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Ulcerative Colitis and Familial Polyposis Oncologic Transformation to Colon Carcinoma: Changes in Carcinoembryonic Antigen Release

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Abstract—*During cell proliferation, several "factors" are released into the microenvironment, or culture medium. The experiments described sought and examined agents that may cause or support malignant cell transformation. The response of colon cells from patients with ulcerative colitis (UC), familial polyposis coli (FPC) and colon carcinoma (CCC) to these agents was monitored by carcinoembryonic antigens (CEA) released into the medium during cell proliferation in a serum-free hormone-defined (SFHDM) medium, oncogenicity in athymic mice and colonigenicity, i.e. the ability of the cells to form colonies in soft agar. When cultured on the extracellular matrix (EM), i.e. footprints from colon carcinoma cells (short term or established cell lines), and in SFDHM, colon cells from patients with UC and FPC showed significant* ($P = 0.001$) *increases in all the three parameters.*

Analyses indicated that EM from cultures of [^{35}S]*methionine-labelled normal epithelial colon cells (NCE) differed from those left by UCC, FPC and CCC cell cultures. EM from NCE cell cultures did not contain* [^{35}S]*methionine-labelled glycoproteins resistant to collagenase action which were not fragments of fibronectin, and which were present in EM from CCC cells. It is concluded that the extracellular matrix from malignnt colon cells contains agents that support colon cell oncogenic transformation.*

INTRODUCTION

Ulcerative colitis (UC), familial polyposis coli (FPC) and colon carcinoma (CCC) are pathological states of the colonic mucosa with strong malignant potential. The described experiments examined responses of cells from colons of patients with such conditions to "factors" released during cell proliferation that may cause or support malignant transformation of human colon cells.

The adenomatous mucosa in FPC invariably shows malignant changes [1, 2]. Ulcerative colitis has a lower potential for such transformation, but the risk increases in direct proportion to the duration of the disease [3, 4]. This progressive malignant transformation is indicative of release by neighboring cells of agent(s) that may cause or support malignant transformation.

Recent studies have indicated that better retention of differentiated cellular expression can be achieved with culture conditions that include the use of serum-free hormonally defined media (SFHDM) [5–7] and the use of substrata enriched in extracellular matrix [7, 8]. Under certain defined *in vitro* conditions, murine calvaeium cells spontaneously transform into transplantable murine carcinoma cells [9].

Many tumors, when grown in cell culture, release polypeptide growth factors into their conditioned media, and these same tumor cells also possess receptors for the released peptides. Three parameters, production and release of carcinembryonic antigens (CEA), oncogenicity [i.e. ability to produce tumors in athymic nude (Nu/Nu) mice] and cell ability to form colonies in soft agar cultures have been used to monitor the effects of agents present in the extracellular matrix, cell footprints of colon cells.

MATERIALS AND METHODS

The objective of the described study was to examine the extracellular matrix (EM) left behind in *in vitro* colon cell proliferation for agent(s) that may cause or support human colon cells to transform to the malignant state. In parallel with primary cell cultures, normal and malignant established colon cell lines provided the extracellular matrix. Colon

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cells from patients with UC, FPC and CCC were used as primary cultures to establish the changes that occur during culture on the extracellular matrix. Malignant cell transformation was monitored by production and release of carcinoembryonic antigen(s) (CEA), oncogenicity to athymic mice and cellular colonigenicity in soft agar.

1. *Patients*

Forty subjects were studied: 10 patients each with UC, FPC and CCC, and 10 normal colon mucosa of surgical specimens obtained at surgical resection for trauma to the colon. Blood samples for plasma CEA levels were drawn prior to biopsies.

2. *Culture media*

Two types of media were used: (A) serum-containing medium consisting of RPMI-1640 containing 10% heat inactivated AB human serum and 10% fetal calf serum (FCS), 1.2 μg/ml sodium bicarbonate, 15 mM hepes buffer, 100 U/ml penicillin, 10 μg/ml streptomycin and 23 μg/ml ampicillin. The medium was used to maintain the short term and the established cell line. (B) The serum-free hormone-defined (SFHDM) medium consisting of 1 : 1 mixtures of Ham's F-12 and RPMI-1640 including 1.2 μg/ml sodium bicarbonate, 15 mM hepes buffer, 5 units/ml insulin, 10 units of epithelial growth factor (EGF), 5 μg/ml hydrocortisone, 25 μg/ml gentamycin, 100 U/ml penicillin and 100 μg/ml streptomycin. This medium was used to culture the cells for CEA determination and colony formation in soft agar.

3. *Cell lines*

A. Established cell lines used included: CCL-239, a normal colonic epithelial [10]; HT-29, a malignant colon epithelial [11]; HCT-BR, a human adenocarcinoma [12]; CaCol, a colon carcinoma [13] and HC-15 a human adenocarcinoma [14] cell lines. All these cell lines were obtained from American Type Cell Cultures, and maintained according to the directions supplied with each cell line. B. The short-term colon cell lines were developed from biopsies as summarized in Fig. 1. These colon cell cultures were carried on as described by Danes [15, 16].

4. *Parameters used to monitor cell transformation*

A. *Carcinoembryonic antigen (CEA) production.* Plasma CEA levels and the CEA released during cell proliferation into the spent media were determined by a commercial immuno-radiometric assay (Abbot CEA-RIA Diagnostic Kit). Each determination was carried out in triplicate and the results are reported as mean ± standard deviation as ng/ml of plasma, or ng/10^6 cells/24 hr.

B. *Oncogenicity.* Aliquots of 10^7 cells were inoculated s.c. in 0.2 ml Hanks balanced salt solution (HBSS) in the supraclavicular region of 20 4–5 week-old female CD-1 Nu/Nu athymic mice. Within 2 weeks, progressively growing tumors were observed in recipients of 10^7 cells of the colon carcinoma. Subsequent histological examination confirmed that the xenografts were morphologically similar to the original tumor cell samples obtained from the patient (data not shown). The tumors have shown sustained growth in the s.c. site. The animals were observed for mortality and tumor appearance for 120 days. Each determination was carried out in duplicate. The results are reported as the ratio of number of animals which developed tumors to total number of inoculated animals.

C. *Colonigenicity.* The ability of the cells to form colonies when grown in soft agar occurred as described by Tucker *et al.* [17, 18]. Briefly, the colon cells were plated at 1500 viable cells/35 mm plates coated with 0.3% agar medium containing the SFHDM. Agar bases (0.5%) were overlayed with 1 or 2 ml of the agar assay medium. Cultures were fed 1 week later with the same medium. The number of colonies that developed after 7 days was quantified using a Quantinet image analyzer (Cambridge Instruments, Morsey, N.Y.). Colonies greater than 60 μm dia. (approx. 50 cells) were scored as positive. The results are reported as the mean ± standard deviation of three separate experiments as the number of colonies per cm^2.

5. *The chemical nature of compounds present in the extracellular matrix*

NCE and CCC cells were cultured in 75 cm^2 Falcon flasks in SFHDM. After reaching confluency in humidified atmosphere of 5% CO_2 in air and at 37°C, the cells were harvested, washed and labelled with [^{35}S]methionine (Fig. 1). After removing the medium and the cells, the proteoglycans in the foot-prints were solubilized using guanidine–HCl. Sequentially were added 8% Zwittergens for 30 min, 8 M guanidine–HCl in 50 mM sodium acetate, at pH 6.0 for 30 min [19, 20] containing the protease inhibitors 20 mM sodium EDTA, 5 mM benzamidine–HCl, 0.1 M 6-aminohexanoic acid and 2 mM phenylmethyl sulfonyl fluoride. The extracts (4 ml) were filtered through a G-25 (PD-10) column, equilibrated and eluted with 4 M guanidine–HCl, 0.1 mM sodium sulfate of pH 7.0 and containing 0.2% Triton X-100. The proteoglycan-enriched samples were resuspended in 0.1 mM Tris sulfate buffer, pH 7.0 and subjected to HPLC as described by Iozzo *et al.* [21]. The HPLC columns were calibrated using proteoglycans from bovine nasal cartilage as well as proteins

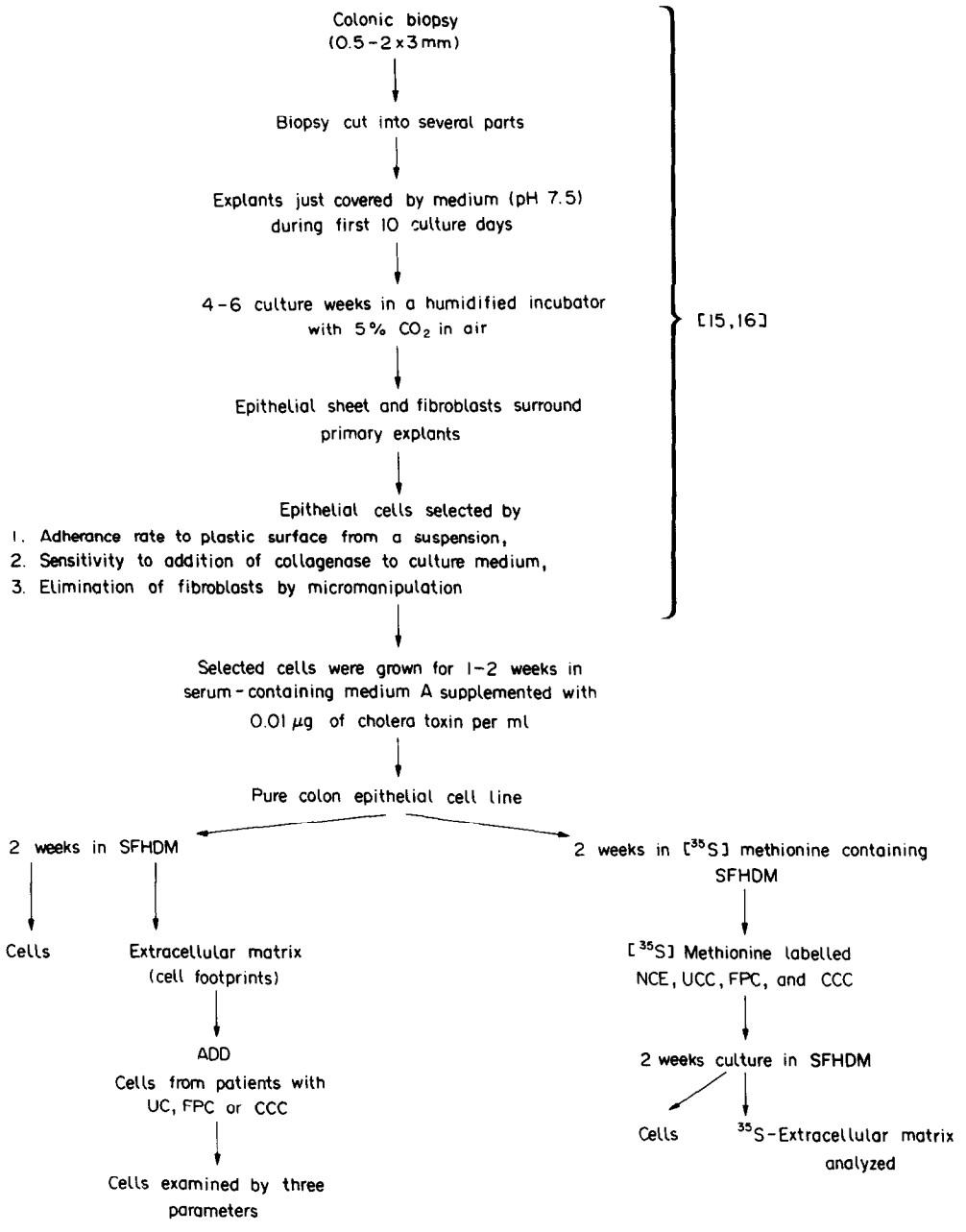

Fig. 1. Method used in the establishment of primary culture from colon muscosal biopsy specimen and preparation of extracellular matrix (EM).

of known molecular weight [22]. The radioeluted macromolecules are shown in Fig. 2.

RESULTS

Characteristics of human colon epithelial cells

When grown on collagen matrix, the normal colon epithelial cells produced duct-like out-growths extending into the collagen gel matrix within 4 days after cultivation in the presence of cholera toxin in the serum-containing medium A. The toxin was included in the culture medium to inhibit the growth of fibroblastic cells [23] and to stimulate epithelial cell proliferation [24]. Electron microscopy of the growing cells (NCE or CCC) showed polarized cells containing many desmosomes, microvilli and cytoplasmic blebs with tight junctions. When plated at high density (1×10^6 cells/cm^2) in a conventional monolayer culture in the serum-containing medium A, the cells showed dome formation [25]. These are well-established characteristics of epithelial [26], and confirm that the colon cells examined in the present investigation are epithelial cells.

Effect of extraellular matrix, i.e. footprints, on carcinoembryonic (CEA) levels released by colon cells

The CEA levels released by colon cells varied from one established cell line to another. The CEA

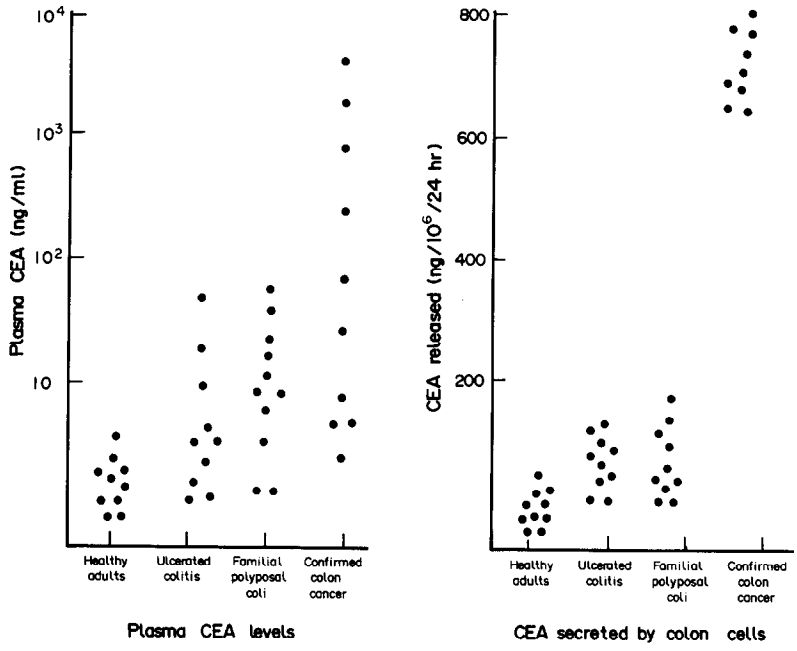

Fig. 2. Correlation between carcinoembryonic antigen (CEA) in plasma and secreted into spent media by human colon cells. Left: Plasma CEA levels of 10 healthy adults and from 10 patients with ulcerative colitis, familial polyposis coli and from 10 patients with confirmed colon cancers. Each reading represents the mean of triplicate assays. Right: CEA secreted into the spent media by cells from colon of healthy adults and from patients with ulcerative colitis, familial polyposis coli and confirmed colon cancer. The cells were cultured in the serum-free hormone-defined medium on footprints of colon carcinoma cells. The cells were grown for 2 months, i.e. three passages, and the media were harvested at confluency on the fourth passage (splitting). Each assay was carried out in triplicate.

Table 1. Release of carcinoembryonic antigens by established colon cell lines

Cell lines	Period of culture (weeks)	Plastic	Substratum* footprints from NCE	Substratum* footprints from CCC
			(ng/10^6 cells/24 hr)	
A. Serum supplemented medium				
CCL-239	12	0.6 ± 0.16	0.8 ± 0.18	2.86 ± 0.43
HT-29	13	11.9 ± 0.95	22.6 ± 1.25	44.75 ± 1.92
HCT-BR	11	15.8 ± 0.77	32.5 ± 2.91	147.6 ± 8.36
HCT-15	10	12.7 ± 0.85	23.7 ± 1.65	43.5 ± 2.15
CaCol	12	14.3 ± 0.97	28.3 ± 1.79	56.7 ± 2.35
B. Serum-free hormone-defined medium				
CCL-239	19	0.8 ± 0.11	1.39 ± 0.12	19.6 ± 0.62
HT-29	18	59.7 ± 2.24	127.7 ± 5.71	825.3 ± 46.3
HCT-BR	15	65.3 ± 4.31	142.5 ± 5.85	736.8 ± 42.8
HCT-15	19	64.7 ± 3.98	135.6 ± 5.38	698.3 ± 39.9
CaCol	21	69.4 ± 5.32	142.8 ± 6.11	918.6 ± 46.2

*The period in weeks of culture of the established cell lines in the standard or SFHDM media prior to assay is shown above. The substratum NCE or CCC footprints were prepared fresh from short term cultures of NCE or CCC.

The above data represent the mean ± standard deviation of three separate assays, each was carried out in duplicate. Differences of 7.6% are statistically significant to the extent of $P < 0.005$.

levels released were highest when the cells were cultured on footprints from CCC-cells, and lowest in cells cultured on plastic substratum (Table 1). The CEA levels released were significantly higher in cells grown in SFHDM than in cells grown in serum-containing medium A.

Short term colon cell lines from patients with UC and FPC released higher CEA levels than the levels released by NCE cells, but significantly lower than the CEA levels released by CCC cells (Table 3), or by malignant colon cell lines. Extracellular matrix from CCC cells significantly increased CEA levels

Table 2. Characteristics of established colon cell lines

Cell lines	Number of passages	Oncogenicity number of animals with tumor to total number of animals inoculated	Colonigenicity number of colonies in agar/cm^2
A. Cells grown on collagen			
CCL-239	12	0/20	Nil
HT-29	11	9 ± 1/20	145 ± 8
HCT-BR	13	11 ± 2/20	273 ± 16
HCT-15	12	10 ± 1/20	178 ± 11
CaCol	14	10 ± 1/20	245 ± 14
B. Cells grown on NCE-footprints			
CCL-239	14	0/20	Nil
HT-29	13	3 ± 1/20	132 ± 7
HCT-BR	12	6 ± 1/20	297 ± 19
HCT-15	14	5 ± 1/20	209 ± 12
CaCol	16	5 ± 1/20	258 ± 16
C. Cells grown on CCC-footprints			
CCL-239	12	1 ± 1/20	14 ± 1
HT-29	12	18 ± 2/20	987 ± 45
HCT-BR	10	18 ± 2/20	1768 ± 89
HCT-15	15	16 ± 2/20	1028 ± 51
CaCol	16	17 ± 3/20	1329 ± 56

The above data represent the mean ± standard deviation of three separate assays, each was carried out in duplicate. Differences of 11.5% are statistically significant to the extent of $P < 0.001$.

released by colon cells from patients with UC or FPC (Table 4).

Effect of extracellular matrix on colon cell oncogenicity in athymic mice

The ability to produce tumors in athymic mice varied from one cell line to another (Table 2), and significantly increased if the colon cells were grown on extracellular matrix from CCC cells.

Effect of extracellular matrix on colon cell colonigenicity

Epithelial cells from normal colons do not form colonies in soft agar (Table 2). The ability to form colonies in soft agar varied from one established cell line to another. Cellular colonigenicity in soft agar significantly increased when the cells were allowed to proliferate on CCC-footprints prior to transfer to soft agar. Colon cells from patients with UC and FPC have greater ability to form colonies in soft agar and to develop into tumors in athymic mice than normal colon (NCE) cells (Table 4). These abilities increase if the cells are grown on extracellular matrix from CCC cells.

Based on the three parameters, release of CEA, oncogenicity and colonigenicity, the results (Table 5) strongly indicate that the extracellular matrix from malignant colon cells can be induced into normal colon cells, and support malignant cell transformation in colon cells from patients with UC and FPC.

Carcinoembryonic antigens (CEA) in plasma of patients with colon diseases

The plasma from healthy adults had approx. 17 ng/ml, whereas three out of 10 patients with UC and seven out of 11 patients with FPC had CEA levels higher than 10 ng/ml. Plasma from five out of 10 patients with CCC had CEA levels higher than 100 ng/ml, two had CEA levels higher than 1000 ng/ml (Fig. 2). There was statistically significant ($P < 0.0001$) correlation between the CEA levels released by the colon cells, the plasma CEA levels and cellular status, strongly indicating that colon cells from patients with UC and FPC could be considered as precancerous.

Differences between the proteoglycans in the footprints from normal colon epithelial (NCE) and colon carcinoma (CCC) cells

[^{35}S]Methionine-labelled proteoglycans from footprints of NCE and CCC cells (Fig. 3) showed that NCE fraction A1 which consists of 25% aggregate and two polydisperse non-aggregating monomes with K_{av} 0.48 and 0.65, respectively. The foot-prints from CCC cells yielded a fraction which was only 12% aggregate with one labelled peak at K_{av} 0.1 [27].

If the labelled peaks were reduced and alkylated with 4 M guanidine–HCl, then chromatographed as described in Materials and Methods, NCE proteoglycans yielded pattern 3C and CCC

Table 3. Release of carcinoembryonic antigen by colon cell cultures in serum-free hormone-defined medium on normal colon epithelial footprints

Colon cell cultures	Period of culture (weeks)	Number of passages	CEA released (ng/10^6 cells/24 hr)
Colon carcinoma	10	4	160.8 ± 2.35
	15	6	164.7 ± 2.19
	27	13	150.8 ± 2.19
	33	16	162.5 ± 2.39
Ulcerative colitis	13	4	29.6 ± 0.57
	18	6	45.8 ± 0.89
	20	8	84.6 ± 1.63
	35	16	98.5 ± 2.72
Familial polyposis coli	8	4	61.7 ± 1.25
	15	5	90.3 ± 2.53
	25	10	148.7 ± 5.47
	38	18	165.8 ± 5.83
Normal colon epithelial	10	3	1.2 ± 0.02
	26	8	1.7 ± 0.02
	34	11	0.9 ± 0.01
	41	13	1.5 ± 0.01

The above data represent the mean ± standard deviation of triplicate per assay sample.
The above represent separate cell cultures from separate donors.

Table 4. Release of carcinoembryonic antigen by colon cell cultures in serum-free hormone defined medium on colon carcinoma footprints

Colon epithelial cells	Period of culture (weeks)	Number of passages	CEA released (ng/10^6 cells/24 hr)
Colon carcinoma	9	4	884.9 ± 25.9
	15	7	798.5 ± 20.3
	27	15	905.6 ± 35.2
	32	19	914.5 ± 36.8
Ulcerative colitis	12	4	35.7 ± 1.23
	17	7	69.8 ± 2.37
	21	10	120.9 ± 4.79
	36	20	195.6 ± 6.57
Familial polyposis coli	8	3	105.4 ± 3.23
	15	4	210.8 ± 7.34
	23	10	365.9 ± 8.25
	35	19	598.5 ± 9.17
Normal colon epithelial	13	4	5.4 ± 0.03
	22	8	14.9 ± 0.09
	28	10	48.6 ± 0.23
	38	13	75.4 ± 0.54

The above data represent the mean ± standard deviation of triplicate per assay sample.
The above represent separate cell cultures from separate donors.

proteoglycans yielded pattern 3D, indicating significant differences between the two proteoglycans.

DISCUSSION

Oncogenic transformation remains a serious problem in both ulcerative and familial polyposis coli. The increased incidence of colonic cancers in patients with UC, although lower than in FPC, indicates a possible relationship between the three colon diseases.

Carcinoembryonic antigens (CEA) are antigenic glycoprotein(s) produced from malignant neo-

Table 5. Malignant cell extracellular matrix supports oncologic cell transformation

Short term colon cell lines	Period of culture (weeks)	Source of extracellular matrix	CEA released	Oncogenicity	Colonigenicity
Colon carcinoma (CCC)	15	CCL-239	175.37 ± 2.20	11 ± 2/20	247 ± 12
		HT-29	917.3 ± 36.5	19 ± 1/20	1289 ± 47
		HCT-BR	998.4 ± 39.8	19 ± 1/20	1697 ± 39
		HCT-15	875.3 ± 27.8	18 ± 1/20	1139 ± 38
		CaCol	932.4 ± 37.6	19 ± 1/20	1543 ± 35
Ulcerative colitis (UC)	18	CCL-239	59.8 ± 1.35	1 ± 1/20	165 ± 9
		HT-29	195.6 ± 6.68	9 ± 2/20	785 ± 19
		HCT-BR	327.8 ± 10.6	10 ± 3/20	897 ± 23
		HCT-15	275.7 ± 8.73	9 ± 3/20	737 ± 17
		CaCol	415.8 ± 14.25	12 ± 3/20	798 ± 18
Familial polyposis coli (FPC)	15	CCL-239	94.4 ± 2.73	2 ± 1/20	227 ± 14
		HT-29	219.8 ± 7.52	10 ± 2/20	973 ± 23
		HCT-BR	432.7 ± 13.85	12 ± 2/20	1085 ± 32
		HCT-15	398.6 ± 11.6	10 ± 1/20	914 ± 37
		CaCol	485.7 ± 16.3	13 ± 3/20	985 ± 22
Normal colon epithelial cells (NCE)	10	CCL-239	0.95 ± 0.11	0	Nil
		HT-29	2.85 ± 0.45	0	Nil
		HCT-BR	8.75 ± 0.55	1 ± 1/20	29 ± 3
		HCT-15	5.96 ± 0.95	0	19 ± 2
		CaCol	7.15 ± 0.75	1 ± 1/20	37 ± 3

The above data represent the mean ± standard deviation of three separate assays, each was carried out in triplicate. CEA released is in ng/10^6 cells/24 hr. Oncogenicity is the ratio of number of animals developed tumors to the total number of animals treated. Colonigenicity is the number of colonies formed/cm^2 in soft agar. A difference of 9.6% is statistically significant to the extent of $P < 0.005$.

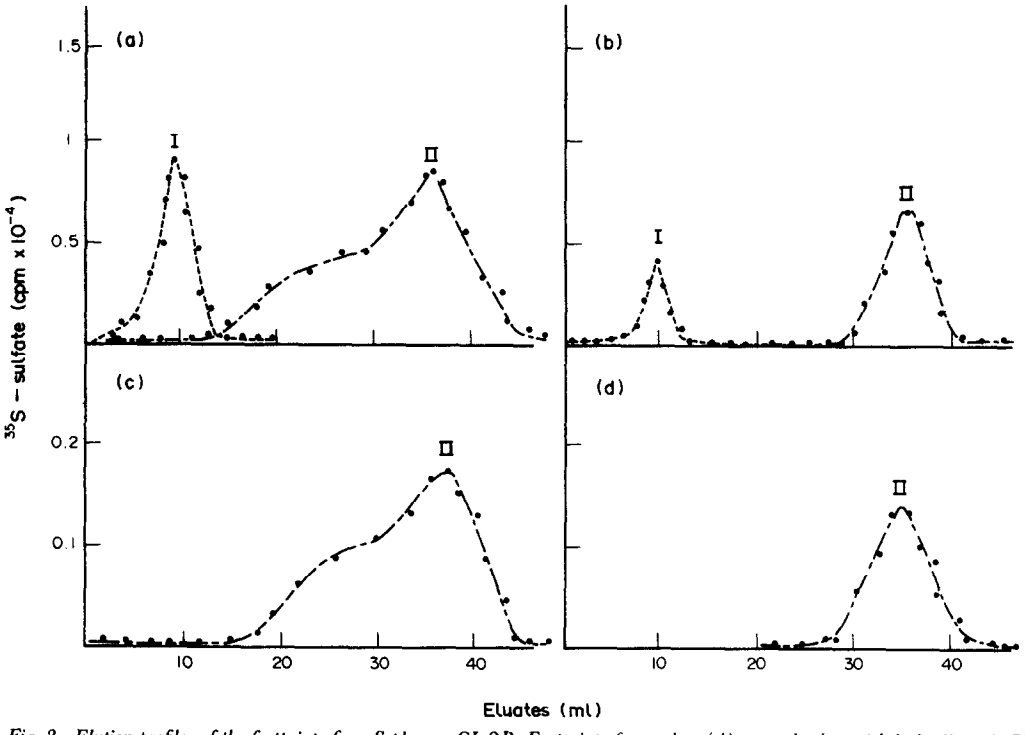

Fig. 3. Elution profiles of the footprints from Sepharose CL-2B. Footprints from colon: (A) normal colon epithelial cells and (B) colon carcinoma cultures labelled with [^{35}S]*sulfate plus* 30 30 µl *of carrier protoglycan aggregate* (1 mg/ml) *from bovine nasal cartilage were applied to CL-2B columns* (0.6 × 110 cm) *eluted with* 0.5 M *sodium acetate buffer*, 2.5 mM *EDTA*, *pH* 7.0. *The excluded radioactive peaks* (.....) *were reduced and alkylated in the presence of* 4 M *guanidine*-HCl, *dialyzed against* 0.5 M *sodium acetate, and rechromatographed on CL-2B* (*C and D*). *Recovery* [^{35}S] *sulfate is greater than* 95%.

plasms, which arise from endodermally derived epithelium. CEA is proved to be a product of cancerous cells becaue it is present in all carcinomatous tissue in greater amounts than normal adjacent mucosa. Although CEA tissue is present in all malignant colorectal specimens, elevation of plasma CEA is observed only in a certain percentage (i.e. 65%) of patients with proven cancer of the colon or rectum. The possible explanation is that, while CEA is produced by malignant cells, the quantity of CEA released into the circulation depends on the tumor mass and the activity of the malignant cells, e.g. small tumors do not produce adequate amounts of CEA to cause any significant elevation in plasma. The present investigation showed significant variation between the CEA levels produced by various esablished colon cell lines (Table 1). This variation suggests possible variations, either in cellular growth rate or in oncogenic ability. Short-term colon cell lines also showed variations in the CEA levels produced. The difference in the CEA levels produced by NCE, UCC, FPC and the levels released by CCC cell lines indicate differences in cellular oncogenic ability (Table 4).

There is substantial evidence that the plasma membrane of transformed cells differs from that of normal cells, and differences in morphological characteristics of tumor cells are associated with changes in cell surface conjugates [23, 24]. Significant differences in the cell surface architecture of sialoglycoproteins also have been detected between variant cell lines differing in their metastatic ability [25] and specific changes in sialylation of a major membrane sialoglycoproteins could be related to the lung cell implantability [26].

The present studies demonstrate that the characteristics of "initiated" preneoplastic cells, i.e. cells from UC and FPC, as well as colon carcinoma (CCC) neoplastic cells is determined not only by the genetic makeup of the cell, but also by its microenvironment. Of particular interest is the possibility that surrounding cells or cell products might be capable of limiting proliferation and/or expression of the neoplastic phenotype of the neoplastic cell populations.

Both *in vivo* [28–21] and cultured [32–34] preneoplastic as well as neoplastic cells are influenced by surrounding normal cell populations. It is not clear whether the influence of normal cells on neoplastic cell behavior is mediated via a direct effect requiring close contact and/or an indirect effect involving stable diffusible factors. Both modes of interactions have been shown to be involved: the first involves formation of gap junctions between two cell populations [35]; whereas the second consists of the diffusible growth factors associated with normal cell population such as liver [36], mammary gland [37], kidney epithelial cells [38] and placenta [27].

Proteoglycans are the major extracellular nonfibrous macromolecules of connective tissues. These highly anionic structural compounds are considered to be important in maintaining a wide variety of cellular tissue functions [39, 40]. Any changes in the structure, composition or distribution of these compounds within the extracellular matrix will have a direct effect on the appearance and function of tissues. The present studies compared the effects of extracellular footprints left on the culture dishes by NCE and CCC cells on cellular growth and differientation. The described experiments demonstrated for the first time the *in vitro* transformation of NCE cells into oncogenic cells, with the ability to develop into tumors in athymic mice, with the ability to produce and secrete increased levels of CEA, a phenotype characteristic of the carcinoma cells (CCC). On the other hand, if the colon carcinoma cells (CCC) are cultured in the serum-free hormone defined media on footprints from NCE cells, the CCC cells progressively lose their ability to develop tumors in athymic mice, and their ability to produce and secrete CEA weaken, indicating that the CCC cells progressively have been converted into cells with non-cancerous phenotype. The exact mechanism leading to the activation or suppression of the oncogenic expression awaits further investigation. The qualitiative and quantitative differences between the marcomolecules of the two types of footprints with differences in the conditioned media could modulate cell differentiation by altering the expression of certain genes. Further studies are in progress examining possible differences in mRNA and its complementary cDNA in the colon normal epithelial (NCE) cells and the cells in colon from patients with UC, FPC and CCC.

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