Tumour related inhibition of macrophage chemotaxis in patients with colon cancer

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SUMMARY The chemotactic migration *in vitro* of peripheral blood, intestinal mucosal, and mesenteric lymph node mononuclear cells has been assessed in patients with colorectal carcinoma. Peripheral blood mononuclear cells of patients exhibited normal chemotaxis. For control patients with non-malignant, non-inflammatory intestinal disease, the chemotaxis of mucosal mononuclear cells was similar to that of autologous peripheral blood mononuclear cells. The chemotactic migration of mucosal mononuclear cells, however, isolated distant from a colon cancer was less than that of autologous peripheral blood mononuclear cells. Chemotactic migration was progressively impaired with increasing closeness to the tumour itself. Chemotaxis of mucosal mononuclear cell was independent of the site of tumour and the Dukes' grading. Mononuclear cells from mesenteric lymph nodes, however, exhibited impaired migration only in patients with Dukes' C tumours. Supernatants of the collagenase digestion of either tumour or adjacent mucosa contained macrophage directed inhibitors of chemotaxis and these inhibitors were not produced by tumour mononuclear cells. The presence of such inhibitors in the digestion supernatants and the demonstration that proximity to the tumour was associated with impaired mononuclear cell motility suggest that the production of macrophage directed chemotactic inhibitors is by colon cancer cells and that this may be occurring *in vivo*.

Macrophages are often a prominent part of the inflammatory response to tumours and may play an important role in the immunopathogenesis of neoplasia.¹ The major mechanism by which they accumulate in and around tumour tissue is probably by the chemotaxis of macrophages from surrounding tissue and of monocytes from peripheral blood. Over one half of patients with various forms of cancer have depressed chemotaxis of peripheral blood monocytes.² In animal models, such a depressed chemotactic migration is reflected in a diminished capacity to mobilise macrophages into inflammatory infiltrates.³⁻⁶ One mechanism of such inhibition appears to be *via* macrophage directed inhibitors of chemotaxis which have been shown to be synthesised by murine tumour cells⁷⁻⁹ and are present in human malignant pleural effusions.¹⁰

Little is known of the motility of monocytes and macrophages of patients with colon cancer which now ranks as the second most common cancer in the Western World. The purpose of this study was to examine the relationship between the chemotaxis of macrophages and colon cancer by determining (a) the chemotactic migration of peripheral blood monocytes and macrophages isolated from histologically normal intestinal mucosa from patients with colon cancer, (b) the effect of proximity to tumour tissue on macrophage chemotaxis, and (c) whether tumour related macrophage directed inhibitors of chemotaxis are present.

Methods

PATIENTS AND SPECIMENS

Specimens of intestinal mucosa, tumour, and mesenteric lymph nodes were obtained from patients undergoing intestinal resection for colorectal carcinoma. The tumours were staged according to

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Dukes' classification.¹¹ For all specimens, mucosa was removed distant from the tumour but, for some studies, tissue adjacent to the tumour was also examined. This was dissected leaving a clear margin of at least 5 cm of macroscopically normal mucosa around the tumour. Histological examination of adjacent mucosa revealed no evidence of infiltration with malignant cells. One half of each mesenteric lymph node studied was also examined histologically. For some patients with tumour involving the ascending colon, ileal tissue was obtained.

A small number of histologically normal intestinal specimens from patients with non-malignant non-inflammatory intestinal disease was also examined. The reasons for surgery were diverticulosis coli (two), multiple tubular adenomata (one), angiodysplasia (one), redundant sigmoid loop (one), and penetrating benign gastric ulceration (one).

Peripheral blood was taken from all patients pre-operatively and from healthy volunteers. Details of patients and controls are presented in Table 1. Of the patients with colonic carcinoma, four were receiving digoxin therapy and one was taking phenytoin. None of the control patients were receiving medication apart from the patient with gastric ulceration who was receiving ranitidine.

ISOLATION OF MONONUCLEAR CELL POPULATIONS

Intestinal mononuclear cells were isolated by a modification of the method of Bull and Bookman¹² as previously described.¹³ Briefly, the intestinal mucosa was treated with EDTA to remove epithelial cells then digested overnight with 25 U/ml collagenase (CLSPA, Worthington) in RPMI 1640 with 25 mM HEPES and supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 units/ml penicillin. The resulting suspension was centrifuged to separate the digestion supernatant (collagenase supernatant) from the cells which were then washed twice and purified over a Ficoll-Paque (Pharmacia) gradient. The collagenase supernatant was retained and stored in aliquots at -20°C until used for further study.

Mononuclear cells were isolated from tumour tissue by dissecting tumour from surrounding fat and mucosa and then subjecting it to the same process as for intestinal mucosa. The collagenase supernatant was retained as above.

Mesenteric lymph nodes were removed from the resection specimen, dissected from surrounding fat, and gently pushed through a sterile stainless steel mesh to release the mononuclear cells. The resulting cell population was washed in Hank's Balanced Salt Solution (HBSS).

Heparinised peripheral blood obtained from

Table 1 Details of patients and control subjects

	Controls	Colorectal carcinoma	Miscellaneous group
N	35	22	6
Age (range)	36 (22-44)	62 (27-81)	73 (66-82)
Male/female	27/8	17/5	3/3
Segment of intestine studied:	Ileum	5	2
	Ascending	6	—
	Transverse	2	2
	Descending	3	3
	Rectum	10	—
Dukes' classification:	A	1	—
	B	11	—
	C	10	—

patients and controls was centrifuged over a discontinuous Ficoll-Paque gradient¹⁴ and the mononuclear cell population thus obtained washed three times in HBSS.

All mononuclear cell populations were suspended in Eagle's medium with Earle's salts (Gibco) supplemented with L-glutamine and antibiotics as above. No further purification steps were performed. The cells were counted in a haemocytometer (Neubauer chamber) and the cell viability assessed by 0.1% trypan blue exclusion. The proportion of macrophages or monocytes in the mononuclear cell populations was estimated in cyto-centrifuge preparations stained with May-Grunwald-Giemsa and non-specific esterase (using alpha-naphthyl acetate as substrate).¹⁵ The tumour mononuclear cell population was also characterised by examining toluidene blue stained 0.75 µm sections of cells fixed in 0.4% glutaraldehyde.

ASSAYS OF CHEMOTAXIS AND RANDOM MIGRATION

Chemotactic and random migration of mononuclear cells were determined by a modification of the Boyden chamber method¹⁶ as described by Wilkinson¹⁷ using the leading front technique.¹⁸ 0.2 ml of mononuclear cells at a concentration of 5 million cells per ml was poured into the upper compartment of the modified Boyden chamber. The lower compartment was filled with either chemotactic factor (chemotaxis) or Eagle's medium with Earle's salts (random migration). Both upper and lower compartments were divided by cellulose ester filters with a mean pore size of 8 µm (Millipore). The chambers were incubated for two hours at 37°C in a humid atmosphere with 5% CO₂, 95% air. The filters were then removed, fixed in ethanol, stained with Harris haematoxylin, washed in water, dehydrated in alcohol, cleared in butanol and xylol, and mounted on a glass slide. The distance in micrometres moved by the leading front was measured in 15 randomly

selected high power fields with the micrometre attachment of a Leitz Laborlux II microscope. Every test was run in triplicate so that each result represents the mean of 45 observations. The variation from the mean was always less than 10%. The leading cell front was taken to be the lowest plane through the filter in which three or more cells were in focus. For each assay involving patient mononuclear cells, peripheral blood from at least one healthy subject was also assayed. Chemotaxis of peripheral blood mononuclear cells from three subjects were assayed repeatedly and were found to be reproducible within 10%.

The migrating cell was inferred to be a monocyte or macrophage on the following criteria: (a) it was morphologically phagocytic; (b) polymorphonuclear leucocytes and eosinophils were not present in the mononuclear cell populations; and (c) the distance of migration of lymphocytes through similar cellulose ester filters is much less than that of phagocytes.¹⁹

PREPARATION OF CHEMOTACTIC FACTORS

For most experiments, the chemotactic factor was prepared by activating autologous or heterologous plasma with 5 mg/ml zymosan (Sigma) for 30 minutes at 37°C. It was used undiluted.

For some studies, lymphocyte derived chemotactic factor was used as chemoattractant and was prepared by culturing normal peripheral blood mononuclear cells at a concentration of 2 million cells/ml in Eagle's medium with Earle's salts supplemented with L-glutamine and antibiotics as above with 1% v/v phytohaemagglutinin (PHA, Wellcome) for 48 hours at 37°C in a humidified atmosphere with 5% CO₂. The culture fluid was centrifuged at 1500 g for 10 minutes, the supernatant stored in 4 ml aliquots at -70°C, and used as chemoattractant undiluted. A supernatant was also produced by culturing peripheral blood mononuclear cells under the same conditions but in the absence of phytohaemagglutinin. This together with a 1% solution of phytohaemagglutinin in Eagle's medium with Earle's salts were used as controls for the lymphocyte derived chemotactic factor.

ASSAY OF MACROPHAGE-DIRECTED INHIBITORS OF CHEMOTAXIS

The collagenase supernatant from intestinal mucosa or tumour was examined for the presence of inhibitors of monocyte chemotaxis that may be released during the overnight digestion. Normal peripheral blood mononuclear cells were incubated overnight at 37°C in collagenase supernatant or, as a control, collagenase in supplemented RPMI medium as used in the isolation process. In additional experiments, to elimination of residual debris contamination,

collagenase supernatants were filtered through sterile 0.22 µm filters (Millipore).

To ascertain whether the inhibitors of chemotaxis were produced by mononuclear cells, tumour and intestinal mononuclear cells from four patients were cultured independently for 24 hours at a concentration of 2×10^6 cells/ml in supplemented Eagle's medium (described above), with and without phytohaemagglutinin (1% v/v). The supernatants were centrifuged at 1500 g for 10 minutes and stored in 2 ml aliquots at -70°C.

Control peripheral mononuclear cells were incubated with these undiluted supernatants (10^7 cells in 2 ml) for 30 minutes at 37°C in 5% CO₂, 95% air and then their chemotactic and random migration determined. Undiluted zymosan activated autologous plasma was used as chemoattractant.

STATISTICAL ANALYSIS

Experimental values have been expressed as mean $\pm$ standard deviation where appropriate. Statistical analyses were performed by Student's *t* test or by Wilcoxon's rank-sum-test.

Results

CHARACTERISTICS OF ISOLATED MONONUCLEAR CELL POPULATIONS

The proportion of macrophages or monocytes was similar in peripheral blood, intestinal, tumour, and mesenteric lymph node mononuclear cell populations and comprised 6 to 12% of mononuclear cells. Mononuclear cell viability ranged between 80 and 90% for tumour and lymph nodes and was always above 85% for mononuclear cells isolated from intestine. In no mononuclear cell population were polymorphonuclear leucocytes or eosinophils seen. The cell population from tumour comprised less than 3% tumour cells.

CHEMOTACTIC AND RANDOM MIGRATION

The random migration of mononuclear cells from peripheral blood and intestine was similar in all patient groups and controls. No difference between the chemotaxis of peripheral blood mononuclear cells of the patients with cancer and healthy controls was found. The chemotaxis of mononuclear cells isolated from intestinal mucosa more than 5 cm from tumour was, however, significantly less than that of autologous peripheral blood mononuclear cells ($p < 0.02$, Fig. 1). Similar chemotaxis was observed for intestinal mononuclear cells isolated from the various anatomical regions of the intestine. For patients with miscellaneous intestinal disease, the chemotaxis of intestinal mononuclear cells was

Fig. 1 Chemotactic migration of peripheral blood (●) and colonic (○) and ileal (□) mononuclear cells (MNC) stimulated with zymosan activated plasma in patients with colorectal carcinoma and miscellaneous intestinal diseases and in normal subjects. Bars represent mean $\pm$ SD. * Student's *t* test and Wilcoxon's rank-sum-test.

similar to that of autologous peripheral blood mononuclear cells.

CHEMOTAXIS AND PROXIMITY TO TUMOUR

The chemotactic migration of mononuclear cells isolated from within tumour tissue was less than that from colonic mucosa adjacent to (less than 5 cm from) tumour which, in turn, was less than that from colonic mucosa distant (greater than 5 cm) from tumour (Fig. 2). Colonic mononuclear cells distant from tumour also moved significantly more slowly than that of autologous peripheral blood mononuclear cells. The Dukes' grading did not influence the chemotaxis of mucosal or tumour mononuclear cells. The chemotaxis of mononuclear cells from mesenteric lymph nodes draining the tumour depended, however, on the presence of metastases – in patients with Dukes' C cancer, chemotactic migration was significantly lower than that in Dukes' A and B cancer ($p < 0.02$). No difference was observed

in the random migration of mononuclear cells from the tumour itself (48.4 ± 7.8 μm), from mucosa adjacent to 49.0 ± 8.8 μm) or distant from tumour (43.2 ± 7.8 μm), or from mesenteric lymph nodes (45.1 ± 10.4 μm).

CHEMOTAXIS OF TUMOUR MONONUCLEAR CELLS TO LYMPHOCYTE DERIVED CHEMOTACTIC FACTOR

To assess if the depressed chemotaxis of mononuclear cells isolated from tumour was because of a loss of reactivity to complement-derived chemotactic factors, chemotaxis using lymphocyte derived chemotactic factor was compared with that using zymosan activated plasma as chemoattractant. As presented in Table 2, no difference in chemotactic migration was found in five experiments. No chemotactic activity was observed for phytohaemagglutinin in Eagle's medium alone or for supernatants from peripheral blood mononuclear cells cultured without phytohaemagglutinin.

Fig. 2 Relation of chemotactic migration of mononuclear cells (MNC) towards zymosan activated plasma to proximity to tumour. † Indicate lymph node metastases. * Student's *t* test.

PRESENCE OF MACROPHAGE DIRECTED INHIBITORS OF CHEMOTAXIS

Overnight incubation of normal peripheral blood mononuclear cells with the collagenase supernatant from mucosa distant from tumour or from mucosa of the miscellaneous group had no effect on chemotaxis (Fig. 3). Collagenase supernatant from tumour markedly inhibited the chemotactic migration of normal peripheral blood mononuclear cells, however, while that from mucosa adjacent to tumour had a smaller but still highly significant inhibitory effect (Fig. 3). Control experiments incubating mononuclear cells in supplemented RPMI containing collagenase had no effect on chemotaxis. 0.22 µm filtration of collagenase supernatants from tumour did not alter their potency to inhibit chemotaxis in two experiments.

No evidence was found for the production of chemotactic inhibitors by tumour or mucosal mononuclear cells even when stimulated with phytohaemagglutinin. Hence, the random and chemotactic migra-

tion of normal peripheral mononuclear cells was not impaired by prior incubation with the supernatants from phytohaemagglutinin stimulated or unstimulated cultures of either tumour or mucosal mononuclear cells.

Discussion

The chemotactic migration towards complement derived chemotactic factors of mononuclear cells from peripheral blood of patients with colon cancer is normal. This contrasts with previous reports that about 60% of patients with cancer of many different origins have depressed monocyte chemotactic responsiveness.² It is unlikely that this discrepancy is related to the selection or the relatively small number of patients studied but does indicate that colon cancer affects the chemotaxis of peripheral blood monocytes less than many other types of cancer.

Abnormalities of the chemotaxis of mucosal mononuclear cells are, however, present in colon

Table 2 Chemotactic response of mononuclear cells isolated from tumour and from normal peripheral blood to lymphocyte-derived chemotactic factor and to zymosan activated plasma ($\mu\text{m} \pm \text{SD}$)

	Normal mononuclear cells (n=5)	Tumour mononuclear cells (n=5)
Zymosan activated plasma	142.1 $\pm$ 4.0	93.3 $\pm$ 14.7*
Lymphocyte derived chemotactic factor	119.8 $\pm$ 14.2	93.3 $\pm$ 15.8†
Culture supernatant without phytohaemagglutinin	48.4 $\pm$ 5.2	47.3 $\pm$ 3.4
Eagle's medium with 1% phytohaemagglutinin (vol/vol)‡	46.9 $\pm$ 4.2	46.0 $\pm$ 4.6
Eagle's medium (random migration)‡	50.1 $\pm$ 4.5	48.8 $\pm$ 3.5

* $p < 0.001$; † $p < 0.02$; ‡controls for lymphocyte derived chemotactic factor.

cancer. The small group of patients with non-malignant intestinal disease studied suggests that isolated intestinal mononuclear cells exhibit migration similar to that of autologous peripheral blood mononuclear cells. In patients with colon cancer, mucosal cells taken more than 5 cm from tumour demonstrate chemotaxis which is significantly slower than that of autologous peripheral blood mononuclear cells. No evidence that this may be caused by artefactual effects of the isolation process were found since collagenase, overnight culture, and collagenase supernatant from mucosa distant from tumour did not influence the chemotaxis of normal mononuclear cells. As autologous peripheral blood mononuclear cells migrated normally, this depression of mucosal mononuclear cells chemotaxis is not a manifestation of a generalised mononuclear phagocyte defect but is more likely secondary to local mucosal inhibition.

The origin of such inhibition was related to the tumour tissue. Proximity of mucosal mononuclear cells to tumour was directly related to depression of chemotaxis and tumour mononuclear cells exhibited grossly impaired motility. In addition, a macrophage directed inhibitor of chemotaxis was present in the supernatant from collagenase digestion of tumour and, to a lesser extent, mucosa adjacent to tumour. In preliminary experiments, no evidence for the production *in vitro* of an inhibiting factor from tumour mononuclear cells could be found. Thus it is more likely that the tumour cells themselves are producing the factors and that surrounding mucosal macrophage chemotaxis is suppressed by local diffusion of such factors. This is in accord with rodent studies in which potent tumour derived factors inhibiting tissue accumulation and *in vitro* chemotaxis of macrophages have been demonstrated.⁷⁻⁹ Characterisation of this factor has shown it to be a

Fig. 3 Effect of collagenase supernatants on chemotaxis of normal peripheral blood mononuclear cells. Cells were preincubated for 15 h in the supernatant remaining after overnight collagenase digestion of intestinal mucosa or tumour, washed, and then assessed for chemotactic migration towards zymosan activated plasma. The results were compared with the chemotaxis of fresh mononuclear cells and of mononuclear cells preincubated in supplemented RPMI medium containing 25 U/ml collagenase. Results are expressed as mean $\pm$ SD. The number of patients studied in each group are shown with each bar. * $p < 0.001$ compared with cells preincubated with collagenase in supplemented RPMI (Student's *t* test).

19000 dalton protein which is physicochemically and antigenically related to a P15 (E) structural protein of retroviruses.⁹ A protein of similar antigenic specificity and functional effect has been isolated from human cancerous effusions.¹⁰

Characterisation of the inhibiting factors in this study has been limited. It is present in the soluble fraction of collagenase supernatant and produces irreversible suppression of macrophage chemotaxis. It probably does not act *via* specific macrophage receptor inhibition as tumour mononuclear cells demonstrated equally depressed migration towards complement-derived (zymosan-activated plasma) and lymphocyte-derived chemotactic factors. The possibility, however, that the depressed chemotaxis of tumour mononuclear cells is caused by *in vivo* receptor desensitisation with the inflammatory mediators, C5a and lymphocyte derived chemotactic factor has not been excluded. Its mechanism of action may involve suppression of metabolic activity as impairment of adherence and cytotoxicity by mononuclear phagocytes has also been shown in patients with cancer.^{20, 21} Non-specific toxic effects, however, were not evident as collagenase supernatant from tumour tissue did not reduce viability any more than

collagenase supernatant from mucosa distant from tumour (data not shown). More precise characterisation of the inhibitory factors from colonic cancer and their relationship to the retroviral envelope protein, P15 (E), have to be determined.

In conclusion, this study highlights the marked effect colon cancer tissue may have on the motility of mucosal and nodal macrophages without a concomitant effect on peripheral blood monocytes. The release of macrophage directed inhibitors of chemotaxis probably from tumour cells during the collagenase digestion of tumour and adjacent mucosa, together with the association of proximity to tumour with inhibition of macrophage chemotaxis, support the hypothesis that the production by colon cancer cells of such inhibitors is occurring *in vivo*.

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