

# Tumour necrosis factor $\alpha$ stimulated rheumatoid synovial microvascular endothelial cells exhibit increased shear rate dependent leucocyte adhesion in vitro

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## Abstract

**Objective**—To investigate endothelial cell adhesion molecule expression and leucocyte adhesion to endothelial cells isolated from the microvasculature of rheumatoid arthritic synovial tissue (SMEC) in comparison with similar cells isolated from healthy subcutaneous adipose tissue (ADMEC) or from umbilical veins (HUVEC).

**Methods**—Cultured endothelial cells were treated with tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) for 2–24 hours before the assessment of cell surface E-selectin, vascular (VCAM-1) or intercellular cell adhesion molecule-1 (ICAM-1) expression. Neutrophil and T lymphocyte adhesion to TNF $\alpha$  treated endothelial cells was assessed using static and shear dependent assay systems.

**Results**—VCAM-1 expression by SMEC was significantly less sensitive to TNF $\alpha$  stimulation than HUVEC or ADMEC. E-selectin expression by SMEC appeared to be more sensitive to TNF $\alpha$  stimulation and maximal expression was about 30% greater in comparison with HUVEC or ADMEC. Sensitivity to TNF $\alpha$  induction and maximal ICAM-1 expression was similar in all three endothelial cell types. Static neutrophil adhesion to TNF $\alpha$  stimulated SMEC was significantly increased in comparison with HUVEC, however this phenomenon was dependent on the presence of neutralising antibodies to ICAM-1. At shear rates in excess of 2.4 dynes/cm<sup>2</sup> significantly more neutrophils and, predominantly CD45RO+, T lymphocytes adhered to TNF $\alpha$  stimulated SMEC than HUVEC.

**Conclusion**—Rheumatoid synovial endothelial cells differentially regulate E-selectin and VCAM-1. The increased ability of TNF $\alpha$  stimulated synovial endothelial cells to support leucocyte adhesion may help to explain the leucocyte, in particular CD45RO+ T-lymphocyte, recruitment observed in the rheumatoid synovium.

(Ann Rheum Dis 1999;58:573–581)

Rheumatoid synovitis is characterised by a large extravascular accumulation of leucocytes, primarily neutrophils and lymphocytes.<sup>1</sup> It is now well established that T lymphocytes are

the predominant perivascular lymphocytes in the rheumatoid synovium.<sup>2</sup> Although normal numbers of peripheral blood CD4+/CD29+ or CD4+/CD45RO+ “memory” and CD4+/CD45RA+ “naive” T cells are usually found in rheumatoid patients, in the synovial membrane nearly all of the T cells display a CD4+/CD45RO+ “memory” phenotype with very few cells displaying a “naive” phenotype.<sup>3</sup> The mechanisms governing leucocyte, specifically CD4+/CD45RO+ cell, accumulation in inflamed synovial tissue remain to be elucidated.

A critical early event governing leucocyte extravasation is the local expression of cellular adhesion molecules (CAMs) promoting the attachment of circulating leucocytes to small vessel endothelium. Current dogma suggest a sequential cascade of adhesion events in which transient, selectin mediated, cell interactions promote more permanent immunoglobulin-integrin mediated cell-cell adhesion and leucocyte diapedesis. Many hypotheses have been suggested to explain the selective recruitment of leucocyte sub-classes into inflamed tissues, however the prospect of different cellular adhesion molecules specifically mediating interactions between different leucocyte sub-populations has been enduringly attractive. Traditionally, interactions between E-selectin (ELAM-1, CD62E) and its ligands were thought to mediate neutrophil diapedesis specifically.<sup>4–6</sup> However, E-selectin is highly expressed at inflammatory sites where the cellular infiltrate is predominantly mononuclear<sup>7,8</sup> and more recently E-selectin has been implicated as a vascular addressin in the control of CD45RO+ memory T lymphocyte diapedesis.<sup>9–12</sup> Likewise, interactions between vascular cell adhesion molecule-1 (VCAM-1, CD106) and the  $\beta_1$  integrin, very late activation antigen-4 (VLA-4) were initially thought to control monocyte diapedesis,<sup>13</sup> however, VCAM-1 / VLA-4 mediated cell interactions are now known to be common among leucocyte classes and may even perform a similar function to that of selectins in mediating dynamic cell interactions.<sup>14</sup>

Increased E-selectin expression in rheumatoid synovial tissue has been demonstrated by immunohistochemical analysis.<sup>15</sup> T lymphocytes isolated from rheumatoid synovial tissue display increased binding to plastics coated with human recombinant E-selectin or VCAM-1 and analysis of the bound population revealed a predominance of memory T lymphocytes.<sup>12</sup> This study suggested that

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Accepted for publication  
4 May 1999

interaction of CD45RO<sup>+</sup> T lymphocytes with endothelial E-selectin may upregulate VCAM-1/VLA-4 mediated interactions in a similar manner to neutrophil interaction with endothelial cell E-selectin upregulating ICAM-1/MAC-1 (CD18/CD11b) mediated interactions.<sup>16</sup> Other studies have shown that while ICAM-1/LFA-1 interactions were important in the binding of T cells to unstimulated endothelium, VCAM-1/VLA-4 interactions act as the main mediators of T cell adhesion to interleukin 1 (IL1) stimulated endothelial cells.<sup>17</sup>

We have shown previously that isolated synovial microvascular endothelial cells (SMEC) express significantly higher levels of E-selectin on their cell surface than human umbilical vein endothelial cells (HUVEC) in response to stimulation with IL1.<sup>18</sup> The aims of this study were to investigate the effects of tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), stimulation on E-selectin, VCAM-1 and ICAM-1 expression by isolated SMEC in comparison to HUVEC and adipose derived microvascular endothelial cells (ADMEC) and to examine the relative importance of adhesion molecule expression in neutrophil and T lymphocyte adhesion to these cell types. Leucocyte adhesion was measured using standard static binding assays in conjunction with antibody adhesion blockade and also a physiologically relevant dynamic binding assay. Adherent T lymphocyte sub-populations were assessed for expression of the memory cell marker CD45RO by indirect immunofluorescence.

## Methods

Unless otherwise stated reagents were purchased from Sigma Chemical Co Ltd, Poole, UK.

### ENDOTHELIAL CELL CULTURE

HUVEC were isolated and cultured using methods modified from those described by Jaffé.<sup>19</sup> Briefly, endothelial cells were isolated by collagenase perfusion of umbilical cord veins obtained from normal full term deliveries. Isolated cells were grown in Medium 199 (Gibco BRL, Paisley) containing 30  $\mu$ g/ml endothelial cell growth supplement (ECGS), 20% v/v heat inactivated fetal calf serum (FCS) (Biological Industries, Glasgow), 17 U/ml heparin (CP Pharmaceuticals, Wrexham), 50 U/ml penicillin, 50  $\mu$ g/ml streptomycin, and maintained in a humidified atmosphere of 5% carbon dioxide/95% air.

Rheumatoid synovial microvascular endothelial cells were isolated and cultured using modifications of immunomagnetic isolation techniques previously described in detail.<sup>18</sup> Modifications of these techniques to improve cell yields and viability are detailed below. Rheumatoid synovial tissue was obtained at routine joint replacement surgery. The average age of the patients was 58.5 years, nine women and four men, the average disease duration prior to surgery was 7.5 years. Patients were not receiving non-steroidal anti-inflammatory drugs (NSAIDs) nor disease modifying agents immediately before surgery. Synovial membrane material was removed and minced into

approximately 3 mm<sup>3</sup> tissue pieces. With the aid of a dissecting microscope, tissue pieces containing vessels with a lumen diameter >150  $\mu$ m were removed and discarded. The remaining tissue pieces were incubated in 0.2% collagenase (CLS4, Worthington Biomedical Corporation, New Jersey)/0.05% bovine testes hyaluronidase in Hanks's balanced salt solution for 60 minutes at 37°C before brief mechanical homogenisation. Undigested tissue pieces were removed from the homogenate by sequential filtration through 250  $\mu$ m, 100  $\mu$ m and 55  $\mu$ m nylon filters. The final filtrate was repeatedly passed through a flame polished siliconised glass pipette, to disaggregate small aggregates of cells into a single cell suspension. Cells were pelleted by centrifugation (400 *g* for five minutes) before resuspension in 1 ml of PBS/1% BSA containing 1  $\mu$ l of anti-CD-31 antibody (1 mg/ml mouse IgG<sub>1</sub>, R&D Systems, Oxford) for 30 minutes on ice. Unbound antibody was removed by washing the cells three times in PBS/1% BSA. A total cell count was performed and endothelial cell number was estimated as 5% of the total. Endothelial cells were isolated by the addition of antimouse IgG<sub>1</sub> coated paramagnetic Dynabeads (Dyna, Sweden) at a ratio of 3:1 (beads:endothelial cells) for 20 minutes in 1 ml PBS/1% BSA on ice. Endothelial cells bound to Dynabeads were retained in a permanent magnetic field while non-bound cells were removed by aspiration. This aspirate was retained for further endothelial cell isolation. The remaining bound endothelial cells were washed three times with PBS/1% BSA to remove contaminating cells. The procedure was repeated to harvest those endothelial cells that escaped the first isolation by the re-addition of Dynabeads to the aspirate, assuming the endothelial cell proportion to be 1% of the total. Anti-CD31 antibodies were used in preference to Ulex European agglutinin-1 (UEA-1)<sup>18</sup> as although CD31 attached beads were found to be easier to dissociate from cells they offered better selectivity toward endothelial cells than UEA-1 coated beads, ensuring very high purity of sorted cell populations.

Isolated cells were cultured directly and usually shed attached beads after 96 hours in culture. Isolated SMEC were grown on fibronectin coated plastic or glass (5  $\mu$ g/cm<sup>2</sup>) in Medium 199 supplemented with 15% v/v FCS and 15% v/v heat inactivated human AB serum with the addition of 20 ng/ml human recombinant epidermal growth factor (EGF) and 50  $\mu$ g/ml ECGS.

Human microvascular endothelial cells were isolated from subcutaneous adipose tissue (ADMEC) obtained from healthy donors undergoing breast reduction surgery or apronectomies. Subcutaneous adipose tissue (50–100 g) was minced and disaggregated by collagenase (0.1% CLS4) for 60 minutes. ADMEC were isolated and cultured in a similar manner to the methods described in detail for SMEC. At confluence, SMEC and ADMEC cultures were maintained in HUVEC culture medium for 48 hours before assay.

All isolated cells characterised as endothelial in origin displayed all of the following "endothelial" markers; contact inhibited growth with cells adopting a cobblestone morphology at confluence, von Willebrand factor expression, CD31 expression, metabolism of fluorochrome labelled acetylated low density lipoprotein (DiI-Ac-LDL) and the ability to differentiate into "vessel-like" tubes when cultured on the extracellular matrix substitute Matrigel<sup>20</sup> (Collaborative Research, Massachusetts). All cells were used for experimentation at passage 3 and were >95% homogeneous as assessed by the above criteria, the small minority of non-endothelial cells were not characterised further.

#### ADHESION MOLECULE CELL BASED ENZYME LINKED IMMUNOSORBENT ASSAY (CELISA)

Endothelial cell adhesion molecule expression was assessed by a CELISA technique, based on methods developed by Wellicome *et al.*<sup>21</sup> Confluent monolayer endothelial cell cultures were stimulated with human recombinant TNF $\alpha$  (0–100 IU/ml) (Biological Standards Institute, Potter Bar, UK) for six hours before paraformaldehyde lysine periodate (PLP) fixation (10 minutes at 4°C). Adhesion molecule expression was assessed by a standard single layer ELISA technique using mouse anti-VCAM-1 (clone 1.4c3), anti-E-selectin (clone 1.2b6) and anti-ICAM-1 (clone 6.5b5) primary antibodies (kindly provided by Dr D O Haskard, Hammersmith Hospital). PLP fixed cells were incubated in PBS containing 1% BSA for 20 minutes before the addition of primary antibodies. Cells were incubated in primary antibody solutions for 120 minutes at

room temperature before gentle washing and addition of a biotinylated goat-antimouse immunoglobulin secondary antibody. The cells were further washed before the addition of an avidin/biotin/horseradish peroxidase solution (ABC complex) (Dako). The relative binding of the ABC complex was ascertained by the addition of a peroxide solution and the colourimetric conversion of the chromagen 1,2-Phenylenediamine dihydrochloride at 492 nm using a Bio-tek EL12E multiwell plate reader (Anachem, Wycombe, England). Cell monolayers were inspected after the CELISA protocol detailed above, any wells containing damaged (incomplete) cell monolayers were ignored during subsequent data analysis. Adhesion molecule expression was directly compared between SMEC and HUVEC and then SMEC and ADMEC in separate experiments in which absolute optical density varied. To compare results obtained from these different experiments, results are expressed as a percentage of the optical density of SMEC stimulated with 100 IU/ml TNF $\alpha$ .

The possibility that endotoxin contamination of the culture media additives might stimulate CAM expression was investigated by Limulus amoebocyte lysate assay. Results (not shown) quantified SMEC medium endotoxin levels as 200-fold less than that required for HUVEC CAM activation. As an added precaution against differing culture medium inducing false positive adhesion molecule expression, SMEC, ADMEC and HUVEC were maintained in the same culture medium for 48 hours before assay.

VCAM-1 expression kinetics were assessed using a similar method. Cells were stimulated

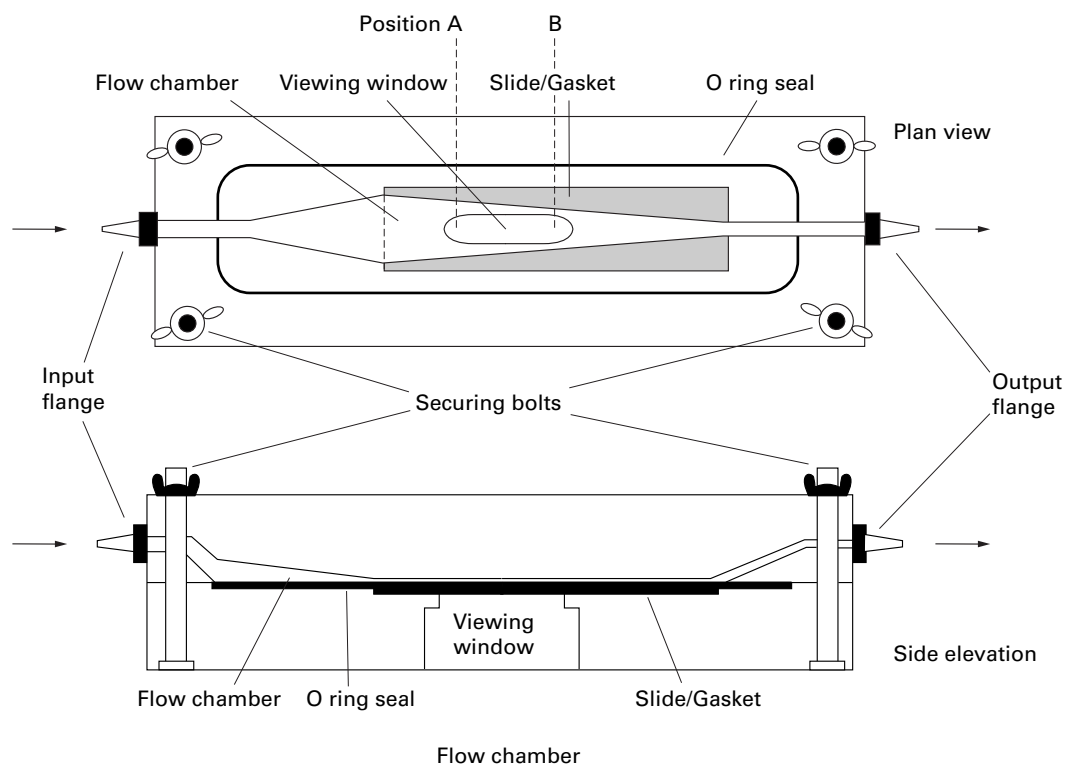
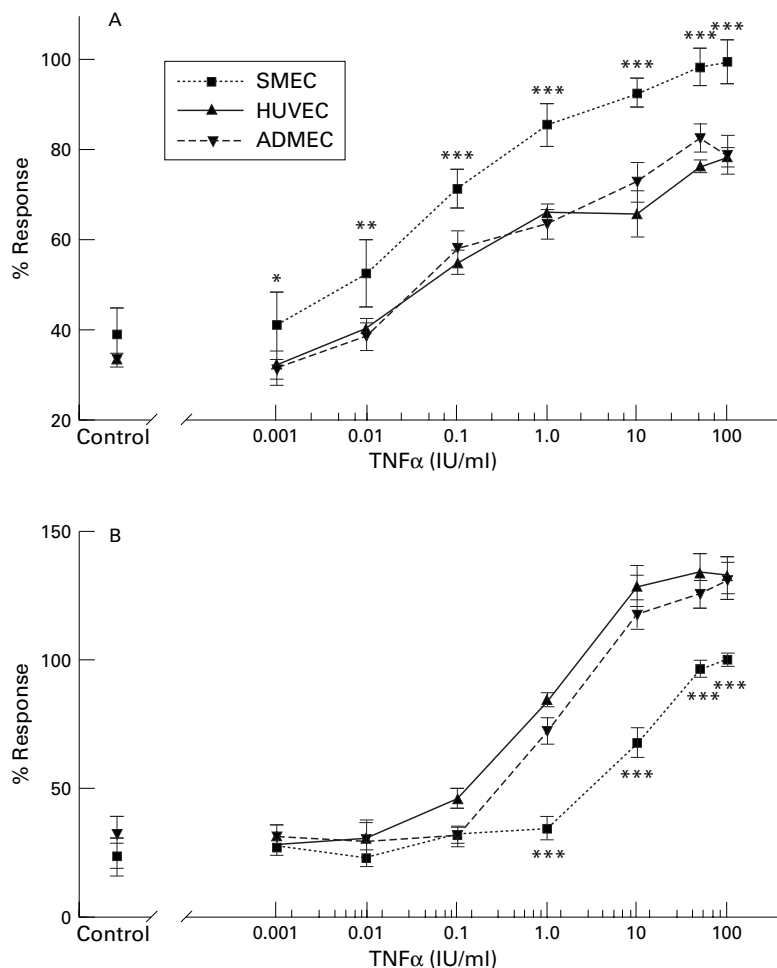


Figure 1 Schematic representation of the parallel plate flow cell used. The convergence of the side walls of the cell increases the velocity of the perfusate as it travels through the cell. This increase in velocity permits measurements to be made on cells exposed to an increasing wall shear stress in the same system without changing the mass flow volume. Wall shear stress at position A ( $\tau_A$ ) = 2.4 dynes/cm<sup>2</sup> and  $\tau_B$  = 4.0 dynes/cm<sup>2</sup> assuming a volumetric flow rate of 0.916 cm<sup>3</sup>/sec (55 ml/min).



**Figure 2** (A) HUVEC, ADMEC and SMEC monolayers were stimulated TNF $\alpha$  (0–100 IU/ml) for six hours before assessment of E-selectin expression. Results are normalised as percentages of the optical density observed in SMEC cultures stimulated with 100 IU/ml TNF $\alpha$  and depict 95% confidence intervals for the mean response. E-selectin expression by SMEC was compared with both HUVEC and ADMEC. SMEC display significantly ( $p < 0.05^*$ ,  $p < 0.01^{**}$ ,  $p < 0.001^{***}$ ) higher E-selectin expression than HUVEC or ADMEC at all concentrations of TNF $\alpha$ , ( $n=3$ ). (B) HUVEC, ADMEC and SMEC monolayers were pre-incubated with TNF $\alpha$  (0–100 IU/ml) for six hours before assessment of VCAM-1 expression. Results are normalised as percentages of the optical density observed in SMEC cultures stimulated with 100 IU/ml TNF $\alpha$  and depict 95% confidence intervals for the mean response. VCAM-1 expression by HUVEC and ADMEC was not significantly different. SMEC display significantly ( $p < 0.001^{***}$ ) lower VCAM-1 expression than HUVEC or ADMEC at concentrations of TNF $\alpha$  in excess of 1 IU/ml, ( $n=3$ ).

with TNF $\alpha$  (50 IU/ml) at two hour intervals for 0–24 hours, before fixation and assessment of VCAM-1 surface expression.

#### LEUCOCYTE ISOLATION

PMN neutrophils were isolated from whole blood, obtained by venopuncture from healthy volunteers, by density dependent centrifugation over Histopaque ( $\rho=1.077$  g/ml), by the methods of Bøyum.<sup>22</sup> Isolated cells were characterised as neutrophils by the presence of multi-lobed nuclei in greater than 98% of the cells.

T lymphocytes were isolated from similar blood samples by the following method. Total mononuclear cells were isolated by density dependent centrifugation. T lymphocytes were obtained from this cell fraction by purging of B lymphocytes and monocytes. Monocytes were removed by adhesion to tissue culture plastic flasks when incubated for 30 minutes at 37°C. B lymphocytes were removed by positive immunomagnetic selection using anti-CD19 coated Dynabeads. After monocyte purging the

isolated cells were counted and B cell proportions were estimated as 30% of the total, anti-CD19 coated Dynabeads were incubated with the B lymphocytes at a ratio of 20:1 for 30 minutes at 4°C. After incubation, B lymphocytes bound to Dynabeads were retained in a permanent magnetic field while T lymphocytes were removed by aspiration. T cell purity was generally greater than 91% as assessed by anti-CD2 (Dako Ltd, High Wycombe, UK) FACS analysis. The proportion of CD45RO+ cells in this population was assessed in a similar manner using FITC conjugated anti-CD45RO+ antibody (Dako).

#### STATIC LEUCOCYTE ADHESION ASSAYS

Static leucocyte adhesion assays were performed according to methods devised by Cavender.<sup>23</sup> Isolated leucocytes were labelled with <sup>51</sup>Chromium (Na CrO<sub>4</sub>) (300  $\mu$ Ci/ $1 \times 10^7$  cells) for 90 minutes at 37°C. These cells were added to endothelial cell monolayers grown in fibronectin (5  $\mu$ g/cm<sup>2</sup>) coated 96 well plates that had been previously stimulated for six hours with 0–100 IU/ml TNF $\alpha$  or 0–1000 IU/ml IL1. After incubation for 30 minutes at 37°C in M199/10% FCS unbound cells were removed by aspiration. The percentage of bound leucocytes was calculated by lysing endothelial cells and leucocytes (1% Triton X-100 overnight at 4°C) and gammacounting the lysate using a Beckman 5500 gammacounter.

Percentages were calculated as:

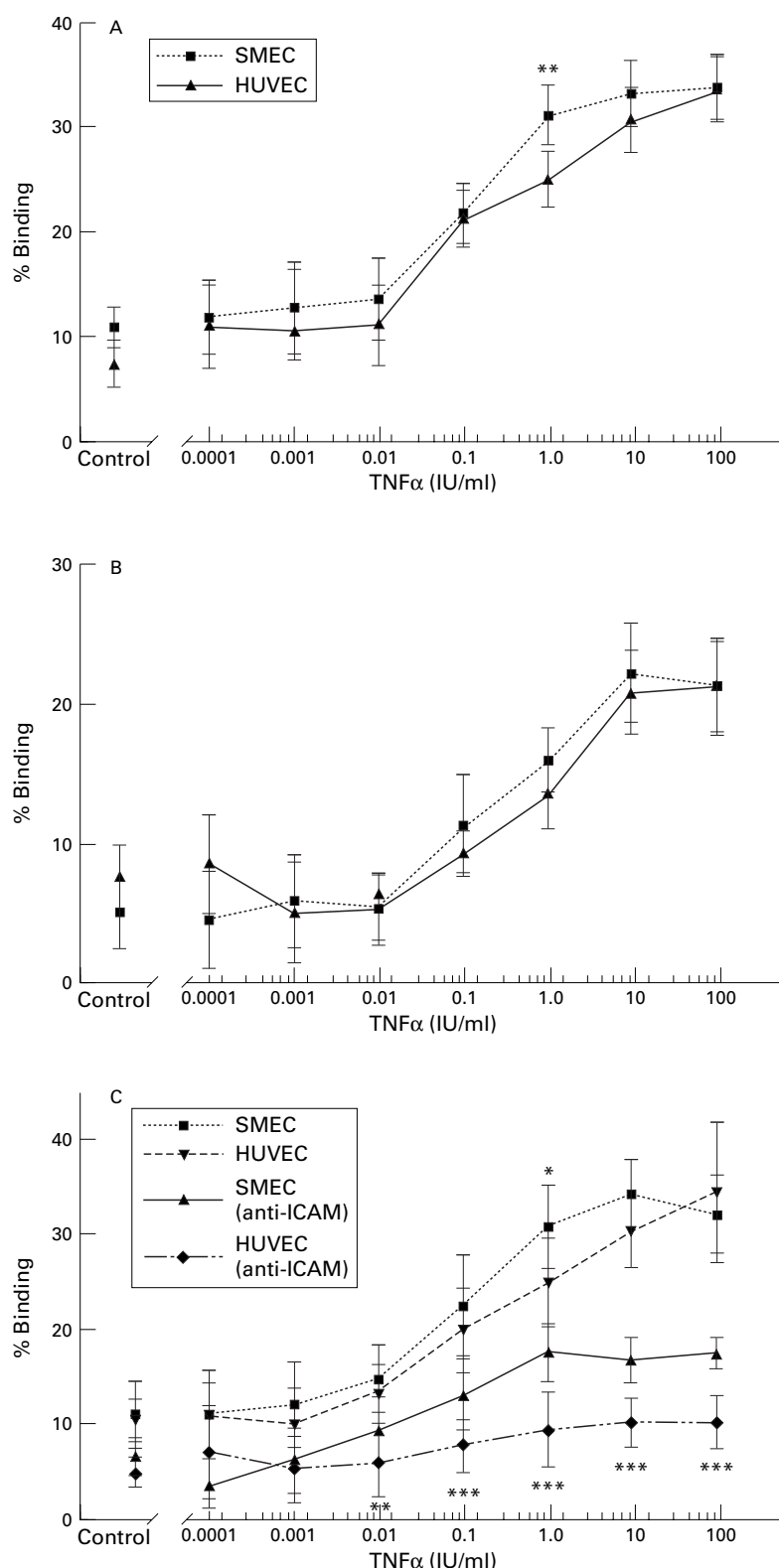
$$\% \text{ leucocytes bound} = \frac{\text{Counts per minute in } 100 \mu\text{l}}{\text{Counts per minute in } 100 \mu\text{l original PMS suspension}} \times 100$$

Antibody neutralisation experiments were conducted with TNF $\alpha$  stimulated endothelial cells that had been exposed to anti-ICAM-1 antibodies (50  $\mu$ g/ml, clone BBIG-II) (R&D Systems, Abingdon, UK) for 30 minutes before the addition of leucocytes as detailed above. Experiments were conducted a minimum of three times using a minimum of three replicate wells in each multiwell plate.

#### DYNAMIC LEUCOCYTE BINDING ASSAYS

Dynamic leucocyte binding assays were performed using a tapering width parallel plate flow cell (fig 1). Endothelial cell monolayers were grown to confluence on fibronectin coated (5  $\mu$ g/cm<sup>2</sup>) glass slides that were incorporated as one wall of the flow cell. Because of the decreasing width of the flow chamber, endothelial cells at different positions within the flow cell experienced a linearly increasing rate of shear (0.6–5.2 dynes/cm<sup>2</sup>) as fluid passed through the chamber at a constant flow rate. Endothelial cells and circulating leucocytes were viewed using an inverted phase contrast microscope. The leucocyte circulating medium was warmed to 37°C by a counter-current heat exchanger immediately upstream of the chamber.

Endothelial cell monolayers were stimulated with TNF $\alpha$  (100 IU/ml) for six hours before use. After stimulation, monolayers were washed with three changes of Dulbecco's modified Eagles medium (DMEM) before



**Figure 3** (A) Neutrophil adhesion to HUVEC and SMEC monolayers pre-incubated with TNF $\alpha$  (0–100 IU/ml). Results show mean binding of neutrophils as a percentage of the initial number added and depict 95% confidence intervals for the mean binding. SMEC occasionally, but not consistently, bind significantly ( $p < 0.01^{**}$ ) higher numbers of neutrophils than HUVEC ( $n=5$ ). (B) T lymphocyte adhesion to HUVEC and SMEC monolayers pre-incubated with TNF $\alpha$  (0–100 IU/ml). Results are expressed as the mean percentage binding of the original number of lymphocytes added and depict 95% confidence intervals for the mean binding. No significant differences were observed between cell types ( $n=5$ ). (C) Comparison of static PMN adhesion to TNF $\alpha$  (0–100 IU/ml) stimulated endothelial cell monolayers. Results show mean binding of neutrophils as a percentage of the initial number added and depict 95% confidence intervals for the mean binding. Results indicate differences in neutrophil binding to the two endothelial cell types before the addition of anti-ICAM-1 antibody (50  $\mu$ g/ml), rarely reaches significance ( $p < 0.05^*$ ). However, in the presence of anti-ICAM-1 antibody SMEC are capable of binding significantly ( $p < 0.05^*$ ,  $p < 0.01^{**}$ ,  $p < 0.001^{***}$ ) more PMN than similarly treated HUVEC at a number of TNF $\alpha$  concentrations ( $n=3$ ).

placement in the flow cell. Isolated leucocytes ( $1 \times 10^7$  cells) were allowed to circulate for 30 minutes (25 ml/min) before the endothelial cell slides were fixed in PLP fixative for 10 minutes. The number of adherent leucocytes per  $\text{mm}^2$  were counted at positions on the slides that experienced wall shear rates of 2.4 and 4.0 dynes/ $\text{cm}^2$ .

The subclasses of adherent T lymphocytes were analysed by direct immunofluorescence using FITC conjugated mouse anti-CD45RO+ (Serotec, Kidlington, England) antibodies. An inverted fluorescence phase contrast microscope (Diaphot II, Nikon) was used to count fluorescent lymphocytes that were expressed as a percentage of the total per field of view, revealed by phase contrast.

#### STATISTICS ANALYSIS AND PRESENTATION OF RESULTS

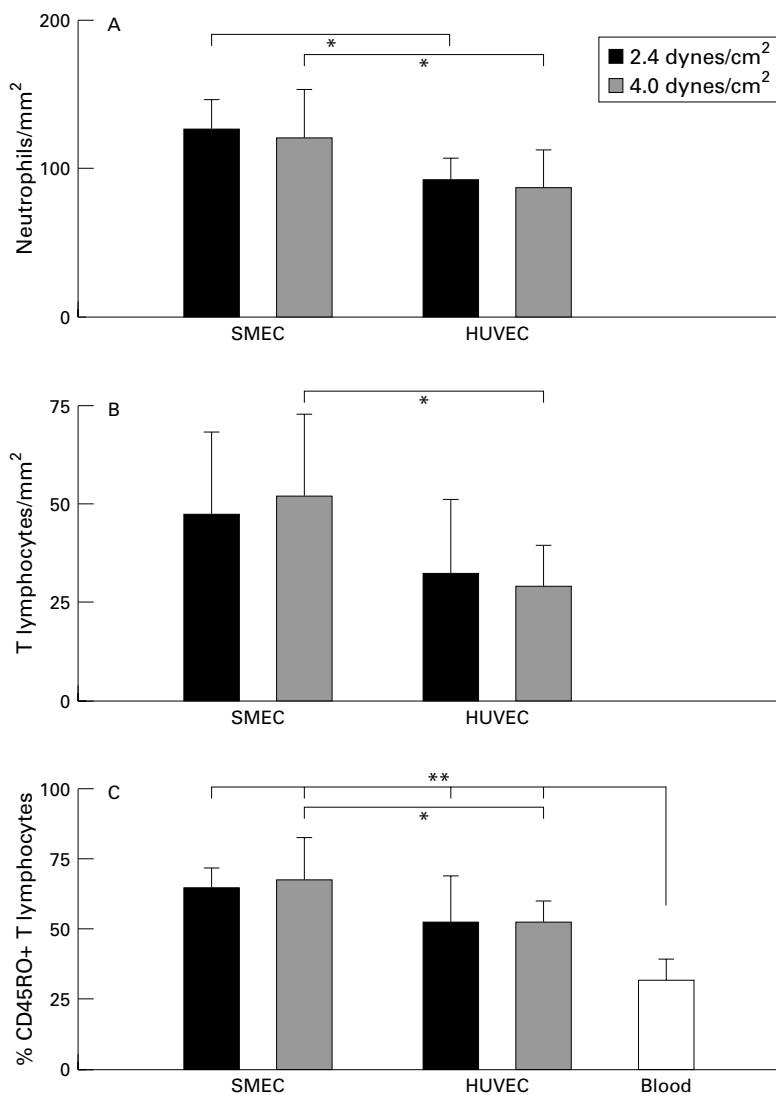
Adhesion molecule expression is presented as percentages of SMEC maximal responses as ascertained by measurement of CELISA optical densities. Changes in optical densities are assumed to be directly proportional to adhesion molecule expression over the range studied, however as this cannot be directly tested as  $EC_{50}$  values for the effects of TNF $\alpha$  have not been calculated. The 'n' numbers presented in figure legends represent the number of individual patient cell populations used to calculate the results. Different patient cell populations were used throughout the course of the experimental work presented.

Results represent mean with 95% confidence intervals assuming normally distributed percentage response at each TNF concentration and using a separate variance estimate at each concentration. Optical density and adherent cell number data were compared at individual cytokine concentrations and shear stress points by ANOVA and Bonferroni post-test analysis using GraphPad Prism 3.0 for Windows 95 (GraphPad Software Inc, San Diego CA). We used the equation built into the program with each replicate value being entered as a separate value. Individual significance thresholds are shown on each figure.

#### Results

SMEC, ADMEC and HUVEC all displayed the following characteristic endothelial cell markers; contact inhibited cobblestone morphology at confluence, discrete granular immunolocalisation of von Willebrand factor (vWf), metabolism of fluorochrome labelled acetylated low density lipoprotein and expression of E-selectin. All cells differentiated into capillary-like structures within 4–12 hours when grown on substrates coated with the basement membrane derivative Matrigel. The microvascular nature of isolated SMEC and ADMEC was guaranteed during the initial tissue dissection by discarding tissue fragments containing vessels with a lumen diameter  $> 150 \mu\text{m}$ .

We have previously demonstrated that IL1 stimulated SMEC display increased E-selectin expression in comparison with similarly treated HUVEC cultures.<sup>18</sup> This study extends these



**Figure 4** Adhesion of (A) peripheral blood neutrophils and (B) T lymphocytes to SMEC and HUVEC monolayers pre-stimulated with TNF $\alpha$  (50 IU/ml) for five hours. The cell counts represents the mean number of leucocytes (per mm<sup>2</sup>) attached to the endothelial monolayers, after 30 minutes of continuous circulation, at points in the flow chamber where wall shear rates were calculated at 2.4 and 4.0 dynes/cm<sup>2</sup> respectively and depict 95% confidence intervals for the mean response. SMEC monolayers bound significantly ( $p < 0.05$ \*) higher numbers of both neutrophils at both 2.4 and 4.0 dynes/cm<sup>2</sup> in comparison with HUVEC at the same rates of shear. Differences in T lymphocytes adhesion to SMEC in comparison with HUVEC were only significant ( $p < 0.05$ \*) at higher (4.0 dynes/cm<sup>2</sup>) rates of shear ( $n = 3$ ). (C) Immunohistochemical analysis of adherent T lymphocyte phenotype and initial proportions in healthy peripheral blood. Results are expressed as the proportion of cells bearing a CD45RO+ phenotype as a percentage of the total and depict 95% confidence intervals for the mean response. Cell populations adherent to both SMEC and HUVEC monolayers at both 2.4 and 4.0 dynes/cm<sup>2</sup> display a significant ( $p < 0.01$ \*\*\*) increase in the proportions of CD45RO+ cells in comparison with peripheral blood. SMEC also display a significantly ( $p < 0.05$ \*) increased capacity to bind such cells in comparison to HUVEC at higher (4.0 dynes/cm<sup>2</sup>) rates of shear ( $n = 3$ ).

observations by comparing E-selectin expression by SMEC to human microvascular endothelial cells derived from subcutaneous adipose tissue. Results demonstrate that ADMEC and HUVEC express similar levels of E-selectin after TNF $\alpha$  stimulation and confirm an approximately 30% increase in maximal expression by SMEC in comparison with either ADMEC or HUVEC (fig 2A). Stimulation of SMEC, ADMEC or HUVEC with IL1 (results not shown) elicited similar responses to those observed after TNF $\alpha$  treatment. Stimulation of each endothelial cell type with 50 IU/ml of TNF $\alpha$  generally induced maximal VCAM-1. However, unlike E-selectin expression, VCAM-1 expression by SMEC was

significantly less sensitive to TNF $\alpha$  stimulation than HUVEC or ADMEC (fig 2B). Comparison of ICAM-1 expression on ADMEC extended previous observations that showed no significant difference between basal or TNF $\alpha$  induced expression in SMEC and HUVEC.<sup>18</sup> None of the cell types displayed significant basal expression (0.85 IU/ml average) and maximal ICAM-1 expression was not significantly different after TNF $\alpha$  stimulation.

We hypothesised that as dose responses to TNF $\alpha$  were measured at one time point (six hours of stimulation), differences between cytokine stimulated VCAM-1 and E-selectin expression in SMEC in comparison with ADMEC or HUVEC may have been attributable to differences in the time required for the respective cell types to display maximal surface expression. However, this hypothesis was not supported by the results of a time course in which endothelial cells were stimulated with 50 IU/ml TNF $\alpha$  for periods of between 0 and 24 hours that demonstrated that maximal E-selectin and VCAM-1 expression occurred between five and nine hours in all three cell types and SMEC consistently expressed lower VCAM-1 and higher E-selectin levels at all time points. Also, while VCAM-1 expression in HUVEC and ADMEC remained near maximal levels for periods up to 24 hours SMEC expression decayed slowly beyond 10 hours (results not shown)

HUVEC and ADMEC demonstrated very similar adhesion molecule expression profiles in response to TNF $\alpha$ . Therefore, because of the ease of isolation and lower cost associated with the isolation and culture of HUVEC, these cells alone were used to compare leucocyte adhesion to SMEC. We predicted that differences between the ability of pro-inflammatory cytokines to induce adhesion molecule expression in isolated SMEC and HUVEC would result in differences in the ability of these cells to bind leucocytes.

Analysis of neutrophil (fig 3A) and T lymphocyte (fig 3B) binding to SMEC and HUVEC in static assays revealed little difference between the level of adhesion (number of leucocytes binding) to either endothelial cell type after stimulation of the endothelial cells with TNF $\alpha$  (0–100 IU/ml). In general, TNF $\alpha$  stimulated SMEC had a tendency to bind more neutrophils and T lymphocytes than similarly treated HUVEC, however this trend did not exhibit consistent statistical significance.

The possibility that ICAM-1/integrin interactions were masking differences in E-selectin and VCAM-1 mediated cell adhesion was assessed in two ways. Firstly, static leucocyte adhesion assays were performed using stimulated endothelial cell monolayers pre-treated with neutralising antibodies to ICAM-1 and secondly, leucocyte adhesion was assessed using a physiologically relevant dynamic assay.

TNF $\alpha$  stimulated endothelial cells were incubated with a neutralising antibody to ICAM-1 for 30 minutes before the addition of leucocytes. Results demonstrated, that while the overall number of neutrophils adhering was

significantly decreased, adhesion of neutrophils to TNF $\alpha$  stimulated SMEC was significantly higher than HUVEC treated in a similar manner (fig 3C).

Dynamic leucocyte adhesion assays were designed to inhibit immunoglobulin/integrin mediated adhesion by subjecting leucocyte/endothelial cell interactions to a shear stress in excess of 2 dynes/cm<sup>2</sup>

Results of dynamic leucocyte adhesion assays demonstrated that TNF $\alpha$  (100 IU/ml) stimulated SMEC monolayers were capable of binding significantly ( $p < 0.05$ ) greater numbers of neutrophils than HUVEC monolayer when exposed to shear stresses of 2.4 and 4.0 dynes/cm<sup>2</sup> (fig 4A). A similar phenomenon was observed with T lymphocyte adhesion when exposed to a shear stresses of 4.0 dynes/cm<sup>2</sup> (fig 4B).

Immunocytochemical quantification of CD45RO+ T lymphocytes adhering to endothelial cell monolayers demonstrated that both SMEC and HUVEC monolayers supported significantly ( $p < 0.05$ ) increased binding of CD45RO+ T lymphocytes when exposed to rates of shear of 2.4 and 4.0 dynes/cm<sup>2</sup> in comparison with CD45RO+ cell proportions found in the initial T cell isolate. In addition CD45RO+ cells comprised up to 69% of the T lymphocyte populations adherent to TNF $\alpha$  stimulated SMEC monolayers subjected to a wall shear stress of 4.0 dynes/cm<sup>2</sup>, this proportion was significantly ( $p < 0.001$ ) higher than that of CD45RO+ cells in the initial T lymphocyte isolate (32%) (fig 4C).

### Discussion

The aims of this study were to examine the ability of TNF $\alpha$ , to induce VCAM-1, E-selectin and ICAM-1 expression and leucocyte adhesion to endothelial cells isolated from rheumatoid synovial microvasculature in comparison with endothelial cells isolated from human umbilical vein or the microvasculature of human subcutaneous adipose tissue.

TNF $\alpha$  stimulated E-selectin expression by SMEC, ADMEC and HUVEC in this study is similar to results of IL1 stimulated expression in our earlier investigations<sup>18</sup> and is consistent with a study by To *et al*<sup>26</sup> that demonstrated increased expression of E-selectin by TNF $\alpha$  stimulated SMEC in comparison with either HUVEC or microvascular endothelial cells derived from neonatal foreskin. A possible explanation of the increased cytokine induced E-selectin expression by SMEC has been highlighted by the recent observations of Chen *et al*<sup>27</sup> that endothelial cell gene expression is not only positively regulated but that a further degree of control is afforded by the actions of an, as yet, unidentified repressor protein. It is therefore possible that the increased E-selectin expression by SMEC, observed both *in vitro* and *in vivo* may be reflect a defect in this repressor protein expression, however this hypothesis requires further study.

VCAM-1 expression by SMEC in this study was significantly less sensitive to TNF $\alpha$  stimulation than that of HUVEC or ADMEC. Time course studies demonstrated that this result is

not because of differences in the kinetics of maximal VCAM-1 induction by SMEC in response to TNF $\alpha$  in comparison with HUVEC or ADMEC. Our present results are however inconsistent with the results of To and colleagues that failed to show any difference in VCAM-1 expression between the endothelial cells studied. To *et al* demonstrated that although the average E-selectin expression by SMEC was higher than in the other endothelial cell types, when individual cell E-selectin expression was analysed by flow cytometry certain cells displayed very low expression. This observation may be explained by either questioning the purity of SMEC cultures used by To or alternatively, if true heterogeneity does exist in SMEC E-selectin expression, similar heterogeneity might be expected in VCAM-1 expression. It is possible that in this study, during cell isolation and culture, we have unwittingly selected for low VCAM-1-expressing SMEC populations. However, while selection and culture methods for ADMEC and SMEC were identical VCAM-1 expression was not. Marui and colleagues have suggested that the transcription of E-selectin and VCAM-1 may be regulated in a similar manner by the oxidant sensitive transcription factor NF $\kappa$ B.<sup>28</sup> Our results suggest that TNF $\alpha$  (or IL1, results not shown) induced VCAM-1 and E-selectin expression by SMEC are differentially regulated and support evidence to suggest that NF $\kappa$ B is not the sole transcriptional controlling mechanism for either gene.<sup>29, 30</sup> Reports that IL4 and TNF $\alpha$  act in synergy to both increase and prolong VCAM-1 surface expression in HUVEC<sup>31</sup> remain to be tested in SMEC. The relative insensitivity of VCAM-1 expression by SMEC *in vitro* may, however, explain immunohistochemical findings that rheumatoid synovial microvessels, unlike the microvasculature in other inflamed tissues, seldom express high levels of VCAM-1.<sup>12</sup> Although synovial microvessels display little cell surface associated VCAM-1, increased levels of soluble VCAM-1 (VCAM-s) have been demonstrated in the serum of rheumatoid arthritis patients.<sup>32</sup> The possibility that the relatively low VCAM-1 expression by cytokine stimulated SMEC is attributable to rapid cleavage from the cell surface remains to be tested.

Analysis of neutrophil and T lymphocyte binding to TNF $\alpha$  stimulated SMEC and HUVEC monolayers in static binding assays revealed little difference in the levels of adhesion (numbers of leucocytes bound) when comparing endothelial cells types. While this result supports previous studies comparing peripheral blood T lymphocyte adhesion to cytokine treated endothelial cells derived from human dermal tissue (DMVEC) and HUVEC,<sup>33</sup> it is inconsistent with reports that neutrophils have a tendency to adhere in higher numbers to C5a stimulated human omental fat derived microvascular endothelial cells than to HUVEC.<sup>34</sup> In addition, results of our static cell adhesion assays failed to support the hypothesis that increased SMEC E-selectin expression would lead to increased leucocyte adhesion. Current dogma suggests that leucocyte

adhesion and diapedesis is a multi-step process involving the sequential action of several families of cellular adhesion molecules.<sup>35, 36</sup> Initially, shear independent selectins mediate the slowing of circulating leucocytes, allowing shear dependent bonds to be formed between members of the immunoglobulin type adhesion molecules and members of the  $\beta_1$  and  $\beta_2$  integrin families when the effective wall shear stress has fallen below 2.0 dynes/cm<sup>2</sup>.<sup>24</sup> Therefore, adhesion during sedimentation in vitro may be predominately mediated by shear rate dependent ICAM-1/ $\beta_2$  integrin interactions. To determine whether ICAM-1/ $\beta_2$  integrin mediated adhesion was masking differences in selectin mediated adhesion in static assay systems, static neutrophil adhesion assays were repeated with the inclusion of a neutralising antibody to ICAM-1. Results demonstrated that while the addition of anti-ICAM-1 antibodies significantly reduced the number of neutrophils adhering to both SMEC or HUVEC monolayers, TNF $\alpha$  and anti-ICAM-1 treated SMEC were capable of supporting significantly higher levels of neutrophil binding than HUVEC treated in a similar manner.

To study the effects of sheer stress on human leucocyte/endothelial cell interactions we developed a parallel plate flow cell that permitted the growth of isolated endothelial cells on one optically clear surface. Results demonstrated that TNF $\alpha$  stimulated SMEC were capable of binding higher numbers of neutrophils and T lymphocytes than HUVEC when exposed to shear rates of >2.0 dynes/cm<sup>2</sup>. Adhesion of both neutrophils and T lymphocytes to SMEC, in comparison with HUVEC correlates well with increased E-selectin expression by cytokine stimulated SMEC, however the absolute contribution of E-selectin to leucocyte adhesion, as confirmed by antibody blockade, was not tested in this study. Recently it has been recognised that the modulation of shear independent leucocyte adhesion is not a property unique to selectins. Under conditions of flow in vitro, peripheral blood derived T lymphocytes have been shown to both roll and firmly adhere to VCAM-1 transfected COS cells,<sup>36</sup> processes that could be abolished by the addition of anti-VCAM-1 or anti-VLA-4 antibodies to the system. These results are intriguing but are unlikely to explain the increased T lymphocyte adhesion to SMEC because of the low levels of surface expression of VCAM-1 on these cells. These observations do not, however, rule out a role for VLA-4 in mediating lymphocyte accumulation in vivo where vascular derangement may expose extracellular matrix fibronectin, providing an alternative ligand for VLA-4.<sup>37</sup> Alternatively, the possibility that  $\beta_1$  integrin interaction with the mucosal addressin MAdCAM-1 may mediate leucocyte rolling<sup>38</sup> on SMEC remains to be tested.

T lymphocytes bearing a CD45RO+ memory phenotype comprise approximately one third of the circulating lymphocyte pool both in health and rheumatoid disease, however, selective recruitment of this class of T lymphocyte within the rheumatoid synovium can increase the proportion twofold.<sup>12, 39</sup> Analy-

sis of the T lymphocytes that adhered to SMEC and HUVEC during dynamic binding assays revealed as significant enrichment in the proportion of CD45RO+ cells in comparison to T cell populations before adhesion. Selectin ligands include E-selectin ligand-1 (ESL-1), P-selectin glycoprotein ligand-1 (PSGL-1) a post-translational modification of PSGL-1 known as cutaneous lymphocyte associated antigen (CLA),<sup>39</sup> together with Sialyl Lewis x and a (SLe<sup>x</sup> and SLe<sup>a</sup>). E-selectin transfected COS cells are capable of binding skin homing memory T lymphocytes,<sup>10</sup> a property not shared by other lymphocyte populations, via E-selectin interaction with the CLA. While it is tempting to suggest E-selectin/CLA interactions may explain our results in vitro, studies have demonstrated a relatively low number of CLA+ve T lymphocytes in rheumatoid synovial tissue sections in comparison with skin sections obtained from patients suffering from psoriatic arthritis.<sup>40</sup> The expression of CLA on migrating T lymphocytes may however be a transient phenomenon. The possibility that T lymphocytes adhere to activated SMEC via the ligand ESL-1 remains a possibility, however, recent studies have demonstrated that only 20–30% of CLA +ve lymphocytes co-express ESL-1.<sup>41</sup>

In conclusion, the results demonstrate that rheumatoid synovial endothelial cell VCAM-1 expression in vitro is relatively insensitive to cytokine (TNF $\alpha$ ) stimulation in comparison to HUVEC or microvasculature derived ADMEC. While these results may help to explain previous observations detailing the immunohistochemical absence of VCAM-1 expression in rheumatoid synovial microvasculature in conjunction with increased levels of pro-inflammatory cytokines in rheumatoid synovial fluid, they do not provide an explanation of the massive leucocyte accumulation generally found within rheumatoid synovial tissue. In contrast, cytokine stimulation of SMEC monolayers in vitro led to increased adhesion of leucocytes at physiologically relevant flow (shear) rates in comparison to HUVEC. The shear independent nature of such adhesion strongly supports a role for selectins in increasing leucocyte adhesion to SMEC. In particular, the selective adhesion of CD45RO+ T lymphocytes to SMEC monolayers under conditions of flow may explain patterns of T cell accumulation in vivo. Although not directly tested, observations of increased E-selectin expression by cytokine stimulated SMEC, in comparison with ADMEC or HUVEC, seem to correlate well with increased leucocyte, particularly CD45RO+ T lymphocyte adhesion to SMEC monolayers. We believe that the evidence presented here supports the hypothesis in which cell specific alteration of mechanisms regulating the expression of cellular adhesion molecules, specifically selectins, by the microvasculature of the rheumatoid synovium facilitates selective leucocyte recruitment and may play an important part in maintaining the chronic synovitis associated with rheumatoid arthritis.



The authors are grateful for the generous gift of anti-CD106 (clone 1.4c3), anti E-selectin (clone 1.2b6) and anti-ICAM-1 (clone 6.5b5) antibodies from Dr D O Haskard, Hammersmith Hospital, London, together with the assistance of Mrs M West and Dr G Scott in the provision of synovial membrane tissue samples.

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