrated that all of the aggregates contained both C-CAM and actin, and suggested that these proteins were associated both on the apical cell surface and at lateral cell borders.

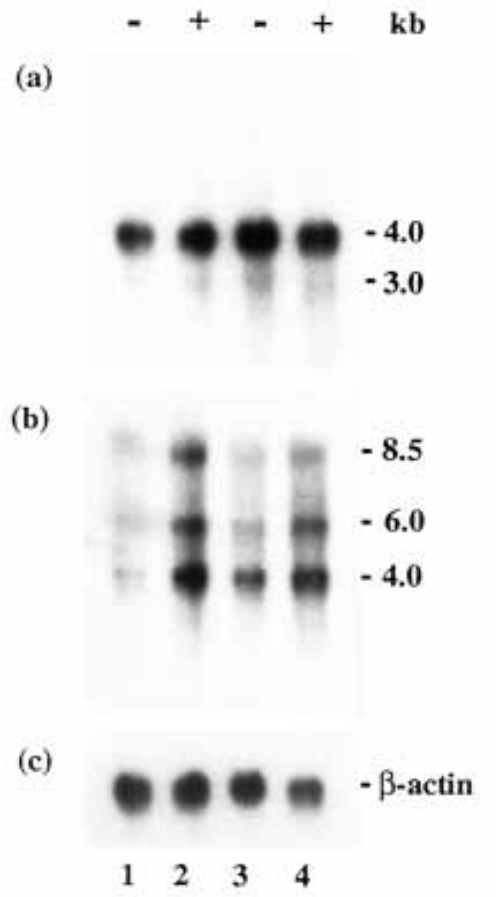
These results thus provide preliminary evidence that at least a subpopulation of C-CAM is structurally linked to the actin cytoskeleton in NBT II cells. Further studies will be required to establish if this is a direct or indirect interaction.

Modulation of C-CAM in NBT II cells

In response to treatment with Ultroser G, a serum substitute rich in growth factors (Boyer et al., 1989), or with individual growth factors (Gavrilovic et al., 1990; Valles et al., 1990a,b), NBT II cell colonies dissociate, lose their epithelial character and give rise to elongated, actively migrating cells expressing mesenchymal markers. This behaviour, which is reminiscent of the epithelial to mesenchymal transitions (EMT) of early embryogenesis, is accompanied by the internalization of desmosomes (Boyer et al., 1989) but no change in the expression of E-cadherin (Boyer et al., 1992). To determine the fate of C-CAM during EMT, we have examined the distribution and expression of C-CAM in NBT II cells treated with either Ultroser G or a-FGF.

In line with previous observations, treatment of confluent cultures with Ultroser G caused no gross morphological changes (Valles et al., 1990b), although compared with untreated cells (Fig. 6a) treated cells appeared to be less tightly packed with widened intercellular spaces (Fig. 6b). C-CAM was predominantly located at sites of cell-cell apposition (Fig. 6e) as it was in untreated cultures (Fig. 1b). In contrast, small colonies of NBT II cells (Fig. 6c) were dissociated by treatment with Ultroser G and the scattered cells had a somewhat elongated morphology (Fig. 6d). In dissociated cells, C-CAM was clearly redistributed and showed a diffuse staining over the entire cell surface (Fig. 6f). The detection of an identical staining pattern in fixed/permeabilized cells suggested that C-CAM was present predominantly on the cell surface. Identical results were obtained with a-FGF-treated cells.

Immunoblotting analysis of equal amounts of cellular protein from subconfluent (Fig. 6g, lanes 1 and 2) and confluent (Fig. 6g, lanes 3 and 4) cultures demonstrated that C-CAM was present in 2- to 4-fold higher amounts after



(d)

Probe	Transcript size (kb)	Subconfluent		Confluent	
		–	+	–	+
G	4.0	1.0	1.1	1.0	1.3
F	8.5	1.0	2.5	1.0	2.5
	6.0	1.0	3.1	1.0	3.5
	4.0	1.0	3.8	1.0	3.1

Fig. 7. Northern blotting analysis of mRNA from Ultroser G-treated cells. Subconfluent (lanes 1 and 2) and confluent (lanes 3 and 4) NBT II cells were grown in either standard medium (–, lanes 1 and 3) or standard medium containing 2% Ultroser G (+, lanes 2 and 4) for 16 hours at 37°C. Poly(A)$^+$RNA (10 μg/lane), isolated from these cells was electrophoresed on agarose/formaldehyde gels, transferred to Hybond N membranes and analysed sequentially with oligonucleotide probes G (a) and F (b). RNA loading was verified by hybridization of the filters with a probe to rat β-actin (c). The expression of individual transcripts relative to β-actin was determined by scanning densitometry and the values obtained for cells grown under standard conditions normalized to 1.0 (d).

treatment with Ultroser G (Fig. 6g, lanes 2 and 4). Increased C-CAM expression was first detected 6-8 hours after Ultroser addition, coincident with the first morphological signs of cell dissociation. a-FGF, which can reproduce the morphological effects of Ultroser G, was also found to upregulate the expression of C-CAM in both subconfluent and confluent cultures of NBT II cells (not shown).

Differential regulation of C-CAM isoforms in Ultroser G-treated NBT II cells

In rat liver, both C-CAM1 and C-CAM2 appear to be coordinately regulated (Cheung et al., 1993; Thomson et al., 1993). Northern blotting analysis using isoform-specific oligonucleotides was used here, to determine if the upregulation in C-CAM protein induced by Ultroser G was correlated with the coordinate upregulation of both C-CAM1 and C-CAM2 isoforms.

Probe G, which recognizes sequences specific to the short cytoplasmic domain isoform (C-CAM2), detected a major 4.0 kb and a minor 3.0 kb transcript (Fig. 7a, lanes 1-4). To correct for differences in loading, the ratio of the major 4.0 kb transcript relative to β-actin (Fig. 7c, lanes 1-4) was determined by scanning densitometry. The results demonstrated that in both subconfluent and confluent cultures of NBT II cells, Ultroser G had essentially no effect on the expression of this short cytoplasmic domain isoform-specific transcript (Fig. 7d, probe G).

In contrast, probe F, specific for the long cytoplasmic domain isoform (C-CAM1), detected a 2.5- to 3.8-fold increase in the levels of the 4.0 kb, 6.0 kb and 8.5 kb transcripts in both subconfluent (Fig. 7b, lanes 1 and 2) and confluent (Fig. 7b, lanes 3 and 4) cells after treatment with Ultroser G (Fig. 7d, probe F).

The upregulation of C-CAM protein by Ultroser G was matched by a corresponding increase in the level of transcripts for the long cytoplasmic domain isoform, indicating that Ultroser G acts to regulate the expression of C-CAM1 at the level of synthesis, processing or stability of the corresponding mRNA.

Upregulation of C-CAM by Ultroser G is dependent upon transcription

In an attempt to determine the mechanism of upregulation of C-CAM, we have examined the expression of C-CAM protein in NBT II cells treated with Ultroser G in the presence of the transcription inhibitor, actinomycin D.

In untreated cells, actinomycin D had no effect on the expression of C-CAM (Fig. 8, lanes 1 and 2). Treatment of cells with Ultroser G for 8 hours caused a 2-fold increase in the level of C-CAM (Fig. 8, lane 3) while cells treated with Ultroser G in the presence of actinomycin D expressed only unstimulated levels of C-CAM (Fig. 8, lane 4). The results suggest that Ultroser G-induced upregulation of C-CAM is primarily due to increased transcriptional activity.

DISCUSSION

Using isoform-specific oligonucleotide probes and affinity-purified polyclonal and monoclonal antibodies against rat liver C-CAM, we have determined the isoform expression pattern and cellular localization of C-CAM in the rat bladder carcinoma cell line, NBT II. This is the first established epithelial cell line reported to express significant amounts of C-CAM, and has provided us with a useful model system in which to study the physiological function and cellular regulation of this protein.

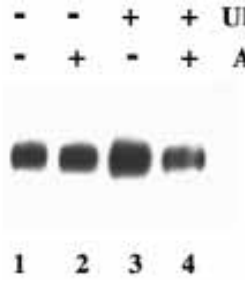


Fig. 8. The effect of actinomycin D on C-CAM expression. Subconfluent cultures of NBT II cells were grown either in standard medium (lanes 1 and 2) or standard medium containing 2% Ultroser G (lanes 3 and 4) in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 0.5 μg/ml actinomycin D. Equal amounts of cellular protein were analysed by immunoblotting and quantified as described in the legend to Fig. 6.

NBT II cells express both Ca^{2+}-independent and E-cadherin mediated, Ca^{2+}-dependent adhesion mechanisms (Boyer et al., 1992). C-CAM is predominantly expressed at sites of cell-cell contact in NBT II cells and when grown in low Ca^{2+} conditions, where the activity of the Ca^{2+}-dependent CAMs is abrogated (Mattey and Garrod, 1986; Kartenbeck et al., 1991), these cells remain extensively associated and continue to express C-CAM at cell contact sites. These results, together with the ability of anti-C-CAM antibodies to cause cell dissociation, argue strongly for C-CAM as a mediator of the Ca^{2+}-independent adhesion of NBT II cells.

The central role played by the Ca^{2+}-dependent CAMs of the cadherin family in the establishment and maintenance of strong cell-cell contacts has been well documented (Takeichi, 1988, 1990) and is supported by the ability of anti-E-cadherin antiserum to disrupt NBT II cell adhesion. It was surprising to find, therefore, that anti-C-CAM antibodies could also dissociate NBT cells. Nevertheless, this effect was specific and reversible and leads us to suggest that the adhesive properties of NBT II cells are dependent upon both C-CAM and E-cadherin, with the abrogation of the activity of either of these systems sufficient to reduce the overall adhesive strength to levels incompatible with the maintenance of cell-cell contacts.

The adhesive properties of the Ca^{2+}-dependent cadherins depend not only on their cell surface expression but also on the association of their cytoplasmic domain with the actin cytoskeleton (Nagafuchi and Takeichi, 1989; Ozawa et al., 1989; Nagafuchi et al., 1991). The ability of both anti-C-CAM antibodies and cytochalasin B to cause the reorganization of both C-CAM and actin suggests that, in NBT II cells, C-CAM is also associated with the actin cytoskeleton. Thus, an alternative mechanism for the action of anti-C-CAM antibodies is that they may, by an indirect effect on the actin cytoskeleton, alter the adhesive properties of E-cadherin, which, together with direct effects on the surface organization of C-CAM, could lead to a decrease in the adhesive properties and subsequent dissociation of NBT II cells. Interestingly, antibodies directed against *N*-acetylgalactosaminylphosphotransferase located on the surface of embryonic chick retinal cells are believed to inhibit CAM-mediated adhesion of these cells by uncoupling N-cadherin from the actin cytoskeleton (Balsamo et al., 1991).

The ability of purified liver C-CAM to interact with itself in solid-phase assays, and to induce aggregation when incorporated into liposomes (Tingström et al., 1990), has demonstrated that the adhesive function of C-CAM is not dependent upon interactions with other cellular proteins. Thus, the finding that C-CAM is associated with the actin cytoskeleton in NBT II cells, suggests that this interaction plays a role in the modulation of C-CAM activity. Two additional CAMs of the Ig family, I-CAM1 (Carpen et al., 1992) and N-CAM (Pollerberg et al., 1987), have also been shown to be associated with the actin cytoskeleton and, as is the case for C-CAM, this interaction is not required for their adhesive function.

The finding in the present study, that differences in the molecular masses of C-CAM in different cell types appears to be the result of differential glycosylation of common protein cores, suggests that changes in glycosylation may also regulate C-CAM function. Changes in the adhesive properties of differently glycosylated forms of N-CAM (Moran and Bock. 1988) and the dependence on glycosylation for CD4- (Fenouillet et al., 1989) and CD2- (Recny et al., 1992) mediated adhesion within the immune system, points to the importance of correct glycosylation for adhesive function. The recent NMR structure of glycosylated CD2 (Withka et al., 1993) provides clear evidence that N-linked glycosylation is necessary to maintain the correct conformation in the binding site of this CAM. Glycosylation has also been implicated in the binding of the adhesion molecules L1 and N-CAM within the plane of the membrane (*cis*-binding) (Kadmon et al., 1990).

In addition to its presence at sites of cell-cell contact in many epithelia (Odin et al., 1988), C-CAM has also been localized to apical cell surfaces such as the bile canaliculi of liver and the brush borders of intestinal epithelium (Odin and Öbrink, 1986; Odin et al., 1988; Hansson et al., 1989). It has been postulated that the role of C-CAM in these locations is to mediate membrane-membrane interactions involved in the organization of microvilli (Öbrink et al., 1988). Re-formation of bile canaliculi-like structures by cultured hepatocytes has been reported (Barth and Schwartz, 1982; Tingström and Öbrink, 1989) and C-CAM has been detected on their surface membranes (Tingström and Öbrink, 1989). However, difficulties in forming and maintaining such structures, together with the rapid de-differentiation of primary cultures of hepatocytes has hindered further studies of C-CAM function in such apical locations. The finding that C-CAM and actin appear to be present in finger-like projections, presumably corresponding to cell surface microvilli in NBT II cells, suggests that this may be a more suitable system in which to investigate further the structure and function of C-CAM at sites where no cell-cell contact occurs. The apparent association of C-CAM and actin on the apical cell surface of NBT II cells is particularly interesting in view of previous observations that C-CAM, located in the brush border microvilli of intestinal epithelium, is associated with a number of proteins, the most prominent of which has an apparent molecular mass of 43 kDa, suggesting that it may be actin (Hansson et al., 1989).

The importance of CAMs in regulating tissue morphology is exemplified by the process of epithelial to mesenchymal transition (EMT), where the conversion of epithelial sheets to loosely associated cells with mesenchymal morphology is accompanied by the disruption of cellular junctions and the altered expression of CAMs at the cell surface. EMT-like changes induced in NBT II cells by a number of soluble factors (Boyer et al., 1989; Gavrilovic et al., 1990; Valles et al., 1990a,b), are, however, not accompanied by any change in the amount or extent of phosphorylation of E-cadherin (Boyer et

al., 1992). In contrast, both subconfluent and confluent cultures of NBT II cells induced to undergo EMT-like changes by the serum substitute Ultroser G or a-FGF showed a 2- to 4-fold increase in their expression of C-CAM. Since almost all of the cells in subconfluent cultures were dissociated by this treatment, the results indicate that C-CAM is upregulated in individual, scattered cells.

In rat liver, the ratio of C-CAM1 to C-CAM2 does not change with time, suggesting that these two isoforms are coordinately regulated (Cheung et al., 1993; Thomson et al., 1993). In contrast, Ultroser G treatment of NBT II cells caused a 2.5- to 3.8-fold increase in the level of C-CAM1 transcripts, while having little or no effect on the expression of transcripts for C-CAM2, clearly demonstrating that C-CAM isoforms can be non-coordinately regulated in these cells. The abrogation of the effects of Ultroser G by actinomycin D suggests that the upregulation of C-CAM1 is primarily due to transcriptional activation.

The findings that C-CAM upregulation can be detected 6-8 hours after addition of Ultroser G, coincident with the detection of morphological changes in subconfluent cultures (Boyer et al., 1989; and this study), and that confluent cultures of Ultroser G-treated cells are highly motile when viewed by videomicroscopy (unpublished observations), suggest the intriguing possibility that increased expression of C-CAM may be a causative factor in cell dissociation. This is a testable hypothesis, and if correct, upregulation of C-CAM1 in NBT II cells by transfection would also be expected to lead to cell dissociation.

Alterations in tissue architecture in colon tumours has been suggested to result from the disruption of normal cellular adhesion by overproduction of the Ca^{2+}-independent CAM, carcinoembryonic antigen (CEA) (Benchimol et al., 1989). In support of this hypothesis, rat myoblasts expressing adhesion-competent CEA on their cell surfaces remain as single cells, completely unable to fuse and differentiate (Eidelman et al., 1993). It has been suggested that CEA-CEA interactions at the cell surface generate signals that compete with the normal pathway of differentiation in myoblasts (Eidelman et al., 1993) and the reported association of CEA and BGP, a related family member, with tyrosine kinases (Afar et al., 1993; Eidelman et al., 1993) has implicated tyrosine phosphorylation in this process. Interestingly, C-CAM is the rat homologue of BGP (Lin and Guidotti, 1989) and dissociation of NBT II cells is associated with the upregulation of C-CAM1, whose cytoplasmic domain, in contrast to that of C-CAM2, contains two tyrosine residues and can be tyrosine phosphorylated (Margolis et al., 1990). Addition of orthovanadate, a tyrosine phosphatase inhibitor, to NBT II cells has also been shown to cause cell dissociation (Boyer and Thiery, 1993).

An alternative possibility is that C-CAM is upregulated in NBT II cells as a consequence of cell dissociation, perhaps in an attempt by the cells to re-establish cell-cell contact. In this respect, it is interesting to note that it is only the C-CAM1 isoform that is upregulated by Ultroser G and that it is this isoform that has been found to be adhesion competent when expressed in Sf9 insect cells (Cheung et al., 1993). However, for the homologous mouse and human proteins, both the long and short cytoplasmic domain isoforms function in homophilic adhesion (Oikawa et al., 1989; Rojas et al., 1990; Turbide et al., 1991; McCuaig et al., 1992).

In addition to cell surface interactions between CAMs, adhesion is dependent upon a complex variety of signalling pathways within the cell, and it is likely that different subsets of pathways may lead to the same adhesion response (Edelman, 1993). Thus, although the morphological effects of Ultroser G and a-FGF are strikingly similar to those induced by anti-C-CAM antibodies, this does not necessarily imply a common mechanism of action. Our present results indicate that NBT II cells are a useful model system in which to investigate further the role of C-CAM isoforms, and current studies are directed towards understanding the molecular mechanisms underlying the effects of Ultroser G, a-FGF and the anti-C-CAM antibodies described here.

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