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CELL ADHESION ACTIVITY OF NON-SPECIFIC CROSS-REACTING ANTIGEN (NCA) AND CARCINOEMBRYONIC ANTIGEN (CEA) EXPRESSED ON CHO CELL SURFACE: HOMOPHILIC AND HETEROPHILIC ADHESION

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Cell adhesion activity of carcinoembryonic antigen (CEA) and non-specific cross-reacting antigen (NCA) has been analysed by using CHO cells which had been transfected with cDNAs and are ectopically expressing each antigen on their surface. CEA expressing CHO tended to aggregate easily within 30 min after being suspended by trypsinization. Cell adhesion assay between ^{51}Cr labelled cells and monolayered cells showed both homophilic and heterophilic interaction, the extent of which was CEA-CEA>>CEA-NCA>NCA-NCA. These reactions were completely inhibited by Fab' fragment of anti-CEA antibody. The results strongly suggested that CEA and NCA function as Ca^{++} independent cell adhesion molecules by homophilic and heterophilic interactions.

CEA first described in colon adenocarcinomas and foetal colon (1) is highly glycosylated glycoprotein and is one of the most widely used human tumor markers, although it lacks absolute tumor specificity because of the presence of a number of immunologically closely related antigens (see 2).

Recently, by molecular cloning of cDNAs and genomic sequences, primary structures of CEA family members were deduced (2-5), which revealed that CEA family belongs to Ig supergene family (6-8). Unexpectedly, it was found later

Abbreviations: CEA, carcinoembryonic antigen; NCA, nonspecific crossreacting antigen; PSβG, pregnacy-specific β1-glycoprotein; Ig, immunoglobulin; CAM, cell adhesion molecule; CHO, Chinese hamster ovary; MEM, minimum essential medium.

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that PSβGs, mainly found in large quantity in the sera of pregnant women, belong to the CEA family (9) and comprise a PSβG subfamily (10, 11).

The physiological roles of CEA family members are largely unknown, but being Ig superfamily members suggested that they might play some important roles in cell-cell or cell-substrate recognitions or they might work as receptors for the effector molecules (7). Adhesive cell-cell and cell-substrate interactions play, as one component of the morphogenetic process, many key roles in the life history of an organism (see 12-14 for reviews), and cellular invasion and metastasis of malignant cells (see 15 for review).

Recently, Benchimol *et al* reported the cell adhesion activity of CEA expressed on the surface of human colon carcinoma cells or rodent cells (16).

We will report here that not only CEA but also NCA expressed on the surface of CHO cells carrying the exogenous cDNA mediate adhesion of cells by homophilic and heterophilic interactions, suggesting their possible role as cell adhesion molecules.

MATERIALS AND METHODS

Culture of CHO Cells. CHOdhfr$^-$ cells (17) were maintained in α-MEM (GIBCO, USA) / 10% fetal bovine serum (GIBCO). CHOdhfr$^+$ transformants were cultured in a selection medium, α-MEM lacking ribonucleosides and deoxyribonucleosides (GIBCO) supplemented with 10% dialyzed fetal bovine serum (HAZLETON, USA).

Construction of expression vectors. Expression vectors were constructed as follows. pdKCR-hIL-5-dhfr (18, 19) kindly provided by Dr. T. Honjo (Kyoto University) was digested with *Kpn* I and *Aat* I, blunt ended with T4 DNA polymerase and the large fragment was self ligated to remove the 127 bp fragment which contained *Eco*R I site and then digested with *Eco*R I. The large fragment was religated to remove the human IL-5 gene. The resulting plasmid, pdKCR-dhfr, contained a unique *Eco*R I site for insertion of foreign sequence, into which the *Eco*R I fragments of full length CEA and NCA cDNAs (3) were inserted (Fig. 1A). The resulting expression plasmids, pdKCR-dhfr-CEA and pdKCR-dhfr-NCA, were used for transfection to the CHO cells.

Transfection. CHOdhfr$^-$ cells were transfected by calcium phosphate mediated coprecipitation with 5 μg of the expression plasmids according to the procedure accompanying CellPhect Transfection kit (Pharmacia, Sweden). After cultured in selection medium for 2-3 weeks, colonies of transformants were trypsinized in cloning cylinders.

Immunoblotting Analysis. Cells were rinsed once with PBS(-) and then scraped off the dish in 0.5 ml of SDS-sample buffer. Extracts were sonicated and 10 μl of each extract was resolved by 7.5% SDS-PAGE. The proteins were electrophoretically transferred to nitrocellulose and visualized with anti-CEA antibody (DAKO, Denmark), which is reactive with both CEA and NCA.

Aggregation Assay. Cells were released from the dish with trypsin (0.125%)-EDTA(0.01%) treatment at 37°C for 5-10 min. $2x10^6$ cells suspended in 2 ml of medium were rotated in a 35mm dish on a rotary shaker (80 rpm) for 30 min at 37°C (20). Extent of cell aggregation was viewed under a microscope and photographed.

Adhesion Assay. 10^7 cells were labeled with 0.1 mCi of $Na_2{}^{51}CrO_4$ in 1.5 ml of medium for 2 hr, washed with medium and incubated for one more hour (21). ^{51}Cr-labeled cells were trypsinized and suspended in 3 ml of medium. 0.1 ml of the ^{51}Cr-labeled cell suspension was added to the monolayer cells in a 24 well dish. After incubated in a 5% CO_2 incubator at 37°C for 15-80 min, cells were washed two times with Hank's balanced salt solution, lysed with 1% NP-40 and the radioactivity was counted in a γ-counter.

Antibody. Fab' fragment of antibody used for inhibition of cell aggregation or adhesion was prepared by digesting a rabbit anti-CEA antibody, which is reactive with both CEA and NCA, with pepsin followed by reduction and alkylation.

RESULTS

Establishment of CHO cell lines expressing CEA and NCA. In order to obtain stable transformants of CHO cells expressing CEA and NCA, we have introduced cDNA fragments containing the entire coding regions of CEA and NCA into CHOdhfr$^-$ cells as described in MATERIALS AND METHODS. After culturing in the selection medium, several dhfr$^+$ transformants were obtained. When cell extracts were subjected to immunoblotting analysis, 12 out of 17, and 15 out of 18 clones were found to express CEA and NCA, respectively. Most of the CEA transformants produced the antigen migrating at about 200 kDa (Fig. 1B, lane 1) except for clone 7 whose products banded at about 130 kDa (not shown), while NCA transformants produced NCA of various sizes ranging from 50 to 130 kDa

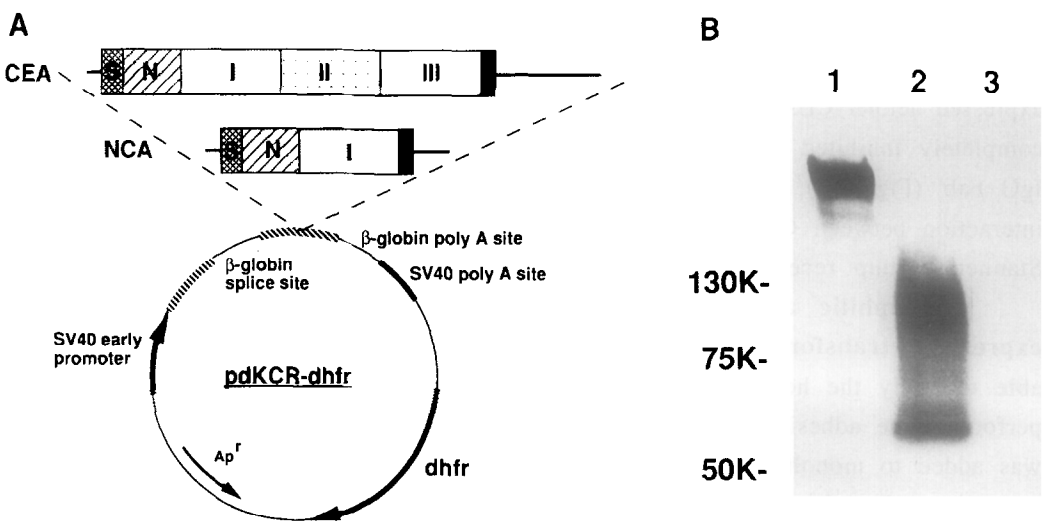

Fig. 1: Expression vector for CEA and NCA (A), and immunoblot analysis of the products of the transformants (B). In (A), CEA and NCA cDNA were inserted into the *Eco*R I site of 3rd exon of rabbit β-globin. ▨, 2nd and 3rd exon of rabbit β-globin. S, N, I, II and III, signal peptide, domains N, I, II and III of CEA and NCA, respectively. ■, M-domain. Arrows indicate transcriptional directions. In (B), extracts of CEA 1 (lane 1), NCA 2-4 (lane 2) and C5 transfected with pdKCR-dhfr (lane 3) were immunoblotted as described in MATERIALS AND METHODS.

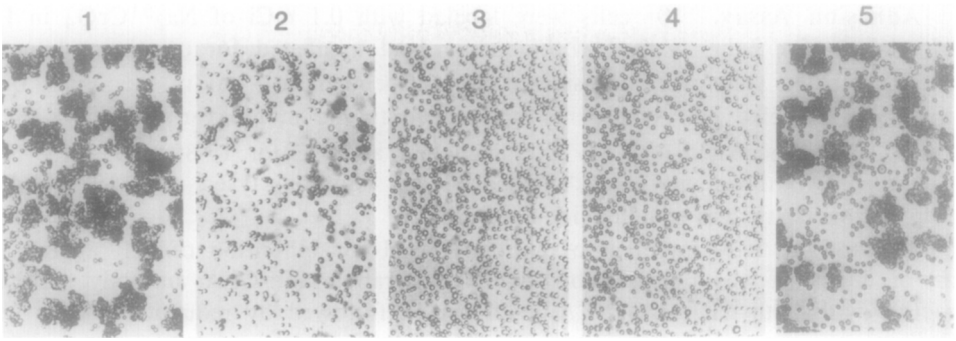

Fig. 2: Aggregation of CHO transformants expressing CEA and NCA. 1, CEA 1; 2, NCA 2-4; 3, C5 cells. 4 and 5, CEA 1 cells in the presence of Fab' fragment of anti-CEA (200μg/ml) and non-immune IgG (500μg/ml), respectively. See MATERIALS AND METHODS for experimental detail.

(Fig. 1B, lane 2). The CEA 1 and NCA 2-4 used for further analyses were shown to contain 1,120 ng and 2,560 ng of the respective antigens/mg of cellular proteins, respectively, by radioimmunoassay (22), the majorities of the antigens being on the cell surfaces as shown by immunofluorescent staining (not shown). Control C5 cells carrying vector pdKCR-dhfr produced neither CEA- or NCA-cross-reacting antigens (Fig. 1B, lane 3).

Aggregation of CHO transformants expressing CEA and NCA. Fig. 2 depicts the results of the aggregation assay. Monolayer culture of the transformants were treated with trypsin and EDTA and dissociated into single cells. When the suspensions were gently rotated, CEA 1 tended to aggregate easily (Fig. 2-1), while NCA 2-4 have much reduced but significant tendency to aggregate (Fig. 2-2). No aggregation was observed in control C5 cells which expressed neither CEA or NCA (Fig. 2-3). The self aggregation of clone CEA 1 was completely inhibited by anti-CEA Fab' (Fig. 2-4) but not inhibited by non-immune IgG Fab' (Fig. 2-5), indicating that the aggregation occurred by homophilic interaction between CEA on the cell surfaces. Similar results were reported by Stanner's group recently (16).

Homophilic and heterophilic adhesion of CEA- and NCA-expressing transformants. In order to be more quantitative and also to be able to study the heterophilic interactions in addition to the homophilic ones, we performed the adhesion assay in which suspension of one cells labeled with ^{51}Cr was added to monolayers of another cells to allow adhesion. Radioactivities remaining after the wash were counted to quantitate the degree of adhesion between the two cells.

As shown in Fig. 3, when labeled NCA 2-4 cells were added to the CEA 1 monolayer or in a reciprocal experiment, labeled CEA 1 cells were added to NCA 2-4 monolayer, about 35% and 40% of radioactive cells were bound, respectively. The difference in bound radioactivity is probably due to the difference in the

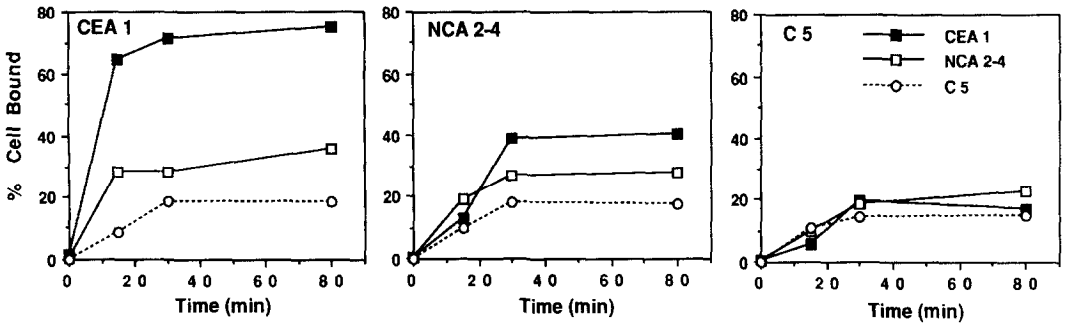

Fig. 3: Homophilic and heterophilic cell adhesion between CEA and NCA expressing transformants. Suspensions of ^{51}Cr-labelled CEA 1, NCA 2-4 or C5 cells were added to monolayers of cells indicated in the upper left corner of the figures. The values are the average of two separate determinations.

degree of aggregation of the radioactive cells, which mediates the indirect binding of the cells to the monolayers. Whereas, less than 20% of ^{51}Cr labelled control C5 cells were bound to monolayers of CEA 1 or NCA 2-4 cells. The reciprocal experiment showed similar results. Apparently, CEA-1 and NCA 2-4 cells adhered to each other by CEA-NCA heterophilic interaction.

When labeled CEA 1 cells were added to the CEA 1 monolayer cells, 75% cells were bound, while 30% of labelled NCA 2-4 cells were bound to NCA 2-4 cell monolayer.

As shown in Fig. 4, anti-CEA Fab' but not control Fab' completely inhibited the binding of the labeled CEA 1 cells to the monolayers of CEA 1 and NCA 2-4 cells, to the level of binding to the monolayer of control cells, indicating that CEA-CEA and CEA-NCA but not other interactions are responsible for the binding.

In summary, the extent of adhesion is that, CEA-CEA interaction is the highest, followed by CEA-NCA, and then by NCA-NCA interaction. This result coincided well with that of aggregation assay, that is, CEA expressing cells aggregated easily, whereas NCA expressing cells aggregated to much reduced extent (Fig. 2).

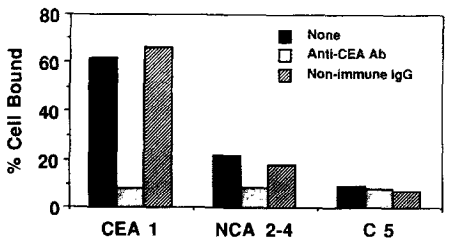

Fig. 4: Inhibition of homophilic and heterophilic cell adhesion by anti-CEA Fab' fragment. Anti-CEA and non-immune Fab' fragments (400 μg/ml) were added to monolayer cultures of cells indicated under the columns for 30 min before addition of ^{51}Cr-labeled CEA 1 cells. The values are the average of two separate determinations.

DISCUSSION

The present study indicated, for the first time, that not only CEA but also NCA can function as Ca^{++} independent homophilic cell adhesion molecule. Also shown was the heterophilic interaction of NCA and CEA.

The possible occurrence of homophilic interaction of CEA and NCA had been already suggested by the biochemical findings that they tended to form homodimers (23) before the recent elucidation of the primary structures of the antigens (2-4), and the finding that they belonged to Ig superfamily whose members are known to participate in various intercellular recognition processes (24). Although the region(s) which participates in the interaction is not at all clear at this time, it is tempting to assume that they may interact with each other in a manner similar to that of domains in Ig molecules. In this mode, interacting chains would have the same N-to-C orientation. In this respect it seems that, N-domains put aside, subdomains A and B are involved in the interaction, for CEA which has 4 extra subdomains (2, 3) tended to interact more intensely than NCA. However, it is also possible that when expressed on cell surface, longer CEA is less susceptible than NCA to steric hindrance and freer to interact to each other. It is known that the extent of expression of the adhesion molecules on the cell surface greatly affects the rate of adhesion (25). However, the amounts of the expressed antigens are excluded as the possible cause of the difference in the extent of adhesion in the present studies for we have used cells which produce enough amounts of the antigens. Furthermore, the amount of NCA produced are about twice that of CEA.

The weak but significant heterophilic interaction between CEA and NCA are in contrast with the case of the cadherins, for there is no binding between the cadherin subclasses (26). Generally, CAMs are homophilic (12). In view of the fact that NCA and CEA are coexpressed in colon carcinoma and colonic mucosa (22, 27), it is possible that CEA-NCA interaction takes place in vivo and plays some important biological roles along with CEA-CEA and NCA-NCA interactions.

It is also interesting to see whether there is any interaction between CEA and PSβG subfamily members, most of the former being anchored on cell surfaces and the latter being apparently not anchored but secreted out of the cells. It should be noted here that, some of PSβGs (10, 28, 29) are shown to have, in N-domain, the RGD sequence which is implicated in recognition between cell surface receptors and extracellular proteins (30). Apart from being Ig superfamily members, it suggests that at least some of the PSβGs may be involved in heterophilic and/or homophilic bindings *in vivo*. In this respect it is interesting to note that proteins containing RGD sequences are usually located in substrata such as exracellular matrices (30) and that in contrast with the CEA subfamily members, PSβGs are apparently secretory proteins and not retained on the cell surface (9-11).

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