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ORIGINAL ARTICLES—Laboratory science

Adhesion molecules in iris biopsy specimens from patients with uveitis

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Abstract
Background/aims—Earlier studies on intraocular tissue have demonstrated that T lymphocytes play a major role in the pathogenesis of uveitis. Adhesion molecules are immunoregulatory molecules for the interaction between T lymphocytes and vascular endothelium and they play an important role in the recruitment of specific T lymphocytes from the circulation into inflamed tissue. In uveitis an increased expression of some of these adhesion molecules may be expected.
Methods—The presence of adhesion molecules was investigated in iris biopsy specimens from 11 patients with uveitis and eight controls (patients with primary open angle glaucoma) immunohistochemically with a panel of monoclonal antibodies: LECAM (CD 62L), ICAM-1 (CD 54), LFA-1 (CD 11a/18), VCAM-1 (CD 106), VLA-4 (CD 49d), and HECA-452, a marker for high endothelial venules.
Results—Positive staining for ICAM-1, LFA-1 and VCAM-1 was found in the iris in a significantly higher number of uveitis patients than in controls. The remaining adhesion molecules were also found in a higher number of uveitis patients than in controls, but this difference did not reach statistical significance.
Conclusion—An increased expression of adhesion molecules was found in the iris of patients with uveitis, indicating an immunoregulatory function for adhesion molecules in the pathogenesis of uveitis.
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In most types of uveitis the aetiology is still unclear. In the past 10 years immunohistochemical studies on intraocular tissue, and the detection of inflammatory mediators in aqueous humour and vitreous, have revealed some mechanisms in the pathogenesis of uveitis. It was demonstrated that the cellular infiltrate in iris specimens obtained from patients with different types of uveitis consisted primarily of T lymphocytes, with a minimal B cell and plasma cell infiltrate.[1-4] Most of these T lymphocytes and even iris resident cells were HLA-DR positive. Also in animal models with experimental uveitis a predominantly T lymphocytic infiltrate was found.[5] A delayed type of hypersensitivity (type IV) immune mediated pathogenesis was therefore suggested for uveitis.[1]

Adhesion molecules are important regulatory molecules for T lymphocytic functions outside the bloodstream and in inflamed tissues. They play a critical role in immunosurveillance and mediate interactions between T cells and vascular endothelium, and T cells and extracellular matrix.[6] Originally, adhesion molecules were discovered as lymphocyte homing receptors, involved in the entrance of lymphocytes from the blood circulation into lymphoid tissue via postcapillary venules with specialised endothelium. These so called high endothelial venules (HEV) express specific surface proteins (vascular addressins) in a tissue specific manner. In the subsequent years, many of these surface proteins have been identified and until now five structural families of adhesion molecules have been described: selectins, integrins, the immunoglobulin superfamily, mucins, and cadherins. They play an essential role in the process of inflammation and recruitment of active and specific T cells and other leucocytes into inflammatory tissue.

A multistep model of sequential leucocyte endothelial cell interactions has been proposed.[7 8] The initial steps, after activation of the endothelium, are mediated to a large extent by selectins resulting in a "rolling and tethering" of the leucocyte to the vessel wall. The second step involves activation of T cell adhesiveness by chemokines (monocyte chemoattractant proteins (MCP) 1, 2, 3, macrophage inhibitory proteins 1α and 1β, RANTES). The third step comprises stable arrest and firm adhesion of the lymphocyte to the endothelium. This is mediated by integrins on the surface of the T cell and immunoglobulin superfamily (IgSF) adhesion molecules on the endothelial cells. Cytokines such as TNF-α, IL-1, IL-4, and IFN-γ strongly upregulate their expression.[6] The fourth step, leucocyte diapedesis, is still poorly characterised; it is likely that integrin-IgSF interactions play an important role.[6] As extracellular matrix component, fibronectin has been demonstrated to interact with integrins resulting in T cell proliferation.[6]

The multistep adhesion cascade of distinct classes of T cell interaction with endothelium, their activation and subsequent transmigration into the extracellular matrix is accompanied by a differential expression of adhesion molecules and cytokines in a tissue specific manner. The eye is known to be an immunologically privileged site[9] and it may be expected that an intraocular homing receptor or a specific combination of adhesion molecules contributes to the immunosuppressive properties of the anterior segment of the eye. In uveitis there may be a disturbance of this phenomenon of anterior chamber associated immune deviation[10] (ACAID) and different adhesion molecules and cytokines may play a role.

Adhesion molecules have been demonstrated in the eyes of patients with uveitis,[11 12] and are also found in other tissue sites involved in autoimmune diseases (synovium in rheumatoid arthritis and skin lesions in psoriasis).[13 14] In an animal model,[15] adhesion molecules are expressed before histological evidence of inflammation, and may therefore be important targets for selective immunotherapies.

We investigated the presence of several adhesion molecules involved in different steps and acting at various moments in the adhesion cascade in the iris of patients with uveitis versus controls (that is, iris from patients with primary open angle glaucoma).

Materials and methods

IRIS BIOPSY COLLECTION

Iris biopsy specimens (from peripheral iridectomies) were obtained from 11 patients (four men and seven women) with uveitis during cataract surgery or trabeculectomy. Their mean age was 60 years (range 27–88 years). Iris biopsy specimens from eight patients (five men and three women) with primary open angle glaucoma, obtained during trabeculectomy served as controls. Their mean age was 68 years (range 28–83 years).

The diagnosis of uveitis was made according to the criteria suggested by the International Uveitis Study Group.[16] Three patients had Fuchs' heterochromic cyclitis, one had recurrent anterior uveitis associated with HLA-B27, and seven patients had chronic idiopathic uveitis (four with anterior uveitis and three with panuveitis, one of whom had a positive mantoux test). At the time of operation the eye was quiet in all cases. Because of the risk of increased inflammation after surgery, uveitis patients are often operated on only during a quiet phase of their disease established by a long preoperative period of anti-inflammatory medication. Moreover, biopsies are not obtained as a procedure to make the diagnosis, which is the case in aqueous humour or vitreous analysis, in which material may be obtained during an active stage of the disease. None of the uveitis patients received systemic anti-inflammatory or immunosuppressive treatment, but five were treated with local steroids and/or NSAIDs, and seven were using local antiglaucoma medication. The diagnosis of primary open angle glaucoma (POAG) was made on the following criteria: (a) open angle

on gonioscopy; (b) elevated intraocular pressure ($\geqslant 21$ mm Hg) with or without antiglaucoma medication; (c) presence of glaucomatous visual field defect(s) and/or optic disc damage; and (d) no signs of intraocular inflammation. None of the patients with glaucoma was treated with systemic or local anti-inflammatory medication, but all received antiglaucoma drops at the time of the operation. We chose patients with POAG as a control group without clinical evidence of inflammation rather than patients undergoing extracapsular cataract extraction for age related cataract, because peripheral iridectomy is an integral part of trabeculectomy. All patients were informed and their consent was obtained.

HISTOPATHOLOGICAL INVESTIGATION

One frozen section from each specimen was stained with haematoxylin and eosin to evaluate the following pathological features: (1) the appearance of a mononuclear cell infiltrate; (2) the presence of high endothelial venules with cuboidal vascular endothelial cells; (3) hyalinisation of the vessel walls; (4) iris stroma atrophy and fibrosis; (5) focal depigmentation of iris pigment epithelium; and (6) the disappearance of stromal melanocytes.

IMMUNOHISTOCHEMICAL TECHNIQUE

All specimens were snap frozen in OCT (optimal cutting tissue) compound within 1 hour of iridectomy. Cryostat sections 4 µm thick were air dried and subsequently fixed in formalin 4% for 10 minutes[17] and were subsequently air dried and rinsed in phosphate buffered saline (PBS) solution (pH 7.4). In order to conserve the morphology of the frozen tissue sections, we tested formalin fixation of frozen tissue sections for only 10 minutes, analogous to a method described earlier.[17] In our positive controls (lymphoid tissue) there were no artefactual staining results or destruction of antigens comparing short formalin fixation and acetone fixation. Moreover, for all monoclonal antibodies, positive and negative controls were tested to determine the optimal concentration of the monoclonal antibodies that had to be applied to the iris biopsies, to ensure that as with the other steps of the immunohistochemical procedures, no artefacts were induced.

Slides were then incubated with 10% normal goat serum in 1% PBS/BSA (bovine serum albumin). After this step, they were incubated for 30 minutes at room temperature (20°C) with a panel of monoclonal antibodies (Table 1) in 1% PBS/BSA solution. After rinsing in PBS, slides were incubated for 30 minutes at room temperature with biotinylated multilink immunoglobulin 1:50 (Biogenex, San Ramon, USA) in 1% PBS/BSA, and after rinsing with PBS, with streptavidin labelled alkaline phosphatase. Subsequently, slides were thoroughly washed in PBS and TRIS-HCl (pH 8.0), and antibody antigen binding was demonstrated using the substrate naphthol-as-mx phosphate (Sigma, USA). Levamisol was added to inhibit endogenous peroxidase and phosphatase. Sections were counterstained with Mayer's haematoxylin to obtain a discrete nuclear staining

Table 1   *Monoclonal antibodies used, function, and family of the adhesion molecules*

Monoclonal antibody/receptor	Specificity, dilution, clone	Source	Family	Expression, function[6] [18–20]	Ligand[20]
LECAM, L-selectin, CD 62L	IgG, 1:75, mouse, clone FMC46	Dako, Denmark	Selectin family	Expressed on most peripheral blood leucocytes. Shed after activation. Involved in adhesion of neutrophils, eosinophils, monocytes to endothelium. Mediates initial rolling and tethering of leucocytes to endothelium.	GlyCAM-1, CD34, sialyl-lewis x
ICAM-1, CD 54	IgG, 1:25, mouse, clone 1H4	Neomarkers, Fremont USA	Immunoglobulin superfamily	Expressed on resting endothelial cells, epithelial cells, mononuclear cells, fibroblasts. Upregulated by IL-1, TNF, and IFN-γ. Mediates firm adhesion and transmigration of lymphocytes to endothelium, and interaction between T cell and extracellular matrix.	LFA-1, MAC-1, CD43
LFA-1β-chain; CD18, LFA-1α-chain; CD11a	IgG, 1:120, mouse, clone NHM23 IgG, 1:800, mouse, clone NHM24	Dako, Denmark	β_2 Integrins,	Expressed on peripheral blood lymphocytes, neutrophils, and monocytes. Mediates adhesion of leucocytes to endothelium.	ICAM-1,-2,-3
VCAM-1, CD 106	IgG, 1:25, mouse, clone 1.4C3	Dako, Denmark	Immunoglobulin superfamily	Expressed on activated endothelium, after upregulation by IL-1, TNF-α, IL-4. Also expressed on macrophages and dendritic cells. Involved in lymphocyte homing and recruitment to sites of inflammation. Mediates firm adhesion of leucocytes to endothelium.	VLA-4, $\alpha_4\beta_7$
VLA-4, CD49d	IgG, 1:10, mouse, clone 44H6	Serotec, UK	β_1 Integrins	Expressed on leucocytes, monocytes, and eosinophils. Mediates adhesion and transmigration of leucocytes to activated endothelium.	VCAM-1, fibronectin (extracellular matrix)
HECA-452, monoclonal antibody against high endothelial venules	IgM, 1:500, rat	Gift from Dr Duijvenstijn, (University of Limburg, Netherlands)		Expressed on activated endothelium, involved in homing of lymphocytes to lymphoid tissue. Expressed on endothelium specialised in highly increased lymphocyte extravasation to sites of severe inflammation.[21]	sial-lewis x

LECAM= leucocyte endothelial cell adhesion molecule; ICAM-1 = intercellular adhesion molecule-1; LFA-1= lymphocyte function associated antigen 1; VCAM-1= vascular cell adhesion molecule-1; VLA-4 = very late antigen-4; HECA-452 = monoclonal antibody against high endothelial venules.

pattern. Positive reactions produced a red colour, which made differentiation from dark brown endogenous melanin possible. Omission of the monoclonal antibodies served as negative control.

SCORING SYSTEM AND STATISTICAL ANALYSIS
Tissue sections were examined using a 25× and 40× magnification by two independent observers, both of whom were unaware of the diagnosis. Vascular endothelium, iris resident cells (stromal melanocytes, iris pigment epithelial cells, fibroblasts, and endothelium), and mononuclear cells were scored separately. A semiquantitative scoring system was used with a scale ranging from zero (-) to 4+ according to the intensity of staining of positive cells. The Fisher's exact test was used to compare the incidence of positive staining (in which only specimens graded as 1+ or more were considered) between specimens from patients with uveitis and patients with glaucoma.

Results

HISTOPATHOLOGICAL EVALUATION
Scattered deposits of infiltrating mononuclear cells, mainly lymphocytes, were seen in haematoxylin and eosin stained sections in 10 out of the 11 iris biopsy specimens from patients with uveitis. A few isolated mononuclear cells were also seen in two of the eight iris biopsy specimens from patients with glaucoma. These were also the only two cases in which an expression of adhesion molecules was found (see below). In the iris of patients with uveitis no evident perivascular accumulation of lymphocytes was observed, nor were lymphocytes found in the vessel walls. Pathological features typical of Fuchs' heterochromic cyclitis[22] were observed in one of the three iridectomies from patients with Fuchs' heterochromic cyclitis: hyalinisation of the vessel walls, iris stroma atrophy and fibrosis, focal depigmentation of iris pigment epithelium, and the disappearance of stromal melanocytes. Some degree of hyalinisation of the vessel wall was observed in two iris biopsy specimens from patients with uveitis, but not in iris biopsies from glaucoma patients. No morphological features of high endothelial venules were observed in any of the patients.

ADHESION MOLECULE EXPRESSION IN UVEITIS PATIENTS
The expression of adhesion molecules in iris biopsy specimens from patients and controls is summarised in Table 2. Because there were only very small differences in intensity of the

Table 2   *Expression of adhesion molecules in iris biopsy specimens from patients with uveitis or glaucoma*

Adhesion molecules	Uveitis (n=11)	Glaucoma (n=8)	p Value†
	Vascular endothelium		
ICAM-1	10/11 (91%)	2/8 (25%)	0.0062
VCAM-1	4/11 (36%)	2/8 (25%)	0.49
HECA-452	3/7 (43%)	1/8 (12.5%)	0.23
	*Iris resident cells**		
LECAM	0/11	0/8	1.0
ICAM-1	11/11 (100%)	2/8 (25%)	0.0013
LFA-1α	6/11 (55%)	0/8	0.017
LFA-1β	1/11 (9%)	0/8	0.6
VCAM-1	7/11 (64%)	0/8	0.013
VLA-4	1/8 (12.5%)	0/8	0.58
	Infiltrating mononuclear cells		
LECAM	0/11	1/8 (12.5%)	0.42
ICAM-1	11/11 (100%)	2/8 (25%)	0.0013
LFA-1α	5/11 (45%)	1/8 (12.5%)	0.15
LFA-1β	1/11 (9%)	1/8 (12.5%)	0.68
VCAM-1	6/11 (55%)	2/8 (25%)	0.21
VLA-4	1/8 (12.5%)	2/8 (25%)	0.5

*Iris melanocytes, iris pigment epithelial cells, fibroblasts.
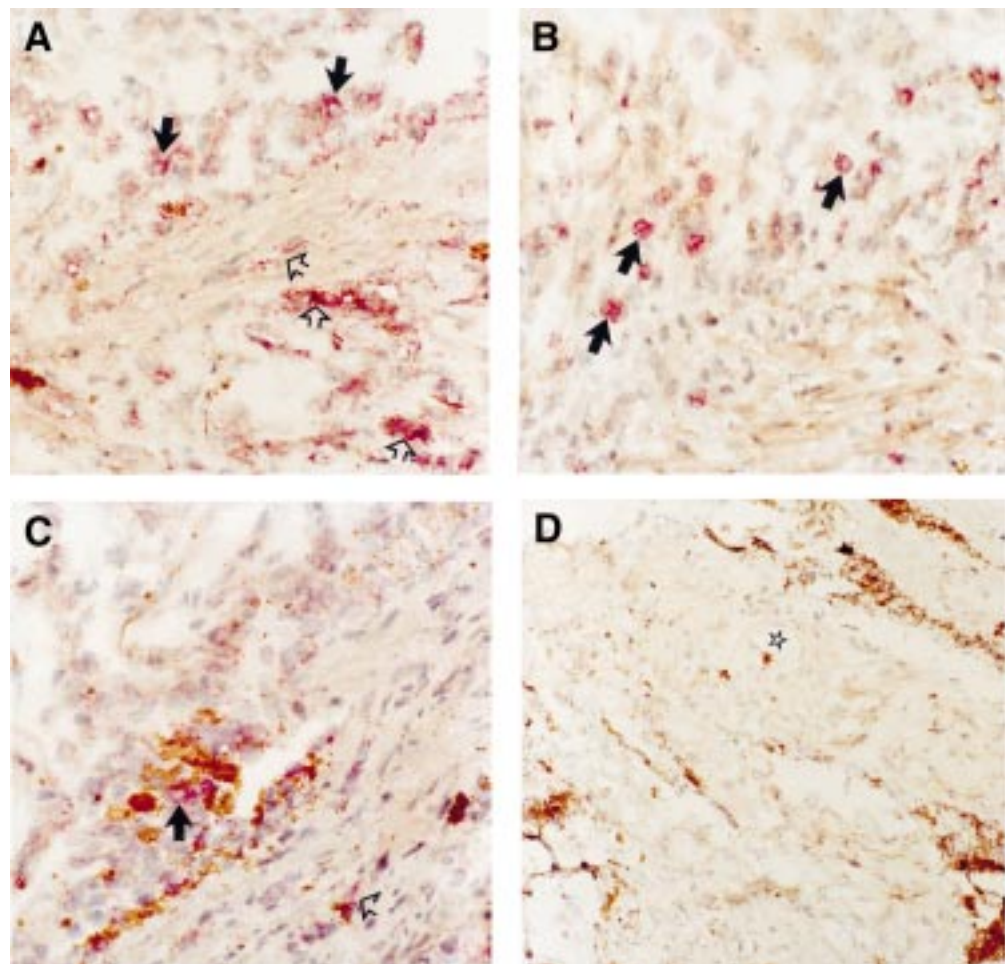†Fisher's exact test, without correction for multiple comparisons.

Figure 1   Light microscopic images (original magnification × 400) of immunohistochemistry giving a red (positive) staining result. Some of the iris resident cells (open arrows), infiltrating mononuclear cells (closed arrows), and a lumen of an iris vessel with a lining of endothelial cells (star) have been marked in the figures. Staining with (A) anti-ICAM-1 and (B) anti-LFA-1α in a patient with Fuchs' heterochromic cyclitis, and (C) anti-VCAM-1 in a patient with anterior uveitis of unknown origin, (D) negative control (original magnification × 250); patient with uveitis, in which all steps of the immunohistochemical technique were applied, except for the specific monoclonal antibodies. These were omitted to demonstrate that there were no non-specific reactions. Only the pigmentation of the iris in iris pigment epithelium (brown colour) is seen, with a counterstaining with Mayer's haematoxylin and eosin showing a discrete nuclear pattern.

staining and the number of positive cells between the various uveitis patients, we only depicted in how many specimens positive cells (1+ or more) were found. Four specimens were excluded from analysis because they were too small. Moreover, as can be seen in Table 2 instead of 11 uveitis patients tested for HECA-452 only seven tissue sections of the biopsies gave a reliable score, and with VLA-4 only eight out of 11 gave a reliable score. The expression of ICAM-1 (on vascular endothelium, iris resident cells, and on infiltrating mononuclear cells), of LFA-1α (on iris resident cells), and of VCAM-1 (on iris resident cells) was found in a significantly higher number of iris biopsies from patients with uveitis than from patients with glaucoma (Table 2). No difference in the expression of any of these adhesion molecules was observed between patients with Fuchs' heterochromic cyclitis and other uveitis entities. No evident correlation was found between those patients treated with local steroids and/or antiglaucoma medication at the time of operation, and the presence or absence of adhesion molecules.

Anti-LECAM (CD 62L) showed a positive staining in only one iris from a patient with glaucoma. Anti-ICAM-1 (CD 54) yielded the highest positive scores; staining with vascular endothelium in 10 out of 11 iris biopsies from patients with uveitis and with iris resident cells and infiltrating mononuclear cells in all cases (Fig 1A). Anti-LFA-1α (CD 11a) antibody reacted with iris resident cells and mononuclear cells, but not with vascular endothelium (Fig 1B). In some cases a few LFA-1α positive mononuclear cells surrounded blood vessels that were ICAM-1 positive. Anti-LFA-1β (CD 18) showed staining of iris resident cells and scattered mononuclear cells in only one patient with uveitis. VCAM-1 reacted positively with vascular endothelium in four out of 11 patients with uveitis, but also with iris resident cells and infiltrating mononuclear cells in seven and six out of 11 patients with uveitis, respectively (Fig 1C). Anti-VLA-4 (CD 49d), functionally the receptor for VCAM-1 and fibronectin, stained only few resident cells and infiltrating mononuclear cells in the iris of one patient with Fuchs' heterochromic cyclitis. HECA-452, a

marker for high endothelial venules, gave positive staining results in three out of seven irises from patients with uveitis.

ADHESION MOLECULE EXPRESSION IN GLAUCOMA PATIENTS

In two iris biopsy specimens from patients with glaucoma, adhesion molecules were expressed; ICAM-1 and VCAM-1 were expressed on vascular endothelium, iris resident cells, and on a few scattered infiltrating mononuclear cells. LFA-1α/β was only weakly expressed on some of these mononuclear cells.

Discussion

We found a statistical significant increase in the expression of the adhesion molecules ICAM-1, LFA-1, and VCAM-1 in the iris of patients with uveitis compared with controls (patients with glaucoma). However, one has to keep in mind that when a correction is applied for multiple comparisons, all p values presented in Table 2 should be multiplied with a factor associated with the number of comparisons made and the sample size. Therefore, owing to the small number of iris biopsies some differences in adhesion molecule expression were not statistically significant in this study. For example, the difference in VCAM-1 expression (36% *v* 25%), HECA-452 (43% *v* 12.5%), and LFA-1α (45% *v* 12.5%) may have become statistically significant with a larger number of iris biopsies (Table 2).

ICAM-1 was expressed in a significantly higher number of iris biopsy specimens from patients with uveitis than in controls. This corresponds with earlier studies.[11] [12] It seems likely that ICAM-1, expressed at low levels on normal endothelium, has been upregulated by chemokines or cytokines. IL-1, TNF-α, and IFN-γ are known to play a specific role in the upregulation of these ICAM-1 molecules.

LFA-1α was also found in a high number of iris biopsies from patients with uveitis, but only reached statistical significance on the population of iris resident cells. A striking difference was found between the expression of LFA-1α (α_L integrin subunit) and LFA-1β (β_2 integrin subunit). Perhaps in patients with uveitis, this α subunit is associated with a different, yet unknown, β chain.

VCAM-1, of which no earlier studies investigated the expression in the iris of patients with uveitis, was found in a significantly higher number of iris biopsies from patients with uveitis than in controls. This points to an activated endothelial state and perhaps a role for IL-1, TNF-α, or IL-4, which are all capable of inducing this adhesion molecule. Moreover, VCAM-1 plays a prominent role in lymphocyte recruitment to sites of inflammation and can be activated by antigen presenting cells.[20] VLA-4 (α_4 subunit), the VCAM-1 counterreceptor, was found in only one iris biopsy specimen from a patient with uveitis. This is a score somewhat below expectations, because VLA-4 is also the counterreceptor for fibronectin, which has been reported earlier to be present in the iris of a majority of patients with uveitis.[3] However, one has to keep in mind that

the absence of VLA-4, which is a late activation marker of T cells, in our iris biopsies was perhaps not surprising since all iris biopsies were obtained in a quiet phase of the disease.

LECAM was not detected in the iris of patients with uveitis. This may be explained by the fact that LECAM (L selectin) is thought to be involved in the initial steps of the adhesion cascade, and all iris biopsy specimens were obtained from patients with a quiescent uveitis, usually with a disease duration of 5–10 years. Moreover, L selectin (LECAM) is shed after activation by protean cleavage near the membrane insertion.[6] This cleavage has been speculated to be a mechanism by which lymphocyte trafficking and transmigration are modulated, producing an attenuation of the immune response.[6] Furthermore, it has recently been demonstrated that various NSAIDs such as indomethacin, ketoprofen, or diclofenac are capable of preventing leucocyte/endothelial cell interaction through the specific effect of inducing L selectin loss by endothelial cells.[23]

HECA-452, a marker for specialised high endothelial venules (HEV),[21] was detected on endothelial cells in the iris of three patients with uveitis, although these venules showed no characteristic morphological changes. Wakefield *et al*,[11] however, did report the morphological appearance of HEV in some of the iris biopsy specimens from their series of patients with uveitis. It is an interesting finding, since HECA-452 is a homing receptor for lymphocytes, and it supports the idea that the eye is capable of processes by which T cells may home to the eye, and it supports its role as an immunocompetent site.

One has to keep in mind, however, that iris biopsy specimens were obtained in a quiet phase of the uveitis in all cases. It may be expected that the expression of adhesion molecules is elevated during the active stages of disease, and perhaps even a different profile of adhesion molecule expression may then be found. Furthermore, the expression of adhesion molecules in the iris of patients with uveitis may be strongly influenced by the fact that most of these patients were treated with local steroids for some time. Corticosteroids have been demonstrated to decrease the expression of adhesion molecules like E selectin and ICAM-1 on stimulated endothelium.[24]

In our study a higher number of control specimens showed an expression of adhesion molecules than in earlier studies.[11] [12] This may be due to the fact that our control population consisted of iris biopsies from patients with glaucoma treated with local antiglaucoma therapy, rather than normal eye bank eyes[12] or patients with cataract[11] who never had any local therapy. Patients with glaucoma cannot entirely be regarded as "normal" controls, as earlier studies and also the current study, reported the presence of scattered infiltrating lymphocytes in the iris of these patients.[2] [3]

In our previous study on iris biopsy specimens from patients with uveitis[25] we found a typical deposition of immunoglobulins and complement in the vessel walls of the iris and not in the surrounding tissue, strongly suggesting a

peripheral blood origin of these immune deposits. Conditions in the endothelial wall of the iris vessels of patients with uveitis may therefore be altered and have an immunoregulatory function. The expression of adhesion molecules on (activated) endothelium found in our current study supports this idea, and suggests the involvement of iris vascular endothelial cells in rolling, adhesion, and transmigration of circulating lymphocytes into the inflamed site in a tissue specific manner. One can, however, not exclude the possibility that the increased expression of adhesion molecules on endothelium in uveitis patients is a non-specific response.

Several therapies that modulate the expression of adhesion molecules have been proposed and some of them have already been carried out in animal models[26]: (1) the use of receptor specific monoclonal antibodies, (2) oligonucleotide based therapies that inhibit the transcription or translation of the adhesion receptor mRNA, (3) small synthetic peptides or peptide analogues that resemble cell ligands and are capable of blocking adhesion receptors, and (4) cytokine directed therapies that decrease the upregulation of adhesion receptors. In animal models with uveitis injections with monoclonal antibodies against ICAM-1 or LFA-1 resulted in a significant decrease of ocular inflammation.[15 27 28]

In conclusion, an increased expression of adhesion molecules in the iris of patients with uveitis was found. However, no specific pattern or combination of expression of adhesion molecules could be detected. Perhaps other, yet unidentified adhesion molecules, specific for the iris, alone or in combination with the adhesion molecules studied here, dictate a tissue specific target for T cells entering the inflamed iris from the circulation. Future studies, preferably in more active stages of the disease, are necessary to search for such iris specific adhesion molecule(s), of which the expression seems obligatory, since the eye is known to be an immunologically privileged site.

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