Abstract

Inflammation and infection of the gut can be followed by reactive arthritis at a distant joint. Leukocyte recruitment into synovium is essential for this process, but nothing is known about the endothelial adhesion molecules in synovial membrane which direct the homing of activated, gut-derived leukocytes to joints. Here we analyzed the expression of the known endothelial adhesion molecules in inflamed synovial tissue (ICAM-1/CD54) and vascular adhesion protein-1 (VAP-1) were most prominently expressed in synovial vessels. All other adhesion molecules were found at lower levels in inflamed synovia, except mucosal addressin which was absent. Binding of macrophages isolated from lamina propria of the gut to synovial endothelium was almost entirely P-selectin-dependent. In contrast, small intestinal lymphocytes and immunoblasts both relied mainly on VAP-1 in recognition of synovial vessels. Thus, endothelial P-selectin and VAP-1 mediate binding of mucosal effector cells to synovium in a leukocyte subtype-selective manner. Antiadhesive therapy against these inducible molecules should ablate the pathogenetic cascade leading to inappropriate homing of leukocytes to joints in arthritis. (J. Clin. Invest. 1997. 99:2165–2172). Key words: arthritis • vascular addressins • vascular adhesion protein-1 • recirculation • inflammation

Introduction

Arthritis is characterized by villous hypertrophy, vascular proliferation, and lymphoplasmacytic infiltration of synovium. The histopathologic picture of idiopathic chronic arthritis (like rheumatoid arthritis, spondylarthropathies, and psoriatic arthritis) and reactive arthritis is practically indistinguishable. In reactive arthritis, the joint inflammation is believed to be triggered by microbial antigens, which are carried to the joint after an infection at a distant mucosal site (intestine, respiratory tree, or urogenital tract). Even less is known about the pathogenesis of reactive arthritis following idiopathic inflammation (e.g., chronic inflammatory bowel diseases). In every case, however, the prerequisite for the inflammation process is the emigration of leukocytes into the synovial tissue (1).

Different subsets of blood-borne leukocytes display distinct interactions with the host tissue (2, 3). Naive lymphocytes continuously patrol between blood and the lymphoid tissues in search of antigens. Two functionally different recirculation routes, one via mucosal lymphatic tissues and the other via peripheral lymph nodes, exist under physiological conditions. After engagement of their cognate antigen lymphocytes selectively accumulate at the sites similar to their original activation to destroy the invading stimulus. Thus, according to the current paradigm, a small lymphocyte recognizing its antigen in organized mucosal lymphatic tissue will mature into an immunoblast in mesenteric lymph nodes, return to the systemic circulation via efferent lymphatics and then enter into lamina propria to exert its effector functions (2–4). Monocytes leave the blood constantly at a low rate to replenish the tissue–macrophage populations, but their extravasation is markedly enhanced at sites of inflammation. Granulocytes, in contrast, only leave the blood at inflammatory foci. At the molecular level, interactions between leukocyte surface receptors and their ligands on the vascular lining regulate the extravasation process (2, 3, 5). Leukocyte emigration is thought to be a multistep phenomenon involving sequential but overlapping contribution of several adhesion molecules on both cell types. The best defined endothelial adhesion molecules belong to selectin and immunoglobulin superfamilies, which mediate initial recognition of leukocytes under shear and stable adhesion and transmigration, respectively. The expression of the majority of these molecules is induced and/or up-regulated at sites of inflammation (2–5).

We have shown earlier that inflamed synovium represents a functionally distinct recirculatory entity (6, 7). We have also dissected the binding of mucosa-derived immunoblasts to different tissues, and found out that these cells have an unexpected dual homing specificity: they adhere equally well both to mucosal and synovial (but not to peripheral lymph node) venules (8). The mucosal homing was readily accounted for by well-defined lymphocyte adhesion receptors, in particular α4β7 integrin and CD44 glycoprotein. In contrast, the synovial adhesion could not be explained by the known lymphocyte adhesion receptors.

In this study, we determined which endothelial adhesion molecules in synovium support the binding of mucosa-derived leukocytes. Blockade of these receptors would be expected to interfere with the harmful accumulation of inflammation-inducing leukocytes in joints.

Methods

Subjects and tissue specimens. Synovectomy specimens from chronically inflamed synovial membranes were obtained from 20 patients and snap-frozen in liquid nitrogen.
Table I. MAbs Used in This Study

<table>
<thead>
<tr>
<th>MAb</th>
<th>Antigen</th>
<th>Function*</th>
<th>Leukocyte-counter receptor†</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAPS12.2</td>
<td>P-selectin/CD62P</td>
<td>Initial</td>
<td>PSGL-1</td>
<td>§</td>
</tr>
<tr>
<td>12B6</td>
<td>E-selectin/CD62E</td>
<td>Initial</td>
<td>PSGL-1, CLA</td>
<td>(37)</td>
</tr>
<tr>
<td>MECA-79</td>
<td>PNAd</td>
<td>Initial</td>
<td>L-selectin/CD62L</td>
<td>(38)</td>
</tr>
<tr>
<td>1B2</td>
<td>VAP-1</td>
<td>Initial</td>
<td>?</td>
<td>(10)</td>
</tr>
<tr>
<td>8C1-12</td>
<td>MAAdCAM-1</td>
<td>Initial/stable</td>
<td>α4β7</td>
<td></td>
</tr>
<tr>
<td>5C3</td>
<td>ICAM-1/CD54</td>
<td>Stable</td>
<td>LFA-1(CD11a/CD18)</td>
<td>(39)</td>
</tr>
<tr>
<td>CBR-1C2/2</td>
<td>ICAM-2/CD102</td>
<td>Stable</td>
<td>LFA-1</td>
<td>(40)</td>
</tr>
<tr>
<td>1G11</td>
<td>VCAM-1/CD106</td>
<td>Stable</td>
<td>VLA-4 (α4β1,CD49d/CD29)</td>
<td>(37)</td>
</tr>
<tr>
<td>2C8</td>
<td>CD31</td>
<td>Transmigr</td>
<td>CD31</td>
<td>(39)</td>
</tr>
<tr>
<td>3G6</td>
<td>Irrelevant control</td>
<td>-</td>
<td>-</td>
<td>(10)</td>
</tr>
<tr>
<td>HB116</td>
<td>Control, HLA class I</td>
<td>-</td>
<td>-</td>
<td>(41)</td>
</tr>
<tr>
<td>TIB 146</td>
<td>Control, mouse B220</td>
<td>-</td>
<td>-</td>
<td>(42)</td>
</tr>
</tbody>
</table>

*Major function in the early, shear-dependent (initial), in the firm sticking (stable) or in the transmigration (transmigr) phase of the extravasation cascade. †The major glycoprotein receptor(s) of the indicated endothelial adhesion molecule on leukocytes. §Gift of Prof. E. Butcher. °Gift of Dr. M. Briskin.

Lamina propria leukocytes from normal small intestine were isolated from nine patients undergoing reconstructive surgery using a previously described methodology (7–9). In brief, lamina propria was dissected free from the muscular layer of gut wall and the epithelium was detached by a short EDTA treatment. Thereafter, the leukocytes were released by an overnight stirring with collagenase type II, and the mononuclear cells collected by a gradient density centrifugation. The enzymatic digestion used to isolate lamina propria does not affect the functional binding capacity of PBL (8).

PBL were isolated from five healthy volunteers, from two patients suffering from chronic arthritis, and from two ulcerative colitis patients by Ficoll-gradient centrifugation.

**Mabs and immunohistochemistry.** The function-blocking mAbs against different endothelial adhesion molecules used in this study are listed in Table I. Acetone-fixed frozen sections were stained with these mAbs and the appropriate second-stage peroxidase conjugated anti–mouse or anti–rat Igs using diaminobenzidine as the chromogenic substrate as described. The number of positive vessels for each antigen was evaluated semiquantitatively using CD31 stainings as a reference (CD31 is constitutively expressed on all types of endothelial cells in synovium): −, no positive vessels in the sample; +, occasional positive vessels in the sample, but < 1 in a ×200 microscopic field; + + 1–4 positive vessels per a ×200 field; + + + 5–10 positive vessels per a ×200 field (practically identical to the number of CD31 positive vessels). Positivity of vessels was only scored from areas with leukocytic infiltrations.

**In vitro adhesion assay.** Stamper-Woodruff binding assay was performed as described (11). Briefly, 8-μm frozen sections from synovia were cut and surrounded by wax-pen circles. The sections were overlying the purified function-blocking anti–endothelial mAbs and with isotype-matched irrelevant control mAbs for 30 min in 100 μl RPMI 1640 containing 10% FCS and 10 mM Hepes at 7°C. Then, 2 × 106 leukocytes isolated from lamina propria were resuspended in 50 μl of the same medium, filtered through 44 μm silk mesh to eliminate aggregates and applied onto the sections. The assay was continued for 30 min at 7°C with constant rotation (60 rpm on an orbital shaker). Thereafter, the nonadherent cells were tilted off, and the adherent cells were fixed to the sections with an overnight incubation in ice-cold phosphate-buffered saline containing 1% glutaraldehyde. The number of mononuclear cells bound to vessels was counted under dark-field microscopy. Under these conditions vessels are easily identified by a dark-appearing basement membrane and different subpopulations of mononuclear cells can be distinguished by their size and morphology. Thus, resting lymphocytes are small clear cells, whereas activated immunoblasts have ~1.7 × larger two-dimensional surface area than the small cells. Macrophages are readily recognized by their large size and ruffled appearance. In preliminary experiments the identity of the bound cells was confirmed by photographing certain venules with the bound cells and then counterstaining the slides with Diff-Quick stains. This enabled us to reevaluate the same cells in a given vessel for their nuclear morphology, which is routinely used for differential counting of leukocytes, and it ascertained that the dark-field image exactly corresponds to the cytological staining. The inhibitory effect of each mAb treatment is given as the percentage of maximal binding, which is defined as the number of adherent cells in the control mAb treated sections. Statistical significance of the inhibitory effects was evaluated using two-tailed Student’s t test.

To study the role of P-selectin in macrophage binding in detail, synovial vessels were overlaid with an FITC-conjugated anti–P-selectin mAb and an FITC-conjugated negative control mAb for 30 min and then washed twice with the assay medium. Lamina propria cells were then applied onto sections and the assays were carried out like the standard high endothelial venule (HEV)-binding assay. The binding was then evaluated by scoring each vessel first by dark-field microscopy to count the number of lamina propria cells bound to the vessel and then changing to epi-illumination to analyze P-selectin positivity of the very same vessel by immunofluorescence.

Synovial HEV adherence of PBL was tested using the standard HEV-binding assay, but only the contribution of vascular adhesion molecule-1 (VAP-1) in binding of small lymphocytes was tested because the other cell types in peripheral blood are too rare for this type of analysis.

Since the endothelial ligand of leukocyte CD44 is hyaluronate (12), the function of which cannot be inhibited by mAbs, its role was analyzed by digesting the target sections with a hyaluronate degrading enzyme, as described (13). Briefly, the synovial sections were incubated with a buffer containing hyaluronidase from Streptococcus dysgalactiae (Seikagaku Corporation, Tokyo, Japan) and a cocktail of protease in-
hibitors for 30 min at room temperature. The slides were then washed before adding the leukocytes. Control sections were treated identically except that the enzyme was omitted from the preincubation buffer. The experiments were then continued as in the normal Stamper-Woodruff assay. The effectiveness of the hyaluronidase digestion was analyzed by stainings with hyaluronate binding protein. Synovial sections were hyaluronidase treated or mock treated (same buffer without the enzyme) as described above, and after washings the slides were reacted with biotinylated hyalurone binding protein (gift of Dr. P. Heldin, Biomedical Center, Uppsala, Sweden [14]) in PBS for 30 min at room temperature. After washings the specific binding was detected using streptavidin-conjugated phycocerythrin, and the slides were mounted in Fluoromount and coverslipped for photographing.

The in vitro binding assay accurately reflects the in vivo homing capacity and specificity of different leukocyte subtypes as shown by numerous animal studies. The results of mAb inhibition studies using Stamper-Woodruff assay have also been readily reproduced in in vivo homing assays (reviewed in [15]). The binding assay mimics the shear conditions under which the initial recognition between blood-borne leukocytes and vascular lining takes place in vivo, and the members from all adhesion molecule families have been shown to work appropriately under these conditions (reviewed in [4]).

**Results**

**Expression of endothelial adhesion molecules in chronically inflamed synovium.** Synovectomy specimens from 20 patients with chronic arthritis were immunohistochemically stained for the expression of the endothelial adhesion molecules known to be involved in leukocyte binding (see Table I for the specific molecules). Representative examples from these stainings are shown in Fig. 1 and the results are summarized in Table II. All adhesion molecules, except mucosal addressin, were found in inflamed synovium. VAP-1 was expressed in practically all noncapillary vessels in all specimens and intercellular adhesion molecules-1 and -2 (ICAM-1 and ICAM-2) were present on most vessels. Vascular cell adhesion molecule-1 (VCAM-1) and the endothelial selectins (E- and P-selectin) showed much more restricted synthesis only in a subpopulation of vessels, often associated with prominent leukocyte infiltrates. Intriguingly, the peripheral lymph node specific addressin (PNAd) was also induced in certain venules in most but not all synovial specimens. PNAd positive venules always displayed morphological features reminiscent of high endothelial venules and were always located within a cuff of infiltrating leukocytes. In synovium, the expression of VAP-1, P- and E-selectin, and PNAd was confined to vessels, whereas ICAM-1 was also synthesized by many leukocytes and synovial lining cells, ICAM-2 by many leukocytes and VCAM-1 prominently by synovial lining cells and also by dendritic cells of organized lymphoid follicles. In endothelial cells, all adhesion molecules showed variable proportion of luminal and cytoplasmic staining. The expression was most prominent in venules, in particular in venules with heightened endothelial morphology, but arterial endothelium also displayed clear positivity with E- and P-selectins, ICAMs, and VCAM-1. Hyaluronate, the only known endothelial ligand of CD44 adhesion receptor, was present luminally in some vessels of the synovium, although it was much more prevalent in the extracellular matrix (Fig. 1 B).

**Function of endothelial adhesion molecules in inflamed synovium.** The role of the endothelial addressins in mediating the binding of gut-derived leukocytes was tested in a frozen section adhesion assay. To explore the potential of each molecule to serve as a ligand for leukocytes, synovial specimens containing high levels of all detectable adhesion molecules were chosen as target tissues for these studies. Adherence of different subclasses of mononuclear leukocytes isolated from lamina propria to synovial vessels in the presence of function-inhibiting and control mAbs was scored separately (Fig. 2).

As shown in Fig. 3, VAP-1 played a prominent role in adhesion of small mucosal lymphocytes (naive cells and memory cells) to venules in inflamed synovium (36% inhibition of binding with an anti-VAP-1 mAb, \( P = 0.004 \)). Anti–VCAM-1 and anti–ICAM-1 and -2 pretreatments had marginal effects at most, whereas any other endothelial adhesion molecules were not required for this interaction.

VAP-1 was also the most important adhesion molecule in binding of mucosal effector cells (large, activated immunoblasts) to synovial vessels (Fig. 3). Anti–VAP-1 treatment abrogated > 50% of the adhesion (\( P = 0.0019 \)). The immunoblasts apparently utilize multiple ligand-receptor pairs in synovial adherence, since other endothelial molecules also contributed to the adhesion. Of these, treatment of tissue sections with anti-ICAMs and E-selectin mAbs resulted in 20–30% inhibitions which, however, did not reach statistical significance. Also MECA-79 mAb against PNAd inhibited immunoblast binding in the synovium expressing the highest levels of this addressin, but when the data from all samples (which expressed less PNAd) were pooled no overall effect was discernible.

Mucosa-derived macrophages displayed a completely different way of recognition of vessels in inflamed synovium (Fig. 3). Their adherence was practically abolished by anti–P-selectin treatment (\( P = 0.0038 \)). In separate experiments, binding of lamina propria cells to P-selectin negative and positive vessels was evaluated in two synovia, which contained 44±1% P-selectin positive vessels, using FITC-conjugated mAbs. Thus, the lamina propria cells had an approximately equal possibility to interact with P-selectin expressing and P-selectin nonexpressing vessels. The results of these assays revealed that P-selectin positive vessels support macrophage binding five times better than P-selectin negative ones. Of the other molecules, E-selectin also significantly supported macrophage binding (\( P = 0.0087 \)). Notably, blocking of the function of VAP-1

**Table II. Expression of Endothelial Adhesion Molecules in Inflamed Synovium**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Percentage of samples in*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−</td>
</tr>
<tr>
<td>E-selectin</td>
<td>0</td>
</tr>
<tr>
<td>P-selectin</td>
<td>0</td>
</tr>
<tr>
<td>PNAd</td>
<td>20</td>
</tr>
<tr>
<td>VAP-1</td>
<td>0</td>
</tr>
<tr>
<td>MAAdCAM-1(^{1})</td>
<td>100</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>0</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>0</td>
</tr>
<tr>
<td>ICAM-2</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^{1}\)Number of positive vessels was quantified from − to ++++ in 20 synovial membranes as described in Methods, and the percentage of samples falling into each category is shown. Only five most heavily infiltrated samples were stained.
and ICAM-2 did not affect the synovial binding of this subset of mononuclear cells at all, and ICAM-1 played a marginal role at best.

We also tested whether PBL from patients with arthritic symptoms and from patients with ulcerative colitis (an inflammatory disease of gut, which has a potential to cause reactive arthritis) use VAP-1 in synovial HEV adherence (Fig. 4). Binding of control PBL was blocked approximately 45% by mAb 1B2 pretreatment. Also, the synovial adhesion of PBL from chronic arthritis patients and from ulcerative colitis patients was VAP-1 dependent. Interestingly, VAP-1 blockade caused more pronounced inhibition of synovial HEV binding of these patient PBL than of control PBL, and the difference was statistically significant ($P = 0.0083$ for normal versus ulcerative colitis, and $P = 0.02$ for normal versus chronic arthritis).

**Discussion**

We have shown here that mononuclear leukocytes isolated from gut have an inherent capacity to bind to vessels in inflamed synovium. Different subsets of these cells utilize remarkably different adhesion pathways in recognizing synovial vessels. In particular, VAP-1 dependent mechanisms are im-
important in binding of mucosal immunoblasts to vessels in synovium, whereas P-selectin is critical to synovial adherence of mucosal macrophages.

Binding of peripheral blood lymphocytes or synovial fluid lymphocytes to inflamed synovium is partially dependent on lymphocyte function associated antigen-1 (LFA-1), very late activation antigen-4 (VLA-4), L-selectin, and CD44 in man (16–18). This has indirectly implicated that the major endothelial ligands of these lymphocyte homing receptors (ICAM-1 or -2, VCAM-1, PNAd, and hyaluronate, respectively) may play a role in synovial adhesion. Indeed, blood vessels in heterogeneous materials of inflamed synovia have been shown to express VCAM-1, PNAd, E- and P-selectin, and ICAMs (16, 17, 19–22). This study is the first systematic effort to analyze the expression of all known endothelial adhesion molecules simultaneously in one series of specimens. This study also for the first time dissects the role of these molecules in binding of gut-derived cells to vessels in joints. We demonstrate abundant synthesis of VAP-1 and ICAMs in synovial vessels. VAP-1 was a dominant endothelial ligand in supporting the adherence of small lymphocytes and, in particular, large immunoblasts of gut. Neither inducible ICAM-1 nor constitutively expressed ICAM-2, two ligands of lymphocyte LFA-1, did statistically significantly support immunoblast binding to synovium. Intriguingly, PNAd, which is normally expressed only in peripheral lymph nodes, was aberrantly expressed in many inflamed synovia, but in most cases it did not appear to markedly contribute to the binding. Instead, synovial vessels completely lack the newly described human mucosal addressin MadCAM-1 (23) (the same anti–MAdCAM-1 mAb brightly stained vessels in tonsil). VCAM-1 was expressed at low levels on many synovial vessels, but it was not required for binding of intestinal lymphocytes. These data are fully consistent with the minimal role of VLA-4 (the lymphocyte receptor of VCAM-1) and lack of function of α4β7 (receptor of MadCAM-1) in synovial binding of gut lymphocytes (8). Finally, hyaluronidase treatment did not markedly diminish synovial adherence of gut-derived lymphocytes. Since anti–CD44 mAbs against nonhyaluronate binding epitopes of CD44 also block the binding (8), our data indicate the existence of a novel endothelial cell counterpart of CD44 in inflamed synovium.

Mucosal macrophages as professional antigen presenting cells are the prime candidates which can transport antigens introduced via mucosal surfaces to joints. P-selectin was required for synovial HEV recognition of these cells. On the other hand, P-selectin has also been shown to be decisive in synovial binding of peripheral blood monocytes (17). P-selectin is released rapidly (within minutes) from intracellular stor-
endothelial cells or inappropriate elimination of the target antigen may aggravate the inflammatory reaction. Sustained activation of the emerging inflammatory response results in further endothelial antigens gaining entrance into the synovial tissue. Thereafter, the P-selectin, macrophages carrying the triggering systemic effects of infection or inflammation (LPS, cytokines) are carried into synovial microcirculation. There, P-selectin may rogradely through the vascular wall (31) and can be carried in different adhesion molecules vary between individual specimens, but when all the data were pooled no apparent contribution was any more detectable.

Based on our findings we propose a working model for the pathogenesis of reactive arthritis. Although, because of ethical reasons, we have measured binding of mucosal leukocytes to synovial tissue from chronic arthritis, we believe that these results are readily applicable to reactive arthritis as well. The two conditions are indistinguishable by histology, and reactive arthritis sometimes leads to a chronic inflammation (1, 25, 26). Moreover, there is evidence of pronounced mucosal involvement also in many chronic arthritides (27–29). Thus, we suggest that in reactive arthritis antigenic load introduced via the gut is processed and engulfed by macrophages in the lamina propria. In this scenario, these cells may then enter the systemic circulation, possibly either via the lymphatics (30) or retrogradely through the vascular wall (31) and can be carried in the blood into synovial microcirculation. There, P-selectin may already be present on the endothelial surface because of the systemic effects of infection or inflammation (LPS, cytokines [32, 33]). Via P-selectin, macrophages carrying the triggering antigens get entrance into the synovial tissue. Thereafter, the emerging inflammatory response results in further endothelial activation. The mucosal effector cells (immunoblasts) are then able to bind to synovial vessels, emigrate into the tissue and aggravate the inflammatory reaction. Sustained activation of endothelial cells or inappropriate elimination of the target antigen may ultimately lead to the development of reactive arthritis in susceptible individuals.

These findings also have direct implications to prevention of arthritis by antiadhesive therapy. This type of therapy has been shown to be effective in animal models of arthritis as well as in preliminary studies with drug-resistant rheumatoid arthritis (34–36). Thus, the initial deposition of macrophage-associated antigens in the synovium can be prevented by eliminating macrophage recruitment into the synovium by blocking the function of P-selectin. After a more prolonged inflammatory history, the circulus vitiosus can still be halted by preventing further influx of preactivated (mucosal) effector cells. This can be done primarily by blocking VAP-1, possibly in combination with ICAMs. Thus, selective interference of endothelial ligands can selectively inhibit synovial homing of different subclasses of mononuclear cells, at least when they originate from the gut. The antiadhesive therapy would appear especially appealing in cases, in which normal disease modifying antirheumatic drugs have failed to alleviate synovial inflammation and to reduce sustained, but inappropriate, expression of endothelial adhesion molecules.

Acknowledgments

We thank Dr. K. Söderlund for the PBL samples from IBD patients; Dr. M. Briskin, Dr. E. Butcher, and Dr. D. Haskard for providing mAbs; Dr. P. Heldin for the hyaluronate binding protein; and Ms. R. Lehven for excellent technical assistance.

This work was supported by the Finnish Academy, Juselius Foundation, Technology Development Center of Finland, Paulo Foundation, and the Farmos Research and Science Foundation.

References


Synovial Homing of Mucosal Leukocytes 2171


