Cellular distribution of human leucocyte adhesion molecule ICAM-3

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Abstract

Aims—To describe the distribution of the recently cloned human leucocyte adhesion molecule ICAM-3 in normal and neoplastic tissues and cell lines.

Methods—A panel of four monoclonal antibodies to ICAM-3 were used to stain cell lines and sections of human lymphoid tissues using the alkaline phosphatase-anti-alkaline phosphatase immunocytochemical method (APAAP).

Results—In peripheral blood ICAM-3 was detected on monocytes, granulocytes, and most lymphocytes. In sections of human lymphoid tissue the antigen was also found on most lymphocytes, but many of the proliferating B cells found in the germinal centres of secondary lymphoid follicles were ICAM-3 negative. ICAM-3 was also found on neoplastic white cells (in chronic lymphocytic leukaemia, hairy cell leukaemia, acute and chronic myeloid leukaemia, and multiple myeloma) with the exception of Reed-Sternberg cells in Hodgkin's disease, many of which were negative. ICAM-3 was consistently absent from cells and tissues of non-haemopoietic origin. Endothelium (which expresses ICAM-1) was negative for ICAM-3, with the exception of vessels in some neoplastic lymphoid samples which showed variable staining for ICAM-3.

Conclusions—These findings suggest that ICAM-3 is essentially restricted to the haemopoietic system and is reciprocal in its expression to ICAM-1, in that it is present on resting cells and its level falls as a result of cell activation.

Methods

Four monoclonal antibodies (designated By44, CG106, KS128, KS118) with identical pan leucocyte reaction patterns have been identified in this laboratory over the past 10 years. These antibodies came from experiments in which murine hybridomas were prepared using standard techniques,¹¹ following immunisation with fresh B cell lymphoma cells (antibody By44), fresh T cell leukaemia cells (antibody CG106) or Karpas 299, a T cell line with a 2:5 translocation (KS128 and KS118). Initial screening was carried out on peripheral blood smears containing immunising cells (By44 and CG106) or on cryostat sections of lymph node affected by Hodgkin's disease (KS118 and KS128). Further screening was performed using normal blood smears and cryostat sections of normal tonsil.

Normal peripheral blood mononuclear cells were separated on Lymphoprep (Nygard, Oslo) and cytocentrifuge preparations were made. All slides were fixed and stored frozen as described before.¹² Tissues were snap frozen in liquid nitrogen, and cryostat sections (8 µm) were cut, fixed, and stored as described before.¹³

All cell lines were cultured at 37°C in RPMI 1640 medium containing 10% fetal calf serum (FCS) (Gibco Biocult Ltd). Cytocentrifuge preparations were made and treated as described before. To promote their differentiation towards macrophages U937 and HL60 cells were incubated with 1.6×10^{-8} M 12-O-tetradecanoylphorbol 13-acetate (TPA, Sigma Chemical Co) in RPMI 1640 containing 10% FCS for up to 12 days.

Antibodies against CD3, CD10, CD11b, CD19, CD68 and cytokeratin were obtained from DAKO a/s (Glostrup, Denmark). Antibodies to ICAM-1 (RR1/1) and ICAM-2 (CBR-IC2/1) were provided by Dr TA Springer.

The APAAP staining technique was carried out as described before.^{12,13} The enzyme reaction was developed using naphthol AS-MX phosphate and Fast Red (TR-Salt, Sigma Chemical Co) as substrate.

Immunoperoxidase staining was performed as follows: tissue sections were incubated first with a monoclonal antibody for 30 minutes, followed by peroxidase conjugated rabbit antimouse immunoglobulin (DAKO, a/s). The peroxidase reaction was developed using diaminobenzidine-hydrogen peroxide substrate.¹⁴

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Lymphocyte function-associated molecule 1 (LFA-1) is a leucocyte integrin which has a major role in cell adhesion.¹² It mediates a wide range of leucocyte interactions in immune and inflammatory responses and acts as a receptor for the intercellular adhesion molecules ICAM-1 (CD54) and ICAM-2.³⁻⁶ It has been suggested that a third form for ICAM exists^{7,8} and recently Fawcett *et al*⁹ and Vazeux *et al*¹⁰ described the molecular cloning of such a gene (designated ICAM-3), the product of which is also a ligand for LFA-1.

Figure 1 (A) Staining of WOP cell line after transfection with an ICAM-3 cDNA clone, using the anti-ICAM-3 antibody By44. Numerous positive cells are seen. Inset: cells transfected with control cDNA, stained under the same conditions.

(B–D) Staining of reactive lymphoid tissue (tonsil) with antibodies to ICAM-1, 2 and 3. (B) ICAM-1 is strongly expressed on germinal centre (GC) cells and also on vascular endothelium (arrowed) in the T cell area. (C) ICAM-2 is absent from the germinal centre but is present on vascular endothelium (arrowed). (D) ICAM-3 is expressed on many cells in the extrafollicular T cell area and on B cells in the mantle zone, but many cells in the germinal centre are negative. Vascular endothelium is negative.

(E–G) Staining of human spleen with antibodies to ICAM-1, 2, and 3. (E) ICAM-1 is seen on cells in the red pulp (RP) and also on vascular endothelium (arrowed) and connective tissue. (F) ICAM-2 is restricted to vascular endothelium (arrowed). Inset: Vascular endothelium at higher power. (G) ICAM-3 is not seen in the red pulp or on endothelial cells but is present on white cells lying within a vessel (arrowed). The inset shows this reactivity at higher power.

(H–J) Human kidney stained with antibodies to ICAM-1, 2, and 3. (H) ICAM-1 is strongly expressed on glomeruli (arrowed). These structures are weakly stained for ICAM-2 (I) and unlabelled for ICAM-3 (J).

(K–M) Staining of a section from a case of multiple myeloma for ICAM-3. (K) Negative control. (L) Strong expression of ICAM-3 on neoplastic cells and also on vascular endothelium, seen at higher power in (M).

(N) Immunostaining for ICAM-3 in a case of Hodgkin's disease showing strong staining of infiltrating lymphoid cells but weak or negative staining of Reed-Sternberg cells (seen in higher magnification below).

All staining was performed on cryostat sections using the APAAP immunoalkaline phosphatase technique, with the exception of (A) in which staining was performed by the immunoperoxidase technique on acetone fixed cytospin preparations.

For the FACS analysis, mononuclear cell suspensions were prepared as described above, and leucocytes were isolated from whole blood by red cell lysis using ammonium chloride.¹⁵ After washing, the cells were incubated for 30 minutes at 4°C with monoclonal antibody. After a further wash the cells were incubated for 30 minutes with fluorescein isothiocyanate (FITC) conjugated goat antimouse F(ab')₂ immunoglobulin (DAKO a/s). The cells were then washed, fixed in 1.5% formaldehyde in phosphate

buffered saline (PBS), and analysed in a FACScan flow cytometer (Becton Dickinson) using Consort 30 software.

In some experiments cells were double labelled by incubation for 30 minutes with FITC conjugated By44, prepared as described by Goding¹⁶ and then, after washing, the cells were incubated with phycoerythrin (PE)-conjugated antibody against CD3, CD4, CD8, CD10 or CD19 (DAKO a/s). After a final wash the cells were fixed and analysed in the FACScan flow cytometer,

Figure 2 Immunoprecipitation of ICAM-3 from surface ^{125}I labelled chronic lymphocytic leukaemia cells with antibody By44 shows a band of 104 kilodaltons, unreduced (lane A) and 110 kilodaltons, reduced (lane B). Positions of molecular weight standards are indicated.

using two colour fluorescence and Paint-a-Gate software.

For the cell transfection experiment, we used a 1.7 kilobase cDNA insert which contains the ICAM-3 gene. This was isolated as described before.⁹ COS cells were transfected with this cDNA.¹⁷ Cytospin preparations were prepared, fixed, and stored as described before, and stained using the immunoperoxidase technique. All four antibodies stained cells transfected with this clone (fig 1).

For the immunoprecipitation, leucocytes prepared from a case of chronic lymphocytic leukaemia were surface labelled with 1 mCi ^{125}I iodide (Amersham) in the presence of 20 μg Iodogen (Pierce). The cells were lysed in buffer containing 2% Nonidet P40 and protease inhibitors. The lysate material was precleared using affinity purified rabbit anti-mouse immunoglobulin (DAKO a/s) bound to *Staphylococcus A* (Staph A, Sigma). Monoclonal antibodies were added to the precleared lysate and after one hour, rabbit anti-mouse immunoglobulin bound to Staph A was added. After a further hour the Staph A was washed in buffer containing 1% NP40 and 150 mM sodium chloride. Immunoprecipitates were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by autoradiography of the dried gel using an enhancing screen.¹⁸

All four antibodies immunoprecipitated an antigen with a molecular weight of 104 kilodaltons in the unreduced state and 110 kilodaltons after reduction (fig 2). The antibodies

also recognised an antigen of the same molecular weight on western blots of unreduced samples (data not shown).

Results

CELL SUSPENSIONS

All four antibodies labelled neutrophils, monocytes, and most (>98%) lymphocytes in peripheral blood. In tonsil cell suspensions the antibodies labelled 85% of cells (the mean of five experiments). Double labelling studies of these cells showed that while all of the T cells (identified as CD3 positive cells) were also ICAM-3 positive, about 18% of B cells (CD19 positive) were unlabelled by the ICAM-3 antibody. These ICAM-3 negative B cells appeared to be germinal centre cells as they also expressed CD10 (fig 3).

TISSUES

The expression of ICAM-3 by normal cells and tissues is summarised in table 1. In the tonsil the antibodies showed extensive labelling of white cells, including mantle zone B cells, plasma cells, and T cell areas. In germinal centres a proportion of the cells were unlabelled and the percentage of these negative cells varied considerably from one germinal centre to another (fig 1). Vascular endothelium did not stain, or rarely, stained very weakly. Epithelium was negative. These results were consistent with those obtained from the flow cytometry studies.

The staining of lymphoid tissue in the spleen was very similar to that of tonsil, but red pulp macrophages were also positive. In the thymus the antibodies labelled more than 90% of cells in the medulla with fewer cells staining in the cortex (about 80% of cells were labelled). In the liver Kupffer cells were labelled, as were scattered lymphocytes.

Apart from scattered leucocytes, no labelling was seen in non-haemopoietic tissues.

We compared the tissue distribution of ICAM-1 and 2 with ICAM-3, especially with regard to endothelial labelling (fig 1).

The pattern of ICAM-3 expression by cell lines is summarised in table 2.

The antibodies labelled neoplastic cells in most leukaemias and lymphomas, as summarised in table 3. In a range of cases, including chronic lymphocytic leukaemia, hairy cell leukaemia, myeloma, immunoblastic lymphoma, and Hodgkin's disease, strong expression of ICAM-3 on vascular endothelium was also observed. This contrasted with the absent or weak staining of endothelium seen in reactive lymphoid tissue (fig 1). This staining was confined to the endothelium of small arteries, and in any one case only a proportion of these vessels were labelled. Reed-Sternberg cells and their variants in samples of Hodgkin's disease tissue were often stained negative, but because of the strongly positive staining adjacent reactive small lymphocytes, which were present around some of the Reed-Sternberg cells and their variants, it was impossible to state that they all remained unstained (fig 1).

Figure 3 Flow cytometric analysis of tonsil cells. (A) Single labelling with anti ICAM-3 shows that most cells (85%) are positive. Double labelling studies to characterise the ICAM-3 negative cells shows that they are CD19 positive (B) and CD3 negative (arrowed) (C). As they also express CD10 (arrowed) (D), they correspond to germinal centre B cells. In keeping with the tissue section staining, only about 50% of the germinal centre (CD10 positive) cells lack ICAM-3.

Table 1 Expression of ICAM-3 in normal cells and tissues

Tissue	Labelling
Peripheral blood	Monocytes, neutrophils and most (>98%) of lymphocytes
Tonsil	Mantle zones, plasma cells, T cells, and some germinal centre cells
Spleen	Mantle and marginal zones, and some red pulp macrophages
Thymus	Medulla and some cells in the cortex
Liver	Kupffer cells and scattered white cells
Lung	Scattered leucocytes
Kidney	Scattered leucocytes and distal tubules
Uterus	Scattered leucocytes
Heart	Scattered leucocytes
Muscle	Scattered leucocytes
Pancreas	Scattered leucocytes
Prostate	Scattered leucocytes
Breast	Scattered leucocytes

Normal tonsil was obtained from the ENT Department of the Radcliffe Infirmary, Oxford.
Other normal tissues obtained from the Histopathology Department of the John Radcliffe Hospital, Oxford.

Table 2 Expression of ICAM-3 by cell lines

Cells	Reactivity
Myeloid	
U937 (with or without TPA)	Positive
HL60	Positive*
Burkitt	
Daudi	Very weak
Raji	Very weak
Pre-B cell	
Nalm-1	Negative
Reh	Negative
T cell	
Jurkat	Positive
Molt-4	Negative
Karpas 299 (lymphoma)	Weak
SU-DHL-1 (lymphoma)	Negative
Erythroid	
K562	Negative
Others	
L428 (Hodgkin's disease)	Negative
Non-haematopoietic	
Kato III (gastric carcinoma)	Negative†
Hela (cervical carcinoma)	Negative
A431 (vulval carcinoma)	Negative
RVH 421 (melanoma)	Negative

*The percentage and intensity of positive cells increased after TPA stimulation.

†Only 5% of cells were stained.

The cell lines were donated as follows: U937, HL60, Daudi, Raji, Nalm-1, Jurkat, Molt-4, K562, Hela, A431, and RVH 421 by the Sir William Dunn School of Pathology, Oxford; Reh by Professor M Greaves; Kato III by Professor P Comoglio; Karpas 299 by Dr A Karpas; SU-DHL-1 by Dr M Cleary; and L428 by Dr V Diehl.

Table 3 Expression of ICAM-3 by leukaemias and lymphomas

Neoplasm	Positive cases/total
Leukaemia	
Hairy cell leukaemia	7/7
Chronic lymphocytic leukaemia	14/14
Prolymphocytic leukaemia	1/1
Myeloma	1/1
Acute myeloid leukaemia	3/3
Chronic myeloid leukaemia	2/2
Acute lymphocytic leukaemia	5/5
Non-Hodgkin's lymphoma (B cell)	
Lymphoplasmacytic lymphoma	2/2
Centroblastic/centrocytic lymphoma	11/12
Centroblastic lymphoma	9/9
Centrocytic lymphoma	3/3
Lymphoblastic lymphoma	5/7
Immunoblastic lymphoma	7/9
Non-Hodgkin's lymphoma (T cell)	
Small cell cerebriform	2/2
Angioimmunoblastic	4/5
Anaplastic large cell	2/2
Pleomorphic, large cell	5/5
Immunoblastic	5/5
Hodgkin's disease	0/5*

*Reactive lymphocytes in the cases of Hodgkin's disease were positive for ICAM-3 but the Reed-Sternberg cells and their variants were negative. (See fig 1).
Leukaemic peripheral blood samples anticoagulated with heparin, from patients attending the John Radcliffe Hospital, Oxford, were used to prepare blood smears.

Discussion

When used to study the pattern of expression of ICAM-3 on human cells, it was evident that this molecule has a novel pattern of distribution. It is constitutively expressed on most leucocytes, but a substantial minority of B cells in germinal centres, and many lymphocytes in the thymic cortex, are ICAM-3 negative. This suggests that proliferation or activation of lymphoid cells may reduce the level of expression of ICAM-3. Most cases of non-Hodgkin's lymphoma that we tested were positive for ICAM-3, but in Hodgkin's disease the antigen was absent from Reed-Sternberg cells and their variants. This is in keeping with the idea that ICAM-3 is lost following cell activation, because these cells express CD30 which is one of the antigens associated with cell activation. We also found a small number (<2%) of lymphoid cells in peripheral blood that were ICAM-3 negative. It may be that these lymphoid cells have lost surface ICAM-3 as a result of in vivo activation. This disappearance of ICAM-3 from lymphoid cells which are dividing or activated is illustrated by its absence from cells in the germinal centre of the tonsil.

ICAM-3 was essentially restricted to cells of haematopoietic origin. This was confirmed by staining cell lines, which revealed only one positive line out of four non-haematopoietic lines tested (KATO III, a gastric carcinoma), and in this exceptional sample only 5% of cells were reactive.

The results of Vazeux *et al*¹⁰ correlate well with our findings, although we could not confirm that ICAM-3 is present on lung epithelium.

The presence of ICAM-3 on resting lymphocytes and its absence from proliferating cells is in contrast to ICAM-1, which shows the reverse pattern.¹⁹ This complementary pattern was also true for endothelium, which was usually ICAM-1 positive, ICAM-3 negative. Although ICAM-3 was absent from, or only weakly expressed, on vascular endothelium in normal tissues, it was expressed strongly on endothelium in samples from a range of cases of lymphoid neoplasia, including chronic lymphocytic leukaemia, hairy cell leukaemia, myeloma and immunoblastic lymphoma. Both ICAM-1 and ICAM-2 are expressed more strongly on the high endothelial venules and other small vessels in malignant lymph nodes,²⁰ and the endothelial expression of ICAM-3 in lymphoid neoplasia possibly occurs via a similar mechanism. It remains to be established whether these concomitant increases in expression of the three members of the ICAM family have implications in terms of altered leucocyte traffic.

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