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Carcinoembryonic antigen: Function in metastasis by human colorectal carcinoma

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Abstract

Carcinoembryonic antigen (CEA) is a glycoprotein that has been useful as a tumor marker to predict recurrence in gastrointestinal malignancies, but whose biological function has not been elucidated. With the recent evidence that CEA is a member of the immunoglobulin supergene family, CEA may be involved in intercellular recognition and binding. This review examines the role that CEA plays in the development of metastases by colorectal carcinoma.

Introduction

Carcinoembryonic antigen (CEA) was first identified in 1965 by Gold and Freedman [1], who described an antigen in extracts of human adult colon adenocarcinoma and fetal colon that was not detectable in similar extracts of normal adult colon. Immunological analysis soon showed that CEA was only one of several glycoproteins that shared carbohydrate and peptide epitopes (for reviews please see references [2–4]. Furthermore, CEA was not tumor-specific, but was expressed in many different normal tissues, although at a lower concentration than in carcinomas and in fetal tissue. With the recent use of molecular biology techniques, CEA was recognized to be a member of a subfamily of at least ten molecules that are also part of the larger immunoglobulin supergene family [5]. It is now almost 25 years since its discovery and the biological function of CEA is still not well defined, although its structural relationship to immunoglobulins suggests a role in intercellular recognition and regulation of the immune response. The purpose of this review is to examine the role of CEA in the development of clinical metastasis by colorectal carcinoma (CRC) and to suggest that CEA may inhibit host defense mechanisms and enhance intercellular adhesion.

The structure of CEA

The CEA originally identified in extracts of colon carcinomas is a 180–200 kDa glycoprotein that consists of approximately 60% carbohydrate. On isoelectric focusing, purified CEA preparations appear heterogeneous due to differences in carbohydrate structure. Most of this heterogeneity is attributable to varying amounts of sialic acid on individual molecules [6]. The oligosaccharide side

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chains are N-linked and of the complex type, 80% tetraantenary structures with the balance being tri- and di-antenary forms [7]. The complete amino acid sequence of CEA is now known and was determined from the nucleotide sequence of the cloned gene [8]. The peptide chain consists of a 108 amino acid N-terminus with three 178 amino acid repeating loop domains held in place by two disulphide bridges in each of the domains. The molecule terminates at the carboxyl end in a short 26 amino acid hydrophobic domain. It is now thought that this C-terminal domain is lost post-translationally from the secreted molecule, and an ethanolamineglycosylphosphatidylinositol is added to a residue in the terminal hexapeptide of the third repeating domain. This lipid facilitates attachment of CEA to cell membranes prior to secretion [9, 10]. There are 28 potential sites for N-linked glycosylation, and the oligosaccharide chains are located to the loop domains with the exception of two potential sites at the distal end of the N-terminal peptide. These carbohydrate structures are similar to those found on many other secreted glycoproteins such as alpha$_1$ acid glycoprotein, which also has highly branched acidic oligosaccharides.

CEA is one member of a family of structurally similar molecules that cross react in standard immunological tests. These molecules have repeating loop domains with a high degree of sequence homology to CEA. The gene family includes the nonspecific cross reacting antigen (NCA 55 kDa, NCA 110 kDa), tumor extractable antigen (TEX 110 kDa) which may be identical to NCA, biliary glycoprotein 1 (BGP-1 83 kDa), NCA-2 (150–170 kDa), and the normal fecal antigen (NFA-1 20–30 kDa). (For reviews of these family members see refs. [2, 3, 4].) More recently, four transmembrane forms of CEA have been described. Two of them, TM2 and TM4, are similar to NCA, while TM1 and TM3 contain the first disulphide bridge domain plus part of the second from CEA. These molecules have a full transmembrane and cytoplasmic domain at the C-terminus and in this respect are similar to BGP-1 [11]. Thompson *et al.* have also described five further members of the CEA gene family, one of which is the human pregnancy specific β1-glycoprotein [12]. The CEA gene family, while extensive, also belongs to a much larger group of molecules: the immunoglobulin supergene family. CEA shares many structural similarities with numerous members of this family, including Thy 1.2 antigen [13], Kappa chain variable region [14], polyimmunoglobulin receptor [15], alpha$_1$β glycoprotein [16], NCAM [17], and MAG myelin associated glycoprotein [18].

The major similarities between these molecules and CEA are in the structure of the disulphide loop domains. These areas of the molecule are composed of antiparallel β strands, and this is the typical feature of the immunoglobulin fold [19]. Circular dichroism studies of CEA showed that 80% of the molecule is made up of β structure and β turn, with the other 20% in random coil conformation [20]. Computer analysis of the disulphide bridge domain structure also suggests that the secondary structure is an antipolar arrangement of β-strands [21]. This similarity in structure between CEA gene family members and the immunoglobulin supergene family also suggests similar functions. It is likely, therefore, that CEA may be involved in cell recognition and possibly in the regulation of the immune response.

The association of CEA with prognosis

Four years after the discovery of CEA, Thomson *et al.* [22] described a radioimmune assay for measuring CEA in serum. When investigators realized that the concentration of CEA in the blood was approximately proportional to the amount of tumor in the patient, attempts were made to demonstrate that CEA may be used to screen individuals for CRC or other gastrointestinal tract malignancy. Unfortunately, CEA is only elevated in the serum of 6%–12% of patients with localized disease, and is frequently elevated in the sera of patients with benign liver disease, renal failure, pancreatitis, biliary obstruction, and chronic obstructive pulmonary disease (for reviews please see references [2, 3, 23]). CEA was not useful in screening for CRC because of these unacceptably high false positive and false negative rates.

In contrast, CEA was useful in assessing progno-

sis. An elevated serum CEA level in the blood of patients prior to operation predicts an increased risk of recurrence in patients operated upon for cure (refs. [24–32], Table 1). There are several caveats to this conclusion. First, each laboratory has had a different threshold for an abnormal elevation of serum CEA. As Fucini *et al.* [31] demonstrated, 95% of normal volunteers in their laboratory had a serum CEA of less than 20 ng/ml in their series. When this concentration was used as the threshold for a positive test, a significant association between serum CEA and the development of metastasis was observed. However, a concentration of 10 ng/ml was not significantly associated with recurrence in this study, although it was a significant threshold value in other studies (Table 1). With the development of standardized, commercially available kits for either radio- or enzyme immunoassays, 95% of serum CEA values from normal volunteers will be less than 5 ng/ml. Consequently, more recent studies have demonstrated an association between clinical recurrence and CEA greater than 5 ng/ml (Table 1). Our own study of 57 patients with CRC operated upon with curative intent at the University of Texas M.D. Anderson Cancer Center also demonstrates an association between a preoperative serum CEA greater than 5 ng/ml and clinical outcome (Table 1).

The site of the primary cancer may also influence the interpretation of elevations in serum CEA. CEA elevations in patients with colon carcinomas are associated with poor outcome, while rectal carcinomas may not be [27]. This discrepancy may reflect different patterns of venous drainage, since rectal carcinomas drain into the systemic circulation, bypassing the liver and its clearance mechanisms (see below). As indicated in Table 1, the ability to predict recurrence when the serum CEA is elevated preoperatively (the sensitivity of a test) ranges from 43%–89%, while the ability to predict that recurrence will not occur if the preoperative serum CEA is normal (the specificity of a test) is higher (a range 70%–99%).

The content and pattern of CEA expression within a carcinoma also appear to be important prognostic factors. The concentration of CEA in sera from patients with poorly differentiated CRCs may not be elevated because poorly differentiated carcinomas produce less CEA. Since poorly differentiated carcinomas are more aggressive than carcinomas that are well or moderately differentiated, serum CEA may not be elevated in this group of patients that will develop metastases. Wiggers *et al.* [33] recently confirmed this in an immunohistochemical analysis of primary CRC by demonstrating that those carcinomas devoid of CEA on immunoperoxidase staining of the primary carcinomas were significantly more likely to be poorly rather than moderately or well differentiated carcinomas. In addition, carcinomas devoid of CEA developed significantly more recurrences than carcinomas that contained CEA. These authors also examined the distribution of CEA within the neoplastic cells. Cancers in which CEA was distributed primarily in the apical cytoplasm (the distribution in normal adult colonic epithelial cells – [34, 35]) had a better outcome than patients whose neoplastic cells had CEA distributed over the entire membrane (the distribution of CEA in the 8–9 week human fetal colon – see [36, 37]). Thus, examination of the pattern of CEA expression improved the association of CEA with the development of metastasis. A fetal pattern of expression was associated with a worse outcome.

The amount of CEA within a tumor also appears to be important. Cosimelli *et al.* [38] have shown that the content of CEA within a CRC measured by a quantitative assay is directly correlated with clinical outcome. Furthermore, the amount of CEA within a primary tumor was independent of either stage of disease at diagnosis or state of differentiation. Prognostic accuracy was improved by measuring the amount of CEA in the adjacent normal mucosa. If both the cancer and the mucosa contained elevated amounts of CEA, patients were at greater risk for recurrence than if either or both contained less than the threshold value of CEA. It is not clear how well the interpretation of CEA expression in immunoperoxidase sections correlates with the quantitative determination of CEA content within a cancer. However, it is clear in the illustrations of Wiggers *et al.* [33] that both the membranous and apical patterns of expression contain similar amounts of CEA. Shi *et al.* [39] showed

that 80%–95% of CEA within colorectal carcinoma cells was associated with the cell membrane. Thus, critical measurement of CEA content within primary CRC may be an important aid in the prospective estimation of prognosis.

Another indication that CEA is associated with recurrence is the observation that the serum level of CEA usually increases prior to the appearance of clinical metastases. While several reviews discuss this point in detail [23, 40, 41], the recent data of Kimura *et al.* [30] illustrate this point well. These authors observed that 26 of 44 patients who were operated on for cure and who recurred after operation had elevations in the serum CEA prior to clinical symptoms, whereas only 8 patients had either no change in the serum CEA or an elevation after clinical symptoms appeared. CEA is more likely to be elevated when visceral metastases are present than if recurrence is limited to local areas. Furthermore, Denstman *et al.* [32] observed that the association between preoperative CEA level and clinical outcome held for those carcinomas that recurred within 2 years of potentially curative surgery. Carcinomas that recurred later tended to be in patients whose preoperative serum CEA had been normal and whose serum CEA did not increase when they recurred. Thus, the association between clinical recurrence and CEA may involve at least two types of recurrences: biologically aggressive carcinomas that occur within 2 years of diagnosis and are associated with CEA; and slower growing carcinomas that recur 2 or more years after primary treatment and are independent of CEA.

Thus, the level of CEA in serum is associated with the development of clinical metastasis. However, these data do not indicate whether CEA is causally related to the development of metastasis. Serum CEA may be associated with other factors (e.g., state of differentiation) that may determine whether CRCs will metastasize. Further, elevations of serum CEA in patients without clinically obvious metastases may occur because microscopic metastases are located in the organs that clear CEA from the blood [42, 43]. As a result, a microscopic metastasis may bypass the clearance mechanisms of the liver and increase the concentration of CEA in the circulation. Thus, CEA may be a passive participant in the metastatic process, merely reflecting tumor mass. In contrast, CEA may have an active role in the formation of metastasis or the proliferation of tumor cells. Unfortunately, correlative studies of CEA serum levels will not establish this point. Based on these observations, we started our investigation into the potential role of CEA in the development of metastasis by asking whether primary carcinomas from patients with an elevated

Table 1. Preoperative CEA levels predict clinical outcome in colorectal carcinoma

Author	Site[a]	Stage[b]	CEA[c]	N	Sensitivity[d]	Specificity[e]	p[f]
Goslin[13]	C,R	C	5	33	89	87	0.0001
Steele[15]	C	B–C	5	223	52	65	0.024
Bartal[16]	C,R	A–C	10	339	82	99	0.0001
Koch[17]	C,R	B2,C	10	93	43	82	0.019
Kimura[18]	C,R	A–C	5	138	49	70	0.039
Fucini[19]	C,R	A–C	20	42	64	82	0.003
Denstman[20]	C,R	A–C	6	214	62	83	0.0001
Jessup[g]	C,R	A–C	5	57	45	81	0.036

[a] Primary site: C-colon, R-rectum.

[b] Stage: A-carcinoma limited to bowel wall, B2-carcinoma invades through bowel wall into adipose tissue but nodes are negative, C-regional nodes contain metastases.

[c] Value of CEA that was considered positive in each study (in ng/ml).

[d] Sensitivity is the percentage of patients who recurred who had a positive preoperative CEA test.

[e] Specificity is the percentage of patients who remained free of disease who had a negative CEA test.

[f] Probability determined by chi-square analysis.

[g] Present series.

serum CEA were biologically more aggressive than carcinomas from patients with a normal serum CEA in a preclinical model.

Serum CEA is associated with the tumorigenicity of human CRC in nude mice

Establishing the biological aggressiveness of human CRCs requires a functional test of the growth potential of a carcinoma. One approach is to place CRCs in tissue culture and correlate growth with the serum CEA level. The problem with this approach is that human colon carcinoma cells are difficult to establish in long-term culture. For example, only 9 (19%) of 46 CRCs were successfully established in our laboratory. This rate of success is similar to the frequency of successful explantation noted in other laboratories [44].

Another approach to the functional measurement of the biological aggressiveness of human CRC is to implant tumors in athymic nude mice and let growth in the nude mouse be a biological assay for the 'aggressiveness' of human carcinomas. Once Rygaard and Povlsen [45] observed that human tumors grow in athymic nude mice, others [46–49] showed that human neoplasms grown in the nude mouse generally maintain the morphological and biochemical characteristics of the original neoplasm. Human melanomas and soft tissue sarcomas have been transplanted to nude mice with a high frequency of success [50], while epithelial neoplasms of breast, stomach, and prostate are more difficult to establish [50]. Human CRC xenografts have been successfully established in nude mice in approximately 60% of the cases [51–56] and several laboratories have either demonstrated [57–59] or suggested [60, 61] an association between growth in nude mice and clinical outcome.

Our laboratory has used tumorigenicity in nude mice to assay the growth potential of human CRC. Eighty-two adenocarcinomas of the colon, rectum, or their metastases were enzymatically dissociated and cells implanted into the flanks of nude mice. Tumorigenicity was defined as the progressive growth of a neoplasm within 6 months at the inoculation site that was successfully passaged into a second set of nude mice. Thirty-two of the 82 surgical specimens were harvested from patients whose serum CEA was greater than 5.0 ng/ml. Eighty-one percent of these carcinomas were tumorigenic in nude mice, compared to only 54% of the carcinomas from patients whose serum CEA was not elevated ($P = 0.017$). CRC from patients with an elevated CEA that were either operated upon for cure or that had localized or regional disease (Dukes A-C lesions) were significantly more tumorigenic than CRC whose serum CEA was less than 5 ng/ml (Table 2), demonstrating that carcinomas from patients with elevated serum CEA are more aggressive than carcinomas from patients with a normal serum CEA. None of the other clinicopathological variables examined were associated with tumorigenicity in nude mice; e.g., stage of disease, differentiation, sex, mucin production, invasion of regional lymphatics, nerves, blood vessels, or age. Serum CEA was the only variable that was associated with clinical outcome. While these data do not suggest that CEA participates in the recurrence of carcinoma, they do indicate that primary CRCs from patients with a serum CEA greater than 5 ng/ml are biologically more aggressive than primary CRCs from patients with a normal CEA level.

If primary CRCs from patients with a high CEA are more tumorigenic in nude mice, is it because CEA has enhanced tumor growth? Does CEA participate in the growth of human CRC, or is the tumorigenicity of a carcinoma still a function of some other neoplastic characteristic? We have tried to answer these questions using an animal model of experimental metastasis by human CRC.

The role of CEA in the development of experimental metastasis by human CRC

Our laboratory has developed assays to study the metastasis of human neoplasms by injecting tumor cells systemically into nude mice so that they form colonies in the liver or lung. While the systemic injection of tumor cells circumvents the first steps of the metastatic sequence in which tumor cells must emigrate from the primary tumor, the cells must survive in the circulation, implant in a capil-

lary bed, extravasate, and proliferate in the organ parenchyma to form experimental metastases. The feasibility of such models of experimental metastasis was first demonstrated by Kozlowski *et al.* [62], who observed that experimental lung and liver metastases are produced by the intravenous or intrasplenic injection of established human tumor cell lines. Giavazzi *et al.* [63] demonstrated that recently isolated primary and metastatic CRC did not form spontaneous metastases when implanted in the subcutis, but formed experimental metastases in lung or liver after intravenous or intrasplenic injection. Cells obtained from clinical hepatic metastases generally grew more efficiently in mouse liver than those from primary tumors [63, 64]. In addition, Morikawa *et al.* [65, 66] observed that the metastatic potential of a Dukes' B2 primary could be enhanced by repeated passage through nude mouse liver. Thus, this experimental hepatic metastasis assay provides a model with which to study the effects of CEA upon metastasis.

Experiments were carried out in which CEA was injected intravenously prior to the intrasplenic injection of human CRC cells, to determine whether it facilitated hepatic metastasis in athymic nude mice. Thomas and Hems [42] demonstrated that CEA injected intravenously into rats was cleared by the liver with a shorter half-life in rats than in patients. Clearance in mice was similar to that in rats [67]. Intravenous injection of CEA produced a serum concentration of 600 ng/ml at 30 minutes, which decreased to 40 ng/ml 24 hours later (H. Wagner, personal communication). Four CRC cell lines were chosen for study: KM-12c [65] and MIP 101 [68], two CRC cell lines that were weakly metastatic in nude mice, mHC 1410, a CRC line that was highly metastatic in nude mice [63], and HC 2998, a CRC that was tumorigenic in nude mice but not metastatic after intrasplenic injection. CRC cells were injected into the spleens of nude mice 30 minutes after CEA was injected intravenously, and liver colonies were examined when the animals were sacrificed 60–90 days later. KM-12c formed liver colonies in 2% of the 45 mice injected with 5×10^5 KM-12c cells and saline. In contrast, when 5–10 μg of CEA (low-dose CEA) was injected prior to KM-12c, 33% of mice formed experimental metastases. Similarly, KM-12c produced experimental metastases in 48% of 25 mice that received 5×10^5 KM-12c cells intrasplenically and 40–60 μg of CEA (high-dose CEA) intravenously (Fig. 1). MIP 101 also formed experimental metastases in 7 of 8 mice pretreated with CEA, compared to none of the mice that were pretreated with saline and 1 of 6 mice pretreated with asialo-CEA. In contrast, CEA pretreatment did not induce a nonmetastatic but tumorigenic CRC line, HC 2998, to form experimental metastases in nude mice. Similarly, CEA pretreatment did not shorten the survival of mice bearing mHC 1410, which was metastatic in 100% of control mice. Thus, CEA enhanced the metastatic potential of two weakly metastatic CRCs in the nude mouse model, but did not make a nonmetastatic CRC metastatic or a highly metastatic CRC more aggressive. This data would suggest that CEA is not acting as a growth factor in this case, but that the enhancement of metastases in its presence must be due to some other mechanism. The experimental hepatic metastasis assay now permitted investigation into alternate mechanisms by which CEA may enhance metastasis by certain CRC cells.

Table 2. Association of serum CEA with the tumorigenicity of CRC in athymic nude mice

Patients	CEA (ng/ml)	N	% Tumorigenic	P
All	<5	50	54	
	≥ 5	32	81	0.017
Dukes' A–D	<5	39	44	
For cure	≥ 5	18	83	0.005
Dukes' A–C	<5	35	43	
	≥ 5	13	77	0.036

P values determined by chi-square analysis.

Mechanisms by which CEA may enhance metastasis

Metastasis is a process in which multiple, linked steps must be performed sequentially in order to enable a malignant cell to leave a primary neoplasm to establish metastases at a distant site [69–

73]. This process may be divided into four phases that may be considered separately: emigration, circulation, implantation, and proliferation (Table 3). During emigration tumor cells leave the primary tumor and enter the circulation. Therefore, this phase involves molecules that mediate tissue invasion and detachment. During the circulation phase tumor cells survive in the circulation while they traverse to a distant site. Immunosuppressive effects of CEA may be important during this phase because malignant cells may be more sensitive to the effects of natural killer (NK) or other host defense effector cells during this phase than any other. Homotypic aggregation may also be important during this phase, allowing tumor cells to arrest more easily in capillary beds. During the implantation phase malignant cells attach to the endothelium of a distant organ and penetrate into the parenchyma. In the proliferation phase malignant cells proliferate within the parenchyma of the metastatic site, often in spite of host cell cytotoxicity and cytostasis. CEA may participate in each of these phases.

The emigration phase

CEA may be involved in intercellular recognition and binding (see below). As such, reduction in the amount or distribution of CEA in the cells leaving the primary tumor may promote the emigration of tumor cells from the primary carcinoma. Intercellular bonds have to be broken so that tumor cells may leave the primary tumor. As reviewed by Gabbert [74], the advancing edge of a cancer is often less differentiated than the bulk of the primary carcinoma. The cells in the advancing edge contain less laminin in their basement membranes. CEA expression parallels that of laminin. CRC cells grown on surfaces devoid of laminin round up, lose their orientation, and produce less CEA [75]. Further, production of plasminogen activators may down-regulate the expression of laminins [76], and be increased in areas of the tumor that are the advancing edge. Poorly differentiated carcinomas do not express mature forms of laminin; however, they do make laminin message [77]. Degradation of laminin may occur and is possibly mediated by plasmin that is activated by the urokinase plasminogen activator secreted by CRC cells. Growth factors enhance laminin expression and increase CEA expression [78]. Laminin added to CRC cells in culture also enhances CEA production [75]. Thus, the reversible loss of differentiation at the advancing edge of a primary CRC may either decrease the amount of CEA or redistribute it over the cell surface. A decrease in laminin expression or an increased rate of degradation may loosen the intercellular bonds between CRC cells and allow them to separate prior to entering the circulation.

Parallel changes in CEA expression may occur *in vivo*. In the normal colon of the adult, CEA is present in the apical cytoplasm of columnar and goblet cells [34, 35, 79]. This pattern also predominates in well-differentiated carcinomas, since CEA is found largely in the apical cytoplasm of the neoplastic cells that line the lumen of a malignant gland. However, in fetal colon [36, 37] and in poorly differentiated carcinomas [33, 80, 81] in which gland structures are not well-developed, CEA is distributed throughout the membrane or over the basolateral surface of the cell. Benchimol *et al.* [36] have suggested that this redistribution may facilitate the loosening of intercellular bonds and enhance the emigration of CRC into the circulation.

The circulation phase

CEA may enhance hepatic metastasis during the circulation phase in two ways. First, as suggested by Benchimol *et al.* [36], CEA may act as a homotypic aggregant. CEA alone was sufficient to cause cell aggregation, since fibroblasts transfected with the gene coding for CEA spontaneously aggregated if they expressed normally glycosylated CEA. Aggregation was inhibited by monovalent Fab anti-CEA antibodies. Lisowska *et al.* have demonstrated that both CEA [82] and NCA [83] form homotypic dimers in solution. Our own studies have shown that fractionation of purified CEA preparations on HPLC using TSK-3000 or TSK-4000 size exclusion columns in the absence of reducing agents or detergents results in molecules

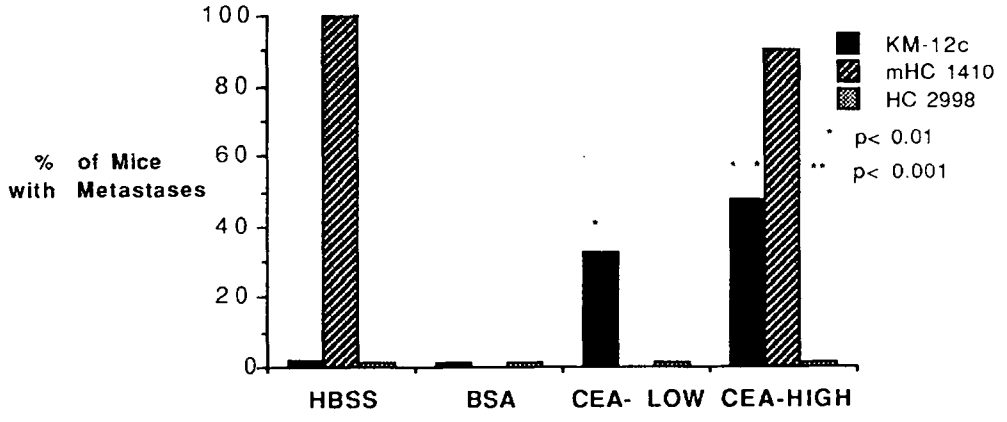

Fig. 1. Effect of CEA upon experimental metastatic potential of CRC. BALB/c athymic nude mice received 0.2 ml i.v. injections in a dorsal tail vein with HBSS, BSA (40 μg per mouse), low-dose CEA (5–10 μg per mouse), or high dose CEA (40–60 μg per mouse). Thirty minutes later 5×10^5 KM-12c, HC 2998, or mHC 1410 CRC cells were injected intrasplenically. Approximately 6 weeks later mice were killed and their livers examined for colonies. Results are expressed as the percent of the cumulative number of mice with liver colonies in seven experiments divided by the total number of mice injected. Both low-dose and high-dose CEAA pretreatment enhanced the production of liver colonies by KM-12c but not by HC 2998 or mHC 1410. * $p < 0.01$ and ** $p < 0.001$ (compared to the HBSS control using Fisher's exact test).

with an apparent MW of 400000. SDS-PAGE of these same preparations gives the expected 180–200 kDa bands. CEA will also form aggregates with other molecules, including a glycoprotein with a close structural relationship to alpha$_1$ acid glycoprotein [84]. Further, Updike and Nicolson [85] have suggested that the ability to aggregate correlates directly with metastatic potential. Lotan and Raz [86] have shown that 14 and 31 kDa galactose-binding lectins may be involved in cell aggregation. Human CRC contains such lectins (R. Lotan, personal communication), and may bind to galactose-containing oligosaccharides expressed in CEA. Thus, CRA may act as a homotypic aggregant and facilitate the entrapment of tumor cells in the microvasculature by causing cells to form large emboli.

CEA may also affect a second aspect of the circulation phase of metastasis. As CRC cells traverse the blood, host effector cells may bind to the carcinoma cells and interfere with the subsequent steps of metastasis. CEA does not appear to be very immunogenic in the cancer patient. While previous studies have suggested that CEA may induce an autoantibody response in patients [87, 88], other studies have not confirmed this finding [89]. Recent work suggests that the development of autoantibodies of CEA is an infrequent occurrence, with a prevalence of 3 responding patients per 500,000 patients studied [90]. Cell-mediated immunity is produced to CEA, since delayed-type hypersensitivity may be elicited to purified preparations of CEA in patients who have CRC [91]. Two of nine patients with CRC who did not have metastases produced a DTH response to CEA, while only 2 of 39 patients with metastases displayed DTH to

Table 3. The phases of the metastatic sequence

Phase	Purpose	Potential role of CEA
Emigration	CRC leave primary	Loosen intercellular bonds
Circulation	Traverse blood stream	Form tumor emboli; resist host defenses
Implantation	Attach to site-specific microvascular beds	Cell attachment
Proliferation	Growth at metastatic site	Resist host defenses

CEA. However, 10%–33% of patients with a variety of benign gastrointestinal tract disorders also displayed a DTH response to CEA, and none of the patients in this study were tested for specificity with skin tests to unrelated antigens. Thus, CEA is unlikely to be a specific target for host effector cells.

However, CEA may be an immunosuppressant that inhibits specific and nonspecific host cell responses that might otherwise prevent metastasis. It has been shown that CEA inhibits both the proliferative responses and the antibody production of lymphocytes incubated *in vitro* with appropriate mitogens [92]. CEA administered to BALB/c mice sensitized with sheep erythrocytes inhibits the plaque-forming cell response [93], a response that requires T-B cell cooperation. In addition, CEA induces suppressor T cells from patients with CRC to release a substance that inhibits the DNA synthesis of normal T cells [94], an effect which may be mediated by sialylated CEA [95]. Recently, Heiskala *et al.* [96] observed that molecules with physicochemical characteristics similar to CEA (mass of 160–180 kDa present in saline extracts of ovarian adenocarcinomas) inhibit natural killer cell cytolysis. Further, the expression of CEA by the CRC may alter their sensitivity to NK-mediated cytolysis. Clark *et al.* [97] reported that the surface expression of CEA by tumor cells correlated directly with sensitivity to natural killer (NK) cell-mediated lysis. The implication of these studies is that tumors that express more membrane-associated CEA will be more sensitive to NK cell lysis, and therefore less likely to be tumorigenic. However, Bagli *et al.* [98] observed that sensitivity to NK cell-mediated lysis was inversely correlated with the state of differentiation of CRC. The induction of a more differentiated phenotype in poorly differentiated CRC by treatment with sodium butyrate decreased sensitivity to NK cell cytolysis. Since well differentiated CRC cell lines and poorly differentiated lines treated with sodium butyrate produced more CEA than do untreated poorly differentiated lines [99], CEA may be involved with resistance to NK cell-mediated cytotoxicity *in vitro*. However, CEA has not been shown to be the target of NK cells. Collectively, these observations indicate that CEA may directly or indirectly inhibit humoral and cell-mediated immunity. Such immunosuppression may be involved during the proliferative phase of metastasis as well as during the circulatory phase. Further, while it is intellectually appealing to suggest a role for host defense mechanisms in the prevention of metastasis, it is not clear that such mechanisms participate in the metastatic process in patients.

The implantation phase of metastasis

Knowledge of how CRC cells traverse the liver may help to determine how CEA facilitates metastasis. Tumor cells enter the organ through the portal veins and traverse hepatic sinusoids, 5–15 μ capillaries lined by fenestrated endothelial cells that connect the hepatic arterioles and portal venules to the central hepatic veins [100, 101]. The endothelial cells form tight junctions with Kupffer cells and have two types of fenestriae: small (100–200 nm) pores that create a sieve plate, and larger gaps (up to 1 μ). Since the hepatic sinusoid does not have a continuous basement membrane, tumor cells within the sinusoid may contact hepatocytes through the fenestriae [100, 101], in addition to interacting with Kupffer and endothelial cells. The space of Disse, located between the endothelial lining of the hepatic sinusoid and the hepatocytes, contains a discontinuous extracellular matrix composed of collagen types I and IV and fibronectin but not laminin, which is present, however, in the basement membranes of the portal and central veins [102]. The composition of the extracellular matrix in rodent liver seems quite similar to that of humans. Thus, in the nude mouse model tumor cells must also bind either to endothelial, Kupffer, or hepatic cells, fibronectin, collagens type I or IV, or a combination of these. This binding phase would be followed by invasion of the space of Disse, and proliferation among the hepatocytes.

We have used the experimental hepatic metastasis model to determine whether CEA enhanced the implantation of CRC cells. For these studies mice were pretreated with either CEA or saline before receiving [125I]Idudr-labeled CRC intrasplenical-

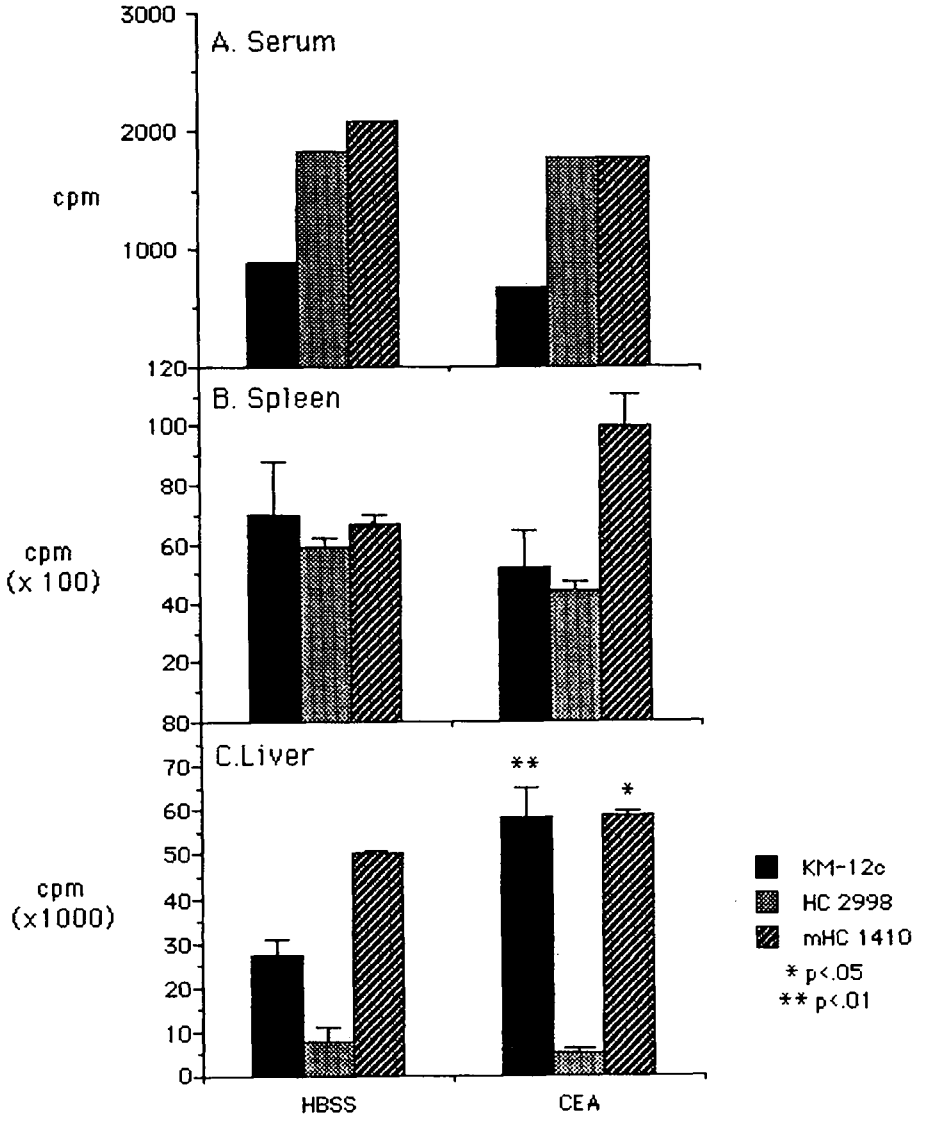

Fig. 2. CEA enhances implantation of CRC in the liver (C) without affecting the amount of radioactivity in the serum (A) or spleen (B). Groups of three BALB/c athymic nude mice were injected intrasplenically with 5×10^5 KM-12c, HC 2998, or mHC 1410 CRC cells that had been labeled with [125 I]IdUrd 30 minutes after HBSS or CEA (40 μg per mouse) had been injected i.v. Four hours later, mice were killed and their sera, spleen, and livers were harvested and counted as described in 'Materials and Methods'. Results are the mean ± SEM cpm for each group. Significance determined by unpaired Student's t test.

ly. The mice were killed 4 hours later and their spleens, livers, and sera were analyzed. All the mice had comparable amounts of radioactivity in their sera and spleens (Fig. 2A and 2B). In contrast, CEA enhanced the retention of both metastatic CRC lines (KM-12c and mHC 1410) (Fig. 2C). CEA did not increase the retention of the nonmetastatic CRC HC 2998 in mouse liver.

Apparent specific binding of CEA to tumor cells

Since CEA enhanced the retention of CRC in liver, CRC must bind CEA if it is to function as an attachment factor. A cell binding assay was performed in which [125I] CEA was added at 4° C to CRC in monolayer culture. The cells were incubated at 4° C for 90 minutes and then washed, and the

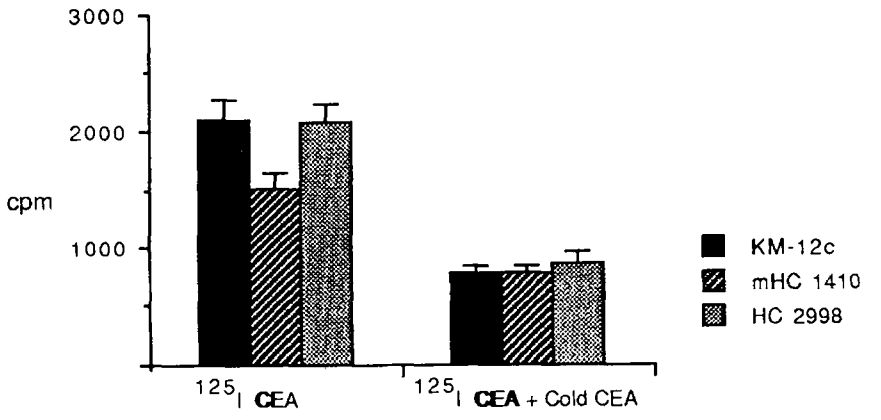

Fig. 3. CRC bind CEA in solution. 1×10^5 KM-12c, HC 2998, and mHC 1410 cells were plated in quadruplicate in microtiter wells for 16 hours at 37° C in 10% FBS and alpha-MEM. Wells were washed once with HBSS and 80 ng/well of [^{125}I] CEA was added to each well. After 90 minutes at 4° C, the wells were washed three times with HBSS and the cells were collected to measure cell-bound radioactivity (^{125}I CEA). In some experiments, a 100-fold excess of unlabeled CEA was added to each well (^{125}I CEA + cold CEA).

amount of [125I] CEA bound by the cells was determined. KM-12c, HC 2998, and mHC 1410 bound 0.3%–0.4% of CEA at 4° C. Similar binding was also noted at 37° C, suggesting that the bound CEA is not internalized, but remains at the cell surface. Titration curves for cell number and amount of CEA demonstrated a linear dose response over the range of 4.5–90 ng/well of CEA with saturation above 450 ng/ml. Competitive inhibition of binding by excess nonradioactive CEA was also demonstrated (Fig. 3). Since a 100-fold excess of nonradioactive CEA causes a 46%–54% reduction in the binding of [125 I] CEA to CRC, CRC demonstrate apparent specific binding of CEA. Further, the binding of CEA free in solution appears to be independent of the ability of CEA to enhance metastasis, since all three CRC tested, and not just KM-12c, bind CEA in solution equally well.

Tumor cells bind preferentially to CEA attached to plastic

Why did CEA enhance metastasis in only two of four CRC if all CRC lines bind CEA equally well? CEA is bound to Kupffer cells and hepatocytes and displayed on cell membranes [103]. This may permit only certain portions of the molecule to participate in tumor cell binding. To model this *in vitro*, we immobilized CEA on 96-well plates and assayed for tumor cell binding in a test similar to that described for fibronectin [104]. mHC 1410, HC 2998, and KM-12c demonstrated similar degrees of nonspecific attachment to either untreated plastic, bovine serum albumin (BSA), or alpha$_1$ acid glycoprotein immobilized on plastic (Fig. 4). However, more than twice as many KM-12c cells bound to CEA attached to plastic as either of the other two CRC lines or as KM-12c bound to the control surfaces (Fig. 4), including alpha$_1$ acid glycoprotein, which has high mannose triantennary oligosaccharides similar to those of CEA. Thus, the binding of KM-12c to CEA attached to a solid phase correlated with the ability of the antigen to enhance experimental metastasis and implantation in the liver. The lack of KM-12c binding to alpha$_1$ acid glycoprotein suggests that the peptide portion of the molecule is critical to the attachment of a CRC cell to a CEA-coated surface. Our studies show that Kupffer cell attachment is not enhanced when plastic plates are coated with CEA. This is in contrast to the increased adherence seen when plates are coated with fibronectin or microaggregated albumin. This would suggest that the Kupffer cell binding site is probably masked because it binds itself to the plastic surface. It seems likely, therefore, that the orientation of the CEA on the plastic surface would be similar to that of CEA

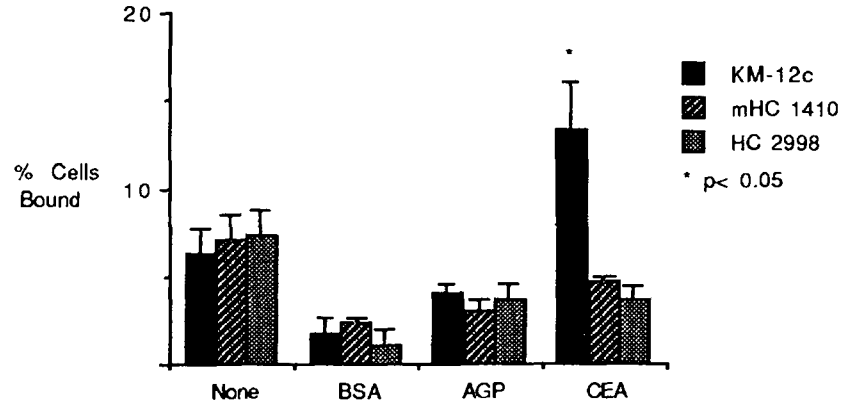

Fig. 4. KM-12c binds to CEA attached to a solid phase. CEA was dried onto 96-well culture plates at a concentration of 20 ng/well in double distilled H_2O (ddH_2O) for 12 hours at 37° C. BSA and alpha$_1$ acid glycoprotein (AGP) were attached in a similar manner as control proteins. The free binding sites on the plastic were blocked with 4% BSA for 2 hours at 37° C. The tumor cell lines were plated in wells at concentrations of 1000 cells/well. After a 1-hour incubation, cells were fixed in 1% paraformaldehyde and 1% sucrose. The unbound cells were removed by three washes with HBSS and the remaining cells were counted in each well in a coded manner. Mean ± SEM of cells per well. * $p \leq 0.05$ (Newman-Keuls test).

when it is bound to the Kupffer cell, with the C-terminal end and the loop domains exposed. This could explain the increased attachment of the metastatic tumor cells in terms of the Kupffer cell/tumor cell interaction model, and could allow plastic adherence to be used in the study of attachment mechanisms.

Structure and function relationships

CEA may promote metastasis through two pathways: modulation of the host immune response, and facilitation of intercellular adhesion. The availability of an *in vitro* assay for cell adhesion permits us to formulate hypotheses about the contribution of the CEA molecule to cell adhesion. Only the weakly metastatic CRC cell lines that were enhanced by CEA bind to CEA attached to a solid phase, while all three CRC lines tested so far bind CEA in solution with apparent binding specificity. Thus, the orientation of CEA on plastic may be similar to its orientation when bound to its receptor, especially if CEA binds to plastic through the oligosaccharide-free NH2-terminus as it does to its receptor on Kupffer cells. In contrast, CEA in solution may be free to bind to CRC in any orientation, with the hydrophobic portion attaching to membrane lipids. This is likely, as most (70%–80%) CEA within CRC is associated with the cell membrane [39]. CEA is released from the cell membrane when phospholipase C cleaves a phosphoinositol-glycerol membrane anchor located at the NH_2-terminus of the molecule [9, 10]. Once released, CEA enters the portal circulation, where it is removed from the blood by the liver. Very little is cleared by the regional lymphatics into the thoracic duct [105]. Immunofluorescence of adult human liver sections has shown that CEA and NCA are expressed on the surface of Kupffer cells [106]. Studies in rodents have shown that CEA is primarily metabolized by the liver, with small amounts removed by spleen and lungs [42]. In the liver, the Kupffer cell binds CEA through a receptor that is distinct from receptors for mannose, fucose, galactose, and N-acetylglucosamine [107]. Once endocytosed, terminal sialic acids are removed [108] and the desialylated CEA is released and transferred to the hepatic parenchymal cell, where it is degraded [67]. Binding of CEA to the Kupffer cell involves the peptide portion of the molecule, because removal of oligosaccharides by multiple Smith degradations still blocks the binding of native CEA to the Kupffer cell receptor [107]. Using peptides pro-

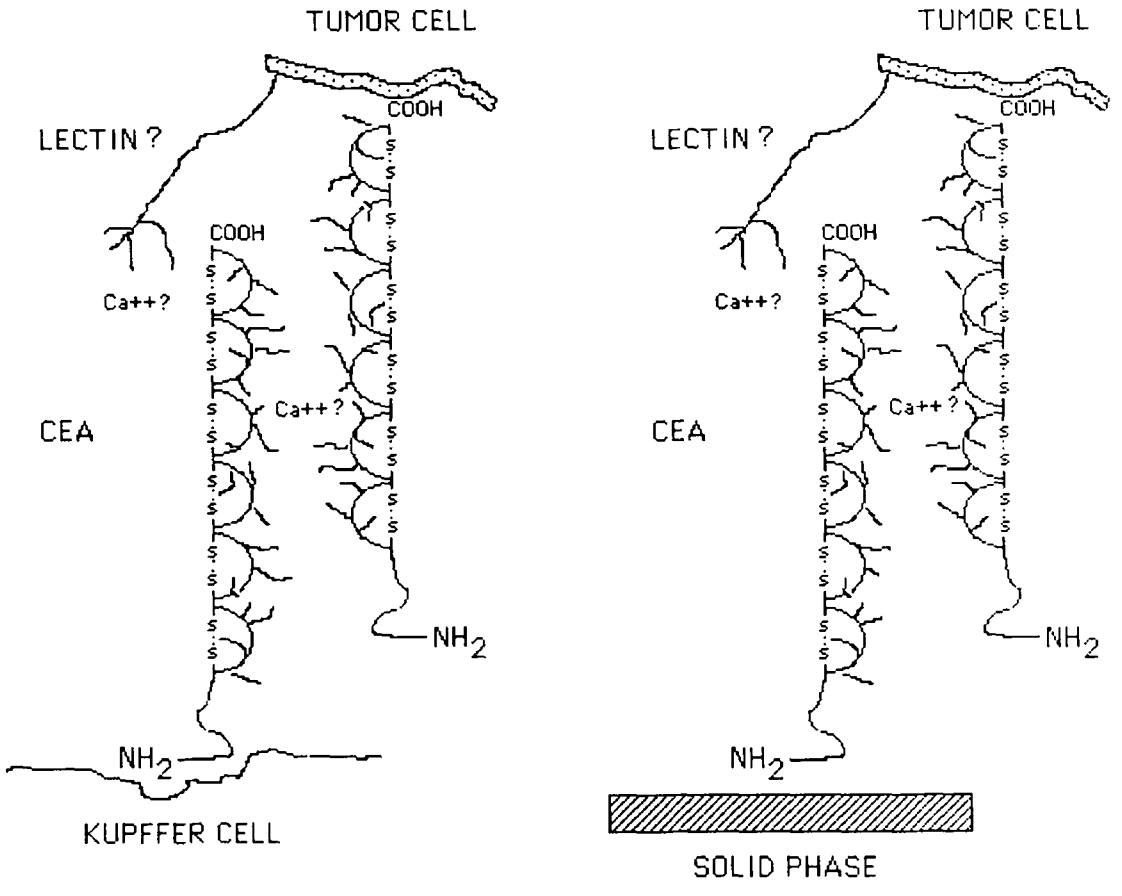

Fig. 5. Schematic representation of possible mechanisms for CEA participation in cell adhesion. The hydrophobic NH_2-terminus binds to the solid phase support and to the Kupffer cell. The actual site of binding may involve part of the N-terminal first loop domain. Homotypic aggregation would then most likely occur between the repeating loop domains, perhaps facilitated by interactions between the oligosaccharide side chains. Oligosaccharides may require divalent cations for noncovalent binding of negatively charged groups. It is possible that other molecules may be involved in the intercellular binding, e.g., lectins that bind specific oligosaccharide epitopes. The positions of the oligosaccharide side chains do not represent any of the actual 28 known N-linked glycosylation sites.

duced from pepsin digests, Thomas and colleagues have identified an amino acid sequence in the NH2-terminus first loop domain junction that binds to the Kupffer cell receptor [109]. The Kupffer cell can display both native and asialo-CEA on its cell membrane, while parenchymal cells will display only asialo-CEA on their membranes. CEA could then bind to itself to form cross-linking homotypic dimers either through peptide interactions in the repeating loop domains or through the oligosaccharides (Fig. 5). In addition, if lectins are important to this interaction they would then be correctly oriented so that the oligosaccharides would be available for binding. However, the removal of terminal sialic acids from CEA abrogated its ability to enhance metastasis by the MIP 101 tumor. Terminal sialic acids may be necessary for homotypic aggregation to occur; this suggests that lectins that do not recognize sialylated oligosaccharides may not be involved in intercellular adhesion. These relationships are suggested in Fig. 5 and are now amenable to evaluation both *in vitro* and *in vivo*.

Conclusion

CEA has been shown to be associated with recurrence in patients with CRC when the serum CEA

concentration is elevated preoperatively in patients whose carcinoma appears to be clinically limited to regional disease. While this association may be a mere reflection of the burden of subclinical disease, it is also possible that CEA actively participates in the development of metastases by CRC. We have shown that CEA may be involved in at least three of the four phases of metastasis. Redistribution of CEA within cells that lose their polarity at the advancing edge of a neoplasm may suggest a loss of intercellular adhesion that facilitates emigration from the primary site. While in the circulation (and possibly during the proliferative phase) CEA may inhibit several host defense mechanisms either by directly inhibiting lymphocyte function or through the induction of immunosuppressive factors. Finally, an important part of the effect of CEA on metastasis may be mediated during the implantation phase. Our laboratory has demonstrated that CEA enhances the implantation of certain CRC cells in the livers and lungs of nude mice. When this observation is added to that of Benchimol *et al.* [36], it is clear that CEA may be an important adhesion molecule. However, CEA is not the sole factor responsible for implantation, but may act as a co-factor for adhesion. The tools of molecular biology that are now available may allow the role of this tumor marker to be fully elucidated.

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