Location of tumour necrosis factor α by immunohistochemistry in chronic inflammatory bowel disease

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Abstract

This study determined the location and tissue density of cells immunoreactive for tumour necrosis factor α (TNF α) in intestinal specimens from 24 patients with chronic inflammatory bowel disease (15 with Crohn's disease, nine with ulcerative colitis) and 11 controls. There was significantly increased density of TNF α immunoreactive cells in the lamina propria of both ulcerative colitis and Crohn's disease specimens, although the distribution of these cells differed in the two conditions. In ulcerative colitis most of the TNF α immunoreactivity was seen in the subepithelial macrophages, with comparatively less in the deep lamina propria, while in Crohn's disease immunoreactive cells were distributed evenly throughout the lamina propria. Increased submucosal immunoreactivity was found only in Crohn's disease, in which TNF α positive macrophages tended to cluster around arterioles and venules, often infiltrating and disrupting vascular endothelium. It is suggested that this degree of TNF α production probably contributes significantly to the pathogenesis of both Crohn's disease and ulcerative colitis, by impairing the integrity of epithelial and endothelial membranes, increasing inflammatory cell recruitment, and by prothrombotic effects on the vascular endothelium.

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Activated macrophages are prominent in the inflammatory infiltrate in both ulcerative colitis and Crohn's disease.¹⁻⁷ There is known to be local production of the macrophage derived cytokines interleukin-1 (IL-1) and tumour necrosis factor α (TNF α),⁸⁻¹⁰ in inflammatory bowel disease, and these may be detected at raised concentrations in the serum in active disease.¹¹ Serum concentrations of interleukin-6 are also raised in active Crohn's disease but surprisingly not in ulcerative colitis.¹² The importance of macrophage products as agents of intestinal inflammation has been shown in animal studies by the induction of intestinal necrosis by TNF α and platelet activating factor¹³ and the protective effect of IL-1 blockade in immune complex colitis.¹⁴ In previous studies of the possible role of TNF α in inflammatory bowel disease we have shown that much higher concentrations are found in the stools during relapse¹⁵ (up to 5-10 μ g TNF α /g) than in serum, thus implying high concentrations in inflamed mucosa. If such high concentration mucosal production does occur, it is probable that specific treatment to reduce TNF α production will be of

clinical benefit, and it is therefore important that the extent of local production is assessed more directly. In this study we have used immunohistochemistry to determine the distribution and density of TNF α containing cells, in the mucosa and submucosa of patients with chronic inflammatory bowel disease.

Methods

PATIENTS

Fifteen surgically resected specimens from patients with Crohn's disease were studied. Four specimens were from the jejunum and 11 from terminal ileum or ascending colon. Twelve of these patients were receiving corticosteroid treatment and three elemental diet at the time of surgery. Nine further specimens, including two colonoscopic biopsy specimens, were obtained from patients with ulcerative colitis, all of whom were receiving steroid treatment. The diagnosis of Crohn's disease or ulcerative colitis had previously been made on the basis of repeated colonoscopy, histological tests, and contrast radiography, and all specimens showed histological evidence of inflammation. The Table gives the clinical details of these patients and treatments received. Six of 11 control specimens were resected from adults (aged 18-64), whose

Clinical details of patients studied

			Activity indices		Treatment		
	Age	Sex	ESR (mm in first hour)	CRP (mg/dl)	Steroid (mg/ kg)	ASA	Location
<i>Crohn's disease</i>							
1	11	F	35	45	E	—	Colon
2	11	M	12	30	0.2	+	Colon
3	13	F	18	26	0.6	+	Colon
4	11	M	70	115	E	—	Ileum
5	14	F	45	35	1.6	+	Colon
6	14	M	26	34	0.4	+	Colon
7	14	M	23	32	0.6	—	Jejunum
8	16	F	20	6	E	—	Jejunum
9	14	M	20	5	0.8	—	Jejunum
10	17	M	35	40	E	—	Jejunum
11	9	F	42	28	0.3	—	Colon
12	16	M	21	36	1.0	+	Ileum
13	13	F	22	14	0.75	+	Colon
14	14	M	24	80	0.7	+	Ileum
15	13	M	35	42	1.0	+	Ileum
<i>Ulcerative colitis</i>							
1	2	F	18	14	1.2	+	Colon
2	13	M	35	10	0.2	—	Colon
3	11	F	45	40	0.3	—	Colon
4	4	M	30	23	1.0	+	Colon
5	11	M	56	56	0.6	+	Colon
6	11	F	25	5	0.2	+	Rectum
7	13	M	49	23	1.5	+	Colon
8	10	F	10	5	0.4	+	Colon
9	9	F	40	18	0.5	+	Colon

E=Elemental diet; steroid=prednisolone dose (mg/kg/day); ASA=treatment with aminosalicylic acid (sulphasalazine or mesalazine); ESR=erythrocyte sedimentation rate; CRP=C reactive protein.

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indications for surgery included polyposis, chronic constipation, and carcinoma (two specimens were of terminal ileum, the rest colonic; all were histologically normal). The other five were histologically normal colonoscopic biopsy specimens taken from children investigated for abdominal pain. All biopsy specimens included muscularis mucosae; however only one contained sufficient submucosa for analysis of submucosal staining. Specimens were frozen in liquid nitrogen and stored at -70°C until analysis, when sections cut at $7\text{ }\mu\text{m}$ were applied to poly-L-lysine coated microscope slides and fixed in acetone before immunostaining.

ANTIBODIES USED

CB0006, an IgG1 $\times$ purified mouse monoclonal antibody to human TNF α (a kind gift from Celltech Ltd, UK) was used throughout (dilution 1/100). This antibody, which neutralises TNF α cytotoxicity on L929 cells and blocks TNF α induced MHC class I expression has been given therapeutically to humans suffering from septic shock,¹⁶ and is used to assay TNF α by enzyme linked immunosorbent assay (ELISA).¹⁷ It displays no cross reactivity with TNF β , interleukin-1 or interleukin-6 in ELISA or cold competition assay.¹⁴ Monoclonal antibodies to HLA-DR (1/100, Dako Ltd, Slough), CD25 (1/100 Dako), and CD3 (UCHT1, an undiluted supernatant) were used to stain serial sections. Control slides were stained with either TRIS buffered saline or an isotype matched monoclonal antibody (anti-cytokeratin, 1/100, Dako). After incubation with primary antibody for one hour at room temperature, staining was developed using the alkaline phosphatase anti-alkaline phosphatase (APAAP) technique with fast red substrate, as previously described.¹⁸

QUANTITATION

The density of TNF α + cells within lamina propria and submucosa was determined with a point counting technique, using a Lennox graticule over at least five randomly chosen high power fields within each region and counting as positive graticule points overlying positively stained cells.¹⁹ As many specimens showed

heterogeneous mucosal staining, with predominant subepithelial immunoreactivity, we subdivided the lamina propria arbitrarily into subepithelial (within one high power field of the epithelium) and deep (within one high power field of the muscularis mucosa). We excluded graticule points overlying the crypts from the initial count and subtracted the density of endogenously positive cells found in the matched control slides (median 29 cells/mm² (range 4–78) in disease specimens; median 3.5 cells/mm² (range 0–18) in controls). Reproducibility of counts on blinded testing was within 10%.

STATISTICAL ANALYSIS

As the resultant percentages within groups were not always normally distributed, group medians were calculated with 95% confidence intervals (derived by the Wilcoxon method) and comparison was made between groups using the two tailed Mann-Whitney U test.

Results

LAMINA PROPRIA

TNF α + cells were present in the lamina propria of six of the 11 normal controls, but these were few in number and largely confined to the subepithelial zone (median density 5.5 cells/mm², 95% confidence intervals 1.6 to 20.9). Subepithelial TNF α + cell density was significantly increased compared with controls in both ulcerative colitis (145.0 cells/mm², 110.3 to 184.5, $p < 0.0001$) and Crohn's disease (148.0 cells/mm², 118.9 to 162, $p < 0.0001$) (see Figs 1A, 2). These large cells had the phenotypic appearance of macrophages, often showing extending processes (Fig 2), and similar cells on serial sections stained HLA-DR+, CD25+, CD3-. In addition the monoclonal antibody showed weak cross reactivity with epithelium.

The pattern of staining differed in the deep lamina propria, where the density of TNF α + cells was significantly greater in Crohn's disease (118.2 cells/mm², 85.1 to 159) than in ulcerative colitis (28.8 cells/mm², 10.9 to 64.3, $p < 0.0005$) or controls (1.9 cells/mm², 0 to 3.8, $p < 0.0001$ – Fig 1B). TNF α + cells were still however higher

Figure 1: Density of tumour necrosis factor α (TNF α) immunoreactive cells, as determined by point counting with a Lennox graticule, in tissue from normal controls and patients with Crohn's disease or ulcerative colitis (UC) in (A) subepithelial lamina propria, (B) deep lamina propria, and (C) submucosa. The filled squares were biopsy specimens and the open squares surgically resected. Among the Crohn's disease specimens, the open circles represent jejunal tissue, the diamonds terminal ileum, and the squares ascending colon.

Figure 2: Tumour necrosis factor (TNF) α immunoreactive cells in chronic inflammatory bowel disease. Top left – lamina propria in Crohn's disease, showing a moderately dense infiltrate of red stained TNF α + cells (magnification $\times 200$); middle left – subepithelial TNF α + macrophages, characteristic large cells with processes, in ulcerative colitis (magnification $\times 400$). The basal epithelial staining is a result of weak cross reactivity (also seen in the crypt in top left panel; top right – a dense infiltrate of TNF α + cells with macrophage morphology in Crohn's disease submucosa (magnification $\times 100$); middle right – a higher power view of submucosal TNF α + macrophages in Crohn's disease (magnification $\times 400$); bottom left – vasculopathy in Crohn's disease submucosa. The small arteriole in the centre is surrounded by TNF α + cells (magnification $\times 400$).

in ulcerative colitis than controls ($p < 0.005$). The morphology of TNF α + cells was more variable within the deeper lamina propria, particularly in Crohn's tissue (Fig 2). In addition to those with macrophage morphology, a population of smaller and more densely stained cells, could be identified. These cells were probably lymphocytes, as similar cells in serial sections were found to be CD3+, CD25+. In addition smaller numbers of TNF α + cells were identified on morphological grounds as polymorphonuclear leucocytes. TNF α + cells were also seen in Crohn's granulomas and in the germinal centres of lymphoid follicles: the second were large CD3– cells and were presumably tingible body macrophages. No evidence of TNF α immunoreactivity was found elsewhere within the follicles.

SUBMUCOSA

Increased submucosal TNF α immunoreactivity

was found in Crohn's disease but not in ulcerative colitis. TNF α + cell density was significantly increased in Crohn's tissue (median density 137.0 cells/mm², 95% confidence intervals 94.6 to 190.2) compared with both controls (2.30 cells/mm², 0 to 15.5, $p < 0.0002$) and ulcerative colitis specimens (7.0 cells/mm², 2.1 to 14.7, $p < 0.0002$) (Figs 1C, 2). All of the Crohn's disease specimens showed evidence of transmural TNF α immunoreactivity, with increased numbers of TNF α + macrophages also found in muscularis externa and serosa.

There was evidence in all Crohn's specimens of accumulation of TNF α + cells, predominantly CD3– cells with macrophage morphology, around submucosal and serosal blood vessels (Fig 2). This perivascular TNF α + accumulation was frequently associated with disruption of vascular smooth muscle and endothelium, and thrombosed or recanalised arteries were seen in eight of 15 specimens. Such perivascular concentration of TNF α + cells was not

seen in ulcerative colitis or in Crohn's lamina propria.

Discussion

We have found TNF α immunoreactive cells at high density within the lamina propria in both Crohn's disease and ulcerative colitis and also within Crohn's submucosa. The extent of this TNF α immunoreactivity confirms that the very high concentrations we have detected in stools during relapse of inflammatory bowel disease give a more accurate estimate of local inflammation than does the modest increase in serum TNF α . Although the difficulty of obtaining true serial sections in frozen tissue prevents formal confirmation of the lineage of these TNF α cells, we have found similar patterns of immunoreactivity in preliminary work with a second monoclonal antibody 52 B 83 (also from Celltech), and have confirmed, with a reverse haemolytic plaque assay, that most mucosal TNF α secreting cells are macrophages (E J Breese, T T MacDonald, submitted data). We suggest that, whatever the lineage of the TNF α producing cells, the effects of such high local concentrations are likely to be adverse.

TNF α produced by subepithelial cells probably impairs epithelial integrity,²⁰ particularly in the presence of interferon γ .^{21,22} Interferon γ may also synergise with TNF α in several proinflammatory ways, increasing expression of adhesion molecules²³ and MHC class I and II,^{23,24} and upregulating macrophage free radical production.²⁵

There is clearly the potential within the intestine for direct activation of subepithelial macrophages by lipopolysaccharides from the luminal flora, particularly after any breach of epithelial integrity. In Crohn's disease epithelial permeability is increased,²⁶ and it is noteworthy that faecal diversion may be protective.²⁷ The subepithelial predominance of TNF α immunoreactivity in ulcerative colitis is additionally concordant with findings of epithelial deposition of immunoglobulin and complement,⁶ now known to be related to expression of the 40 kD putative autoantigen,²⁸ as well as the subepithelial localisation of procollagen RNA production.²⁹

The perivascular production of TNF α in Crohn's disease, also concordant with known patterns of complement deposition,³⁰ is likely to be particularly damaging. It binds to high affinity endothelial cell surface receptors,³¹ and acts to increase inflammatory cell recruitment by upregulating expression of both adhesion molecules³² and the chemotactic cytokine interleukin-8.³³ The vascular effects of TNF α include increase in permeability^{20,34} and powerful promotion of thrombosis³⁵ by reduction of anti-coagulant and activation of procoagulant mechanisms. It reduces the production of thrombomodulin,³⁶ a natural anti-coagulant, which inactivates factor Va^{35,37} and increases synthesis of plasminogen activator inhibitor-1.³⁸ TNF α additionally induces expression of the powerfully procoagulant tissue factor,³⁹ released only at sites of endothelial damage⁴⁰ where it activates the extrinsic limb of the coagulation cascade.⁴¹

Although TNF α infusion causes general activation of procoagulant pathways,⁴² its action seems preferentially targeted towards dividing endothelial cells – that is, at sites of injury, where much lower concentrations will induce this spectrum of prothrombotic change.⁴³ There is now substantial evidence that vascular disruption and focal thrombosis, related to macrophage infiltration, occurs in Crohn's disease.⁴⁴ The production of both the procoagulant TNF α and the potent vasoconstrictor endothelin-1⁴⁵ by perivascular macrophages will contribute powerfully towards thrombosis in this situation.

In view of the high density of TNF α producing cells in active inflammatory bowel disease, and the well characterised effects of this cytokine, it is probable that TNF α makes an independent contribution to inflammatory tissue damage. If this can be confirmed in animal models of intestinal inflammation, clinical use of anti-TNF α monoclonal antibodies or therapeutic inhibition of its production by agents such as transforming growth factor β ,⁴⁶ interleukin-10⁴⁷ or thalidomide^{48–50} may provide potent and selective alternatives to current immunosuppressive treatment.

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