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Lack of Correlation between the Gap Junctional Communication Capacity of Human Colon Cancer Cell Lines and Expression of the DCC Gene, a Homologue of a Cell Adhesion Molecule (N-CAM)

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In many human colorectal cancers, the DCC gene encoding for a homologue of the neural cell adhesion molecule (N-CAM) is found to be deleted. Previous work suggested that gap junctional intercellular communication (GJIC) might play an important role in carcinogenesis and could be regulated by the expression of cell adhesion molecules such as E-cadherin in some epithelial cell systems. In order to examine whether the deletion of the putative cell adhesion molecule DCC is related to the level of GJIC, which might, in turn, be important in human colorectal cancers, we compared levels of expression of the DCC gene with the GJIC capacity of a panel of human colorectal adenocarcinoma cell lines isolated from different stages of tumor progression. While the level of GJIC varied between the cell lines studied, we found no correlation between their communication capacity and DCC expression revealed by a reverse-transcriptase/polymerase chain reaction method. This lack of correlation suggests that DCC is not a crucial regulator of GJIC.

Key words: Gap junctional intercellular communication — Connexin expression — Colon cancer — DCC gene expression — Cell-cell recognition

Several studies have shown defective gap junctional intercellular communication (GJIC) during *in vitro* cell transformation or *in vivo* carcinogenesis.[1,2] The defects observed include: (i) inhibition of GJIC by various kinds of tumor-promoting stimuli, (ii) a progressive decrease of GJIC with increasingly transformed phenotype and (iii) a selective lack of GJIC between normal and transformed cells. Although GJIC can be regulated at various levels, little is known about how this is modulated during carcinogenesis. Recent investigations have suggested that GJIC may be regulated through the cell-cell recognition process. It has been shown that calcium-dependent regulation of gap junction synthesis and control of GJIC in mouse epidermal cells is regulated by the cell-adhesion molecule (CAM) E-cadherin, which itself is calcium-dependent.[3] Moreover, transfection of the E-cadherin (or L-CAM) gene induces GJIC in mouse sarcoma cells and such transfectants are less tumorigenic.[4] In humans, aberrant E-cadherin expression is often associated with late stages of cancers.[5–7] Several metastatic human carcinomas show lower expression of E-cadherin, while *in vitro* transfection experiments suggest that E-cadherin may act as a suppressor of invasion.[8]

In many human colorectal cancers, the DCC gene has been shown to be deleted.[9] This gene has homology with one of the well known cell adhesion molecules, N-CAM,[10] and it has been suggested that DCC gene deletion is associated with the progression stage of colon carcinogenesis.[11] Loss of expression of the DCC gene in ductal pancreatic adenocarcinomas has also been described,[12] but the function of this gene and its precise role in human carcinogenesis are not known. However, it has been shown that the introduction of antisense RNA to the DCC gene transformed rat-1 cells,[13] suggesting that the DCC gene is a tumor-suppressor gene. Since many tumor cells show decreased homologous and/or heterologous GJIC and since cell adhesion molecules, such as E-cadherin, appear to be important for the establishment of GJIC, it was of interest to assess the relationship between DCC expression and GJIC in a series of human cell lines established from colon carcinomas of different grades (from differentiated carcinomas to metastatic cell lines). We examined the ability of these cells to grow in soft agar and characterized their GJIC functional ability as well as RNA and protein expression levels of connexin. When these results were compared with DCC gene expression, no clear correlation was observed, suggesting that the DCC gene may not be directly involved in GJIC regulation.

MATERIALS AND METHODS

Cell culture The human colorectal adenocarcinoma cell lines used in this study were purchased from the American Type Culture Collection and chosen according to the progression grade of the tumor from which they were

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derived (from grade II to grade IV and metastases). In total, seven cell lines were studied: Caco-2 (ATCC no. HTB 37), SW 1116 (ATCC no. CCL 233), SW 403 (ATCC no. CCL 230), SW 1417 (ATCC no. CL 238), SW 480 (ATCC no. CCL 228), SW 620 (ATCC no. CCL 227) and LS 180 (ATCC no. CL 187). All the cell lines were cultivated in Dulbecco's minimum essential medium (GIBCO, Paisley, Scotland), supplemented with L-glutamine, penicillin-streptomycin (GIBCO) and 10% fetal calf serum (Orgenics Ptd., Yavne, Israel). Cell lines cultured in a 37°C incubator, under a humidified 5% CO_2 atmosphere, were routinely sub-cultured by trypsinization once a week and their medium changed twice weekly.

Anchorage-dependent and -independent growth of cells To measure the anchorage-dependent growth curve, cells (5×10^4) of each cell line were seeded in 60 mm dishes and cell numbers counted every day. For the estimation of growth capacity in soft agar, 5×10^4 cells from each line were seeded in 4 ml of complete MEM containing 0.3% agar on a solidified (0.5% agar) basal layer (5 ml) in 100 mm dishes.[14]

Estimation of gap junctional intercellular communication by dye-transfer assay A 5% (w/v) solution of Lucifer Yellow CH (Sigma, St Louis, MO) in 0.33 *M* lithium chloride was transferred to a glass needle prepared from a capillary tube (A.M. Systems Inc., Everett, WA). Cells were impaled with needles and dye injected continuously for 0.8 s under air pressure (400–600 hPa), using an Eppendorf microinjector 5242 (Hamburg, Germany) and after 10 min, the intercellular transfer of fluorescent Lucifer Yellow CH was estimated under an Olympus IMT-2 phase-contrast and fluorescence microscope.[15]

Northern blot analysis of connexin gene transcripts Total RNA of each human colorectal adenocarcinoma cell line was extracted from confluent cultures (100 mm dishes), according to the single-step technique described as the RNAzol™-B method (Cinnax/Biotecx Lab. International, Inc., Friendswood, TX). Separation of the RNAs (20 μg/well) was performed by electrophoresis (30 V, overnight) in denaturing formaldehyde agarose gels (1%). Gels were then capillary-blotted onto Hybond-N^+ nylon membranes (Amersham, Buckinghamshire, UK). [γ-^{32}P]dCTP-radiolabeled cDNA probes for connexin 26,[16] connexin 32,[17] connexin 43[18] and the DCC gene[10] were prepared by using multiprime DNA labeling systems (Amersham). Blots were pretreated with 50% formamide, 0.1 *M* Na_2HPO_4 (pH 7.4), 1% sodium dodecyl sulfate (SDS) and 10 μg/ml herring sperm DNA for at least 2 h at 42°C. Hybridizations were carried out for 24 h under high-stringency conditions (formamide 50%, 42°C). Blots were then washed twice in 2×standard saline citrate and 1% SDS at 65°C for 30 min before exposure at −70°C to Hyperfilm-MP (Amersham) with an intensifying screen.

Detection of DCC transcript by the reverse polymerase chain reaction Total RNA (8 μg) from each cell line was reverse-transcribed (1 h at 42°C) into the first-strand cDNA with DCC antisense primer (600 ng): 5′-AGCCTCATTTTCAGCCACACA-3′ (synthesized by Genosys, Cambridge, UK), using 200 units of Moloney murine leukemia virus (M-MuLV) reverse transcriptase (Boehringer Mannheim GmbH). The primers used in this study originate from two adjacent exons (Fragment O, P) as described.[10] The PCR amplification was carried out by adding 600 ng of sense primer 5′-TTCCGCCATGGTTTTTAAATCA-3′ to 10 μl of the cDNA aliquots. Optimal PCR conditions included denaturation for 1 min at 95°C, annealing for 1 min at 60°C, and extension for 2 min at 72°C using 4 units of *Taq* polymerase (Promega); amplification was carried out for 35 cycles.[12] The reaction mixture (10 μl) was analyzed after separation on 3% agarose gels by Southern analysis with a 1.65 kb DCC cDNA containing nucleotides 591 to 2250 of the DCC

Table I. Characterization of the Phenotype of the Human Colon Carcinoma Cell Lines Used in this Study

Cell line	Tumor type[a] (grade)	Growth in soft agar (% of cells forming colonies)	GJIC[c] (±standard deviation)	Cx 32[d] mRNA level
Caco-2	II	0.02	7.2±2.1	+
SW 1116	II	0.01	1.7±0.3	±
SW 403	III	0.1	5.7±0.6	+
SW 1417	III (metasasized)	7.8	2.4±0.4	+
SW 480	III, IV	22.4	5.9±1.1	++
SW 620	metastasis	56.9	1.6±0.6	++
LS 180	II-III[b]	28.1	2.3±0.3	−

a) Information supplied by ATCC.
b) Deduced from ref. 19.
c) GJIC (gap junctional intercellular communication), number of communicating cells.
d) Relative expression of connexin 32 gene summarized from Fig. 2.

sequences as already described.[10] In order to exclude the possibility that positive signals could be the consequence of contamination with any DNA recognized by the DCC primers, we performed a similar analysis, as described above, of our RNA samples before reverse transcription.

RESULTS AND DISCUSSION

Characterization of the transformed phenotypes We tested the ability of seven human colorectal cancer cell lines derived from tumors of different cancer progression stages (Table I), to grow in an anchorage-independent manner. Generally, a good correlation between their anchorage-independent growth capacity and the grades of the original tumors was observed (Table I), except for the LS 180 cell line which was derived from a low-grade tumor (grade II-III).

Gap junctional communication capacity of human colon adenocarcinoma cell lines The seven colorectal adenocarcinoma cell lines showed variable degrees of gap

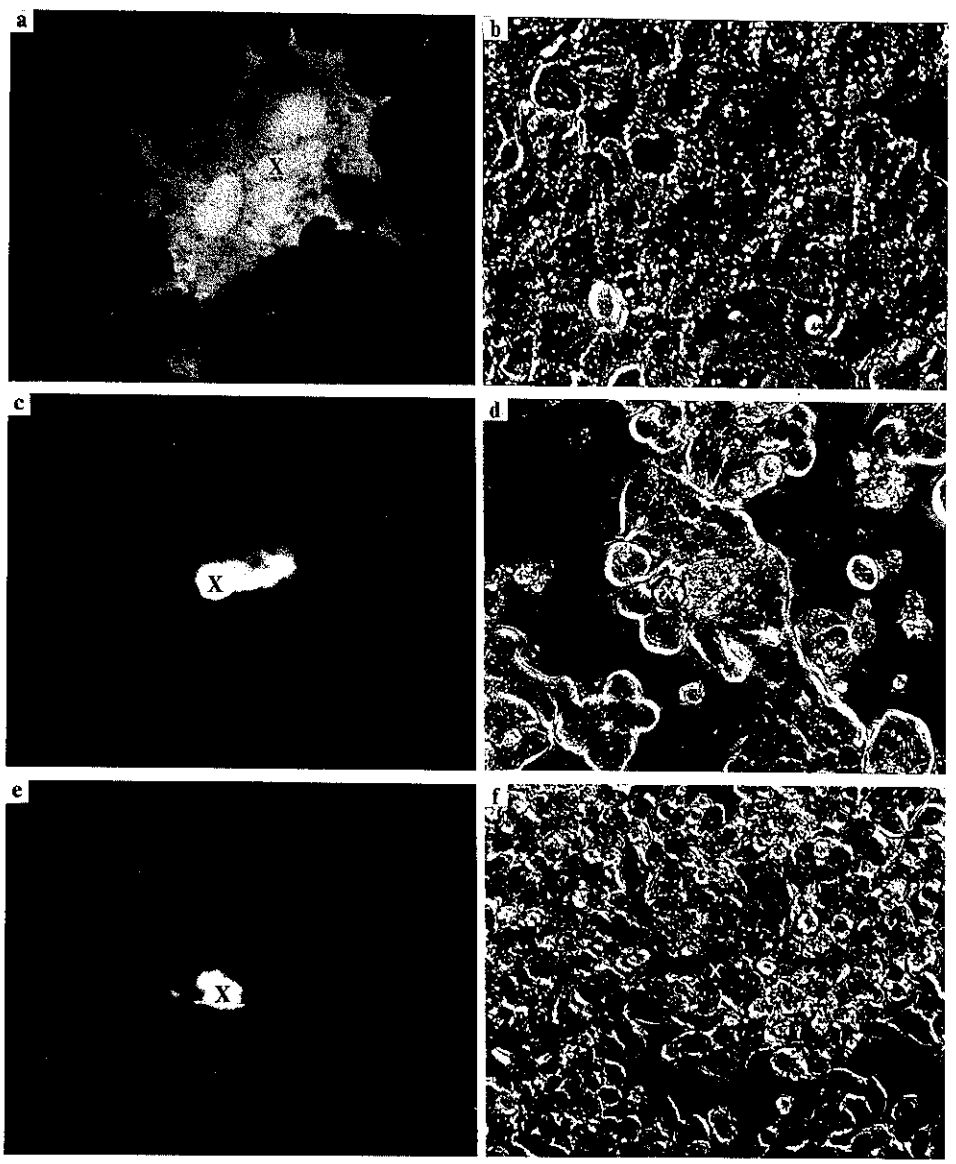

Fig. 1. Gap junctional intercellular communication capacity tested by the dye-transfer assay of human colon carcinoma cell lines: a, b: Caco-2; c, d: LS 180; e, f: SW 620. Cells with x are those injected with Lucifer Yellow. a, c, e: fluorescence micrographs showing the extent of communication; b, d, f: corresponding phase-contrast micrographs showing the morphology of various cell lines.

junctional communication capacity that could be divided into two groups, one in which the microinjected cells communicate with all their immediate neighboring cells (Caco-2, SW 403, SW 480), and a non-communicating group in which the microinjected cells only communicate with one or two of their neighbors (LS 180, SW 620, SW 1116, SW 1417). Some typical examples are shown in Fig. 1.

No clear correlation was observed between the degree of growth in soft agar and the communication capacity of the cell lines. These results contrast with those obtained from previous studies with other tumor cells, in which the acquisition of anchorage-independent growth capacity was associated with progressive loss of GJIC.[20] It is possible that the tumor stages of the cell lines used in this study are later than those used previously, so that the level of GJIC is lower in all cell lines, making the comparison of degrees of tumorigenicity and GJIC difficult. Furthermore, previous studies in which a correlation was found between tumorigenicity (and/or expression of transformed phenotypes) and lower gap junctional intercellular communication capacity mostly used experimental systems,[20, 21] in which defined carcinogens were used to induce tumors or transformation in homogeneous populations of animals or cells. In human carcinogenesis, including that of colon, various carcinogens are involved and the subjects are genetically heterogeneous. These factors may influence the biology of human cancers and result in expression of more heterogeneous phenotypes.

Northern analysis of the major connexin mRNA The amount of transcripts encoding for the major connexins (Cx 26, Cx 32 and Cx 43) in confluent cultures was analyzed. The only detectable connexin transcript in some cell lines was the Cx 32 transcript (Fig. 2).

The amount of Cx 32 transcript differed among the cell lines. The highest amount was found in the SW 480 and SW 620 cells which are communicative and non-communicative cell lines, respectively. A similar lack of correlation between the amount of Cx 32 transcript and the communication capacity of the cell lines was also observed for the communicating Caco-2 and non-communicating SW 403 cells, in which the Cx 32 transcript is hardly detectable. The results are more consistent for the non-communicating cell lines such as SW 1116, SW 1417 and LS 180, in which the Cx 32 transcript was hardly detectable.

This apparent lack of correlation with the level of transcription probably reflects different post-transcriptional regulation of the Cx 32. An immunohistochemical analysis indicated that all the cell lines express very low levels of Cx 32 protein (data not shown). This lack of detection of Cx 32 protein is consistent with the low communication capacity (less than 10 communicating cells/injection) of all the cell lines; in our experience, most immunopositive cell lines communicate much more.[22]

Analysis of expression of the DCC gene The amount of the DCC transcript was too low to be detected by classical Northern analysis and such a study requires amplification by PCR of the cDNA synthesized from the transcript by a reverse transcriptase reaction.[10, 12] Such analysis of 8 μg of total RNA showed that only the Caco-2 cells expressed the DCC gene at a detectable level (Fig. 3).

There is, however, no correlation between the DCC gene expression and the degree of the gap junctional communication capacity of colon carcinoma cell lines tested in this study. We detected a DCC transcript only in Caco-2 but not in SW 480 cells. Since these two cell

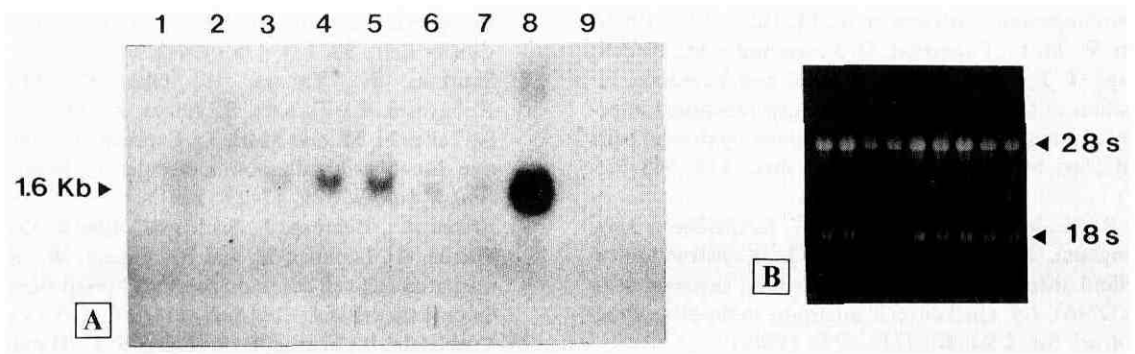

Fig. 2. Detection of connexin 32 (Cx 32) mRNA in the human colon carcinoma cell lines by Northern analysis. Lane 1, LS 180; lane 2, SW 1116; lane 3, SW 403; lane 4, SW 480; lane 5, SW 620; lane 6, Caco-2; lane 7, SW 1417. Human liver total RNA (lane 8) has been used as a positive control for Cx 32 mRNA detection (at 1.6 kb) and human heart total RNA as a negative control (lane 9) (A). The quality and the comparative quantity of total RNA of the different samples loaded for Northern analysis has been estimated by ethidium bromide staining of ribosomal RNA bands (28S and 18S) on the gel (B).

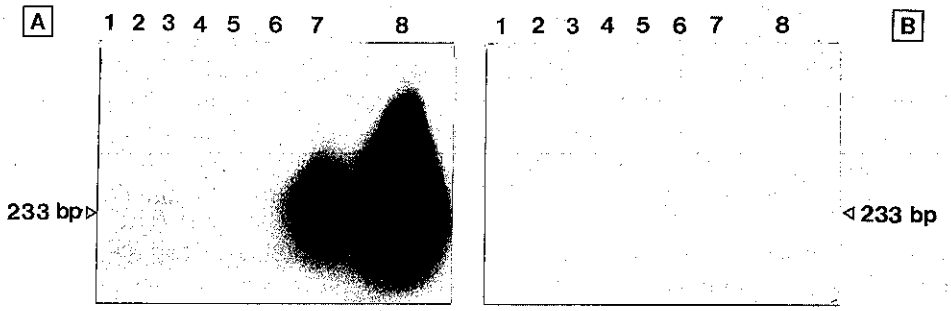

Fig. 3. DCC gene expression estimated by reverse transcriptase-PCR. Detection of the amplified DCC fragment (233 bp) by Southern analysis with (A) or without (B) reverse transcriptase reaction of total RNA from various human colon cell lines (see "Materials and Methods"). Lane 1, SW 403; lane 2, SW 480; lane 3, SW 620; lane 4, SW 1116; lane 5, SW 1417; lane 6, LS 180; lane 7, Caco-2. Total RNA from human frontal brain was used as a positive control for DCC gene expression (lane 8).

lines have similar communication capacity, and since SW 403 cells communicate without a detectable level of DCC gene expression, it appears that DCC is not involved in the regulation of the gap junctional communication of human colon cells. In conclusion, our study suggests that although loss of DCC gene expression and of gap junctional intercellular communication is common in human colon cancers, they occur independently.

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