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Expression of adhesion molecules in human intestinal graft-*versus*-host disease

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SUMMARY

The distribution of three cellular adhesion molecules, ICAM-1, ELAM-1 and VCAM-1, was studied in normal rectal mucosa and in graft-*versus*-host disease (GvHD) using immunohistological and morphometric techniques. In normal controls, ICAM-1 was demonstrable on endothelial cells and leucocytes within the lamina propria, ELAM-1 on endothelial cells only and VCAM-1 on lamina propria leucocytes, many of which exhibited long dendritic processes surrounding the glands. In GvHD, the enterocytes became positive for ICAM-1 and this was often associated with the presence of intra-epithelial LFA-1$^+$ lymphocytes and macrophages, the latter containing debris of apoptotic cells. The staining was, however, restricted to the luminal membrane of the epithelial cells, raising doubts about the role of ICAM-1 as a ligand for LFA-1 on mucosal leucocytes in rectal GvHD. ELAM-1 expression was increased in GvHD both in terms of the length of positive endothelium and staining intensity. VCAM-1 was increased on endothelial cells but not leucocytes in the lamina propria in contrast to our previous findings in cutaneous GvHD where VCAM-1$^+$ dendritic cells were increased and endothelial cells remained negative. Normal patterns of adhesion molecule staining were seen in two biopsies exhibiting no morphological evidence of GvHD, from patients who had strong clinical evidence of the disease, indicating that immunostaining for these molecules is unlikely to be of help in improving the sensitivity of histological diagnosis. However, the possibility that adhesion molecule staining may be useful in improving diagnostic specificity by helping to distinguish GvHD from identical histological changes produced by irradiation and cytotoxic drugs is worthy of further investigation.

Keywords GvHD bone marrow transplantation adhesion molecules

INTRODUCTION

Acute graft-*versus*-host disease (GvHD) of the large intestine following allogeneic bone marrow transplantation is characterized clinically by diarrhoea and, in severe cases, by haemorrhage and mucosal sloughing. The most consistent histological feature seen in rectal biopsies is individual cell necrosis (or apoptosis) at the base of crypts, which may progress to total glandular loss and ulceration. However, similar histological changes may be seen in cytomegalovirus infections [1] and for up to 3 weeks following the administration of cytotoxic drugs and irradiation to condition patients before transplantation [2]. The histological diagnosis of rectal GvHD is thus limited and more sensitive and specific markers of the disease are required. Studies of the early cell and molecular events occurring in the tissues affected by GvHD may identify changes of possible diagnostic value in addition to giving insights into local mechanisms of tissue damage.

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HLA-DR expression has been shown to be increased on enterocytes in GvHD [3] and reported to be a useful early diagnostic marker, but others have found more variable results [4]. Immunophenotypic studies of leucocytic infiltrates in rectal GvHD have shown characteristic patterns of infiltration but identified no features of diagnostic value [4]. However, these studies have been of interest in showing that the composition of leucocytes is different in the rectum, skin and liver, raising the possibility that local pathogenetic mechanisms may vary from tissue to tissue.

Cellular adhesion molecules (CAMs) mediate leucocytic infiltration of tissues and, like HLA-DR antigens, may be upregulated by local cytokine release. We have recently undertaken studies of the adhesion molecules ICAM-1, ELAM-1 and VCAM-1 in cutaneous GvHD [5,6] and found increases on various cells which could interact with donor T cells.

The present study was therefore undertaken: (i) to determine if the expression of these adhesion molecules is increased in the rectal mucosa in GvHD; (ii) to determine whether the pattern of expression differs from that in the skin; and (iii) to identify any

changes which would be worthy of further investigation as diagnostic markers of the disease.

PATIENTS AND METHODS

Eleven rectal biopsies were obtained from 10 bone marrow recipients between days 28 and 55 post-transplantation, for the investigation of diarrhoea. The patients included seven males and three females with a mean age of 29 years (range 4–42 years). The underlying disease was acute myeloid leukaemia in six cases, chronic granulocytic leukaemia in one, acute lymphoblastic leukaemia in two and lymphoblastic lymphoma in one. Pre-transplantation conditioning regimens varied according to the primary disease but included total body irradiation and melphalan or cyclophosphamide and busulphan. GvHD prophylaxis included cyclosporin A alone or with methotrexate.

Four specimens of normal large bowel were selected as controls, two from the uninvolved margins of resections for colonic carcinoma and two from rectal biopsies taken for investigation of diarrhoea unrelated to immunosuppressive therapy or bone marrow transplantation.

The rectal biopsies were divided in half. One half was immediately snap frozen in liquid nitrogen, and the remainder fixed in formalin and embedded in paraffin in the routine manner.

Seven of the 11 rectal biopsies from bone marrow recipients showed histological evidence of GvHD, three Grade I, three Grade II and one Grade III [7]. The remaining four biopsies showed no histological abnormality, although all were performed for the investigation of diarrhoea. Two patients had cutaneous GvHD and their skin and gut problems resolved on immunosuppressive therapy with corticosteroids. The other two had no other evidence of GvHD and their gastrointestinal symptoms eventually settled without treatment.

Serial 5-μm cryostat sections were cut, air dried for 1 h at room temperature and fixed in formol calcium/chloroform. Immunostaining was performed by the biotin–streptavidin alkaline phosphatase technique using the following monoclonal antibodies: 1.2B6 against ELAM-1 and 1.4C3 against VCAM-1 [8], both 1/50; 15.2 against ICAM-1 (supplied by Dr Nancy Hogg, ICRF, London, UK) used neat; L243 against HLA-DR (Becton Dickinson, Oxnard, CA) at 1/20; LFA-1 against the alpha chain of the leucocyte function associated-1 protein, CD11a antigen (Dako, High Wycombe, UK) at 1/50; and EBM11 against macrophage antigen CD68 (Dako) at 1/50. Rabbit polyclonal antibody to Factor VIII related antigen (Dako) was used at 1/100 dilution.

The sections were sequentially incubated with: (i) monoclonal or polyclonal antibodies at their optimal dilutions; (ii) biotinylated anti-mouse IgG 1/100 (Vector Laboratories, Peterborough, UK) or biotinylated anti-rabbit IgG 1/400 (Amersham International, Amersham, UK); and (iii) streptavidin alkaline phosphatase conjugate 1/100 (Amersham International). Each incubation lasted 1 h and was followed by 5 min wash in Tris-buffered saline 0·05 M pH 7·6. The colour reaction was developed with the substrate naphthol AS-B1 phosphate and fast red TR salt.

Morphometric studies were undertaken using Colour-Morph, a video-based, computerized semi-automated image analysis system (software supplied by Precision Instruments Ltd) attached to a Leitz Laborlux microscope. This allowed measurement of the total length and mean intensity of positively stained vascular endothelium in each section. The length of ELAM-1 and VCAM-1 positive endothelium was expressed as a percentage of the total length of Factor VIII positive endothelium in adjacent 5-μm sections in which variation in endothelial length or any other histological feature is negligible. Densitometric measurements were performed with standardized microscope settings and illumination. The amount of light absorbed is proportional to the concentration of the absorbing substance (Bouguer–Beer absorption law). The light transmitted through the positive vascular endothelium is compared to a standard grey scale and expressed as a mean grey level for the area covered by the reaction product. Results in Table 1 are expressed as transmission, i.e. the percentage of incident light which passes through the specimen. Thus, lower values are obtained where staining intensity is greater.

Table 1. Endothelial staining intensity for ICAM-1 and ELAM-1

	Mean percentage of light transmitted through stained endothelial cells	
	ICAM-1	ELAM-1
1 Non-transplanted controls	58·4 (53–66·2)	74·1 (68·2–76·3)
2 Post-transplant normal histology	55·4 (47·6–59·6)	71·38 (67·6–73·2)
3 Post-transplant GvHD	53·5 (38–58·5)	55·4 (48·1–67·6)

1: (ELAM-1): GvHD *versus* groups 1 & 2 $P<0·02$. No other differences significant.

2: Values are percentages of light transmitted, i.e. lower values reflect greater intensity of staining.

3: VCAM-1 excluded as there was little or no staining in control groups.

Statistical analysis

Statistical analysis was performed using the rank sum test.

RESULTS

ICAM-1

In histologically normal large bowel mucosa from bone marrow transplant recipients and non-transplant controls, ICAM-1 immunostaining was seen on leucocytes in the lamina propria and on the vascular endothelium but not on the glandular epithelium. Biopsies from bone marrow recipients with histological features of GvHD differed from the two control groups by showing positive staining of the luminal surface of the glandular epithelium (Fig. 1a). This was sometimes focal and variable in intensity but was present in all cases. Staining of cells in the lamina propria in GvHD appeared to be increased subjectively but was difficult to quantify.

No significant increase in the intensity of vascular endothelial ICAM-1 staining was found using densitometric measurements (Table 1). Measurements of length were not undertaken as virtually all endothelium was positive in the normal state.

ELAM-1

ELAM-1 staining was confined to vascular endothelial cells. No positivity of glandular epithelium or lamina propria leucocytes

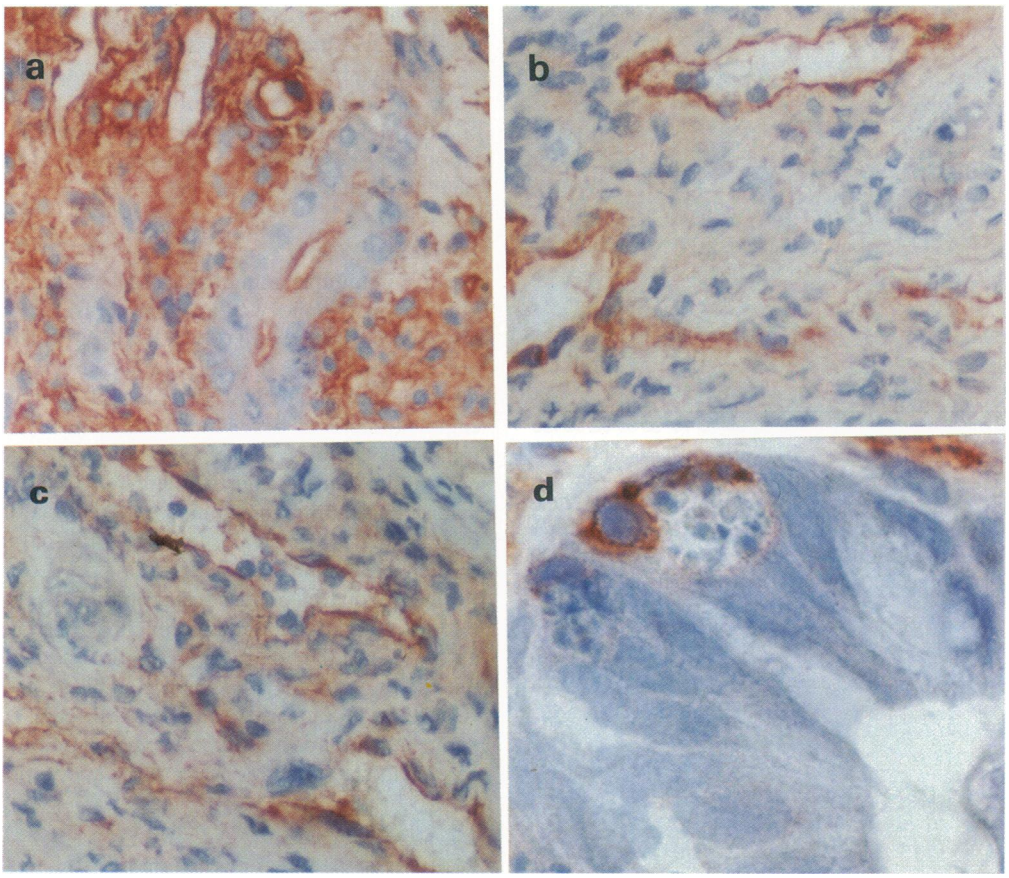

Fig. 1. Immunohistochemical staining of rectal biopsies with GvHD. (a) ICAM-1; positive staining of vascular endothelium (top left), lamina propria leucocytes and the luminal surface of a gland (right). (b) ELAM-1; positive staining of vascular endothelium. (c). VCAM-1; positive staining of vascular endothelium and a few dendritic cells in the lamina propria. (d) LFA-1; apoptotic cellular debris within the cytoplasm of an intra-epithelial LFA-1 positive macrophage.

Table 2. Endothelial staining for ELAM-1 and VCAM-1

	Length of stained endothelium as percent of Factor VIII rag	
	ELAM-1 (%) Median (range)	VCAM-1 (%) Median (range)
1 Non-transplanted controls	13·9 (5–22·8)	0
2 Post-transplant normal histology	26·5 (6·6–39)	0·8 (0–2·7)
3 Post-transplant GvHD	46·2 (36–79·4)	16·7 (8·3–46)

1: (ELAM): GvHD *versus* groups 1 and 2 $P<0{\cdot}02$. Differences between groups 1 and 2 not significant.

2: (VCAM-1): GvHD *versus* group 1 $P<0{\cdot}01$. GvHD *versus* group 2 $P<0{\cdot}02$. Differences between groups 1 and 2 not significant.

3: ICAM-1 excluded as virtually all endothelium was positive in controls.

was seen in any of the cases studied. In biopsies from transplant recipients without GvHD and non-transplant controls, there was weak, patchy endothelial staining of a proportion of mucosal blood vessels. There was no significant difference between these two groups. In GvHD, however, endothelial ELAM-1 expression was increased in terms of both the length of positive endothelium (measured as a percentage of that stained for Factor VIII rag) and the intensity of staining (Fig. 1b; Tables 1 and 2).

Factor VIII rag is expressed on vascular endothelium and lymphatics, so a measurement of total length of positive vessels in a section will include both. The morphological features of ELAM-1 and VCAM-1 positive vessels suggested they were blood rather than lymphatic in type but a lack of suitable markers does not allow determination of the precise nature of the vessels involved. Values for total length of ELAM-1 or VCAM-1 positive vessels thus fall short of 100% of Factor VIII length, but comparisons betwen groups can be made, and statistical analysis applied.

Comparing densitometric measurements of the intensity of ELAM-1 staining with the length of positively stained endothelium showed a significant positive correlation using Spearman's rank correlation test ($P=<0{\cdot}05$) (Fig. 2).

Where GvHD changes within a biopsy were patchy, ELAM-1 staining was noted to be most intense on blood vessels in the worst affected areas of the mucosa.

VCAM-1

Biopsies with normal histology from both transplant and non-transplant control groups showed VCAM-1 positivity of cells in

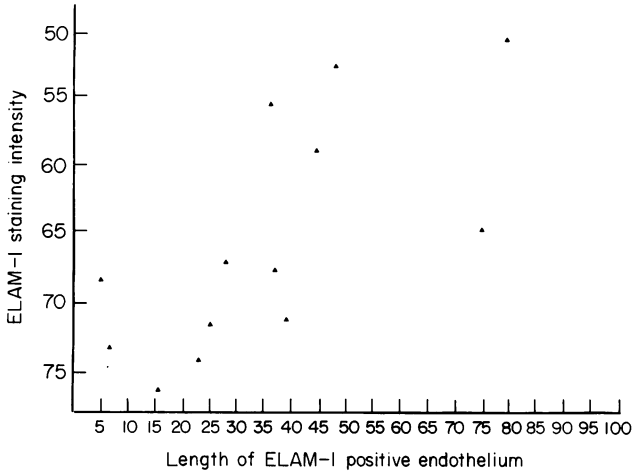

Fig. 2. Comparison of the intensity of ELAM-1 staining (expressed as mean percentage of light transmitted through stained cells) with the length of ELAM-1 positive endothelium (expressed as a percentage of Factor VIII positive endothelium) in control biopsies and those showing GvHD.

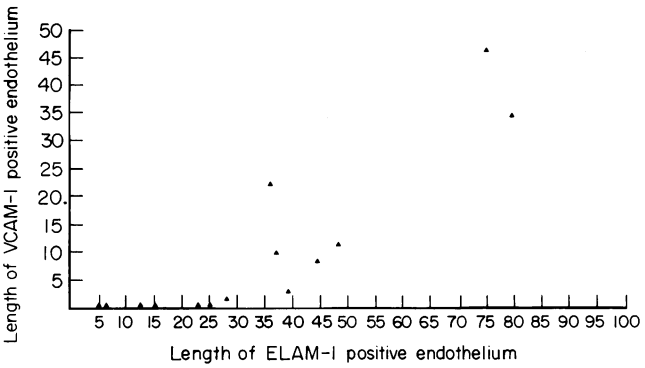

Fig. 3. Comparison of the length of endothelium stained with VCAM-1 and ELAM-1 (both expressed as a percentage of Factor VIII positive endothelium) in control biopsies and those showing GvHD. The control biopsies show little or no VCAM-1 positivity.

the lamina propria with fine dendritic cytoplasmic processes. These were frequently seen around the basement membrane of rectal glands. Occasional lymphocytes were also VCAM-1 positive. No epithelial staining was seen. Non-transplant controls showed VCAM-1 negative vascular endothelium, whereas post-transplantation normal biopsies showed occasional weakly VCAM-1 positive endothelial cells.

In GvHD there was a significant increase in VCAM-1 positivity on mucosal vascular endothelium (Fig. 1c). Results were expressed only as percentage length of positive endothelium in view of the almost complete negativity of control groups (Table 2). As with ELAM-1, staining was greater in areas where the histological changes of GvHD were most severe.

The staining of dendritic cells in the lamina propria in GvHD was similar to that in the control groups. Cell counting showed that VCAM-1 positive cells comprised a similar percentage of total lamina propria cells in GvHD as in all biopsies with normal histology (29% *versus* 32%). No epithelial staining with VCAM-1 was present in GvHD.

For each biopsy studied, the percentage length of ELAM-1 positive endothelium was plotted against the percentage length of VCAM-1 positive endothelium. A significant positive correlation was shown using the Spearman rank correlation coefficient ($P = < 0{\cdot}05$) (Fig. 3).

LFA-1 and EBM11

Positive immunostaining for LFA-1 was seen in normal rectal mucosa on lymphocytes and macrophages. In biopsies with histological features of GvHD, LFA-1 identified increased numbers of intra-epithelial lymphocytes, together with many macrophages situated at the base of the glands containing engulfed debris of degenerate cells (Fig. 1d). These apoptotic fragments characteristic of GvHD were frequently seen to be not within vacuoles, but in the cytoplasm of LFA-1 positive macrophages. Immunostaining of serial sections with an antibody to EBM11 (CD68) confirmed the nature of the intra-epithelial macrophages.

Debris of fragmented rectal epithelial cells in GvHD was also occasionally seen either engulfed by adjacent enterocytes and shed into the lumen, or free within the lamina propria just outside the glandular basement membrane.

Although increased numbers of LFA-1 positive intra-epithelial lymphocytes were seen in GvHD, they did not have a consistent spatial relationship to the apoptotic epithelial cells.

HLA-DR antigens

Positivity of glandular epithelium was seen in all cases showing GvHD, in three out of four post-transplant specimens with normal histology but none of the non-transplanted cases.

DISCUSSION

In this study we have shown increased expression of three cellular adhesion molecules on various cells in the large bowel mucosa in acute GvHD. In normal controls, ICAM-1 was demonstrable on vascular endothelial cells and lamina propria leucocytes, ELAM-1 was weakly expressed on vascular endothelial cells and VCAM-1 on dendritic cells and other leucocytes in the lamina propria. In GvHD, glandular epithelial cells became positive for ICAM-1 with staining restricted to the luminal membrane. There was increased endothelial ELAM-1 expression as measured by staining intensity and percentage length of positive endothelium. VCAM-1 positivity was significantly increased on vascular endothelium in GvHD, but no increased numbers of VCAM-1 positive dendritic cells were seen in the lamina propria.

ICAM-1 (CD54) is a member of the immunoglobulin supergene family of adhesion molecules and binds the $\beta 2$ integrin LFA-1 (CD11a/CD18) [9]. It is constitutively expressed on many cells and may appear on others in inflammation [10]. Expression can be induced *in vitro* by the addition of interferon-gamma (IFN-γ) or tumour necrosis factor-alpha (TNF-α) [11]. We have previously shown increased expression on epidermal keratinocytes in cutaneous GvHD [5]. In the present study, ICAM-1 was increased on rectal epithelial cells in GvHD but only on luminal membranes. Its role as a ligand for LFA-1 on intramucosal leucocytes in rectal GvHD is thus questionable.

ELAM-1 is a member of the selectin family, and can be induced *in vitro* by the addition of IL-1α, IL-1β or TNF-α to endothelial cell cultures. In cardiac and renal allograft rejection, however, ELAM-1 expression has been found not to be increased [12,13]. We found increased endothelial ELAM-1 in rectal GvHD in a fashion similar to that we have previously reported in the skin [6], raising the possibility that it may be important in leucocytic infiltration of both tissues in this disorder. As sophisticated and time-consuming morphometric techniques were required to demonstrate significant changes, any value for anti-ELAM-1 antibodies in the clinical diagnosis of GvHD would be very limited.

VCAM-1 is another member of the immunoglobulin supergene family which binds to VLA-4 (CD49d/CD29) on lymphocytes and monocytes [14], and is inducible *in vitro* on cultured endothelial cells by the addition of TNF or IL-1 [8]. Immunohistological studies have shown expression on endothelium in a variety of tissues in inflammation as well as other cells including dendritic cells, macrophages, synovial and mesothelial cells [15]. The increased expression of VCAM-1 on post-capillary venules in cardiac allografts in the presence of T cell infiltration [12] suggests a role in mediating lymphocyte influx during transplant rejection.

In the normal rectum, we found VCAM-1 positive dendritic cells in the lamina propria, many with long cytoplasmic processes extending around glands. These cells were similar to those we have previously noted encircling blood vessels and accessory structures in the skin [6]. Their anatomical location and morphology suggests a role in regulating immune responses, possibly in presenting antigen to donor lymphocytes. It is worth noting that VCAM-1 positivity is also seen on interdigitating reticular cells in lymph nodes [8,16].

Whereas cutaneous GvHD was associated with a striking increase in VCAM-1 positive dendritic cells in the dermis, no such increase was seen in the lamina propria of the rectum. Furthermore, the increase in VCAM-1 endothelial staining in rectal GvHD was not seen in the skin where increases were detected only in perivascular dendritic cells. Previously, it has been noted that the composition of the leucocytic infiltrate is different in rectal and cutaneous GvHD [4]. Whether this is a consequence of differences in endothelial function in the skin and rectum is not clear but these two studies clearly show that the cell and molecular changes are different in the two organs involved by the same disease. The possibility that the mechanisms of tissue damage may not be the same has to be considered.

Immunostaining with antibodies to LFA-1 and EBM11 (CD68) in rectal GvHD confirmed lymphocytic infiltration of glandular epithelium and also showed the majority of degenerate cellular fragments at the base of crypts to be within macrophages, not in 'vacuoles' or 'empty spaces' as previously described [2,17]. Occasional fragments were also seen within adjacent enterocytes towards the glandular lumen, arguing that the macrophages were merely phagocytosing debris from degenerate cells rather than inducing cell death.

Two of the post-transplant control biopsies in the present study were from patients who exhibited strong clinical evidence of GvHD and positive staining of enterocytes for HLA-DR antigens. It is not clear why such patients lack morphological evidence of GvHD. Possibilities include a minor or early form of the disease or that the changes are focal and have not been included in the biopsy. In both cases, the pattern of adhesion molecule expression did not differ from that of the other controls and this is consistent with the observation that in cases with morphological evidence of GvHD, staining for adhesion molecules is greater in the areas of epithelial damage. It therefore seems unlikely that immunostaining for ICAM-1, ELAM-1 or VCAM-1 can improve the sensitivity of histological diagnosis. However, staining for these molecules may have some value in improving diagnostic specificity by helping to distinguish GvHD from similar changes induced by irradiation and cytotoxic drugs in the early period after transplantation, particularly as in our experience enterocyte HLA-DR staining is not entirely specific for GvHD during this period. No biopsies taken within 21 days of transplantation were available for evaluation in the present study but this is worthy of further investigation.

Any diagnostic value is likely to concern ICAM-1 expression on epithelial cells and VCAM-1 on endothelium as these were the only changes that were readily observed without the need for very elaborate and time-consuming morphometry. Given that adhesion molecules and HLA-DR antigens are induced by various cytokines, the demonstration *in situ* of these molecules in tissues affected by GvHD would give further insights into the complex molecular changes occurring in the early phases of the disease and possibly be of greater diagnostic value.

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