Histol Histopathol (2011) 26: 725-733

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Induction of epithelial migration of lymphocytes by intercellular adhesion molecule-1 in a rat model of oral mucosal graft-versus-host disease

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Summary. To elucidate the involvement of intercellular adhesion molecule-1 (ICAM-1) in the migration of lymphocytes to the oral mucosal epithelium in a rat model of acute graft-versus-host disease (AGVHD), we investigated (1) ICAM-1 and major histocompatibility complex (MHC) class II expression by keratinocytes (KCs) and their role in the epithelial infiltration of $CD8^+$ cells, (2) the tissue expression of interferon-γ (IFN-γ) mRNA and expression of IFN-γ receptor by KCs, and (3) the ability of KCs to direct $CD8^+$ cells into the epithelial layers. We classified the oral mucosal lesions into three consecutive temporal phases on the basis of increased epithelial ICAM-1expression: basal- (phase I), parabasal- (phase II), and pan-epithelial except for the cornified cell layer (phase III). Basal ICAM-1 expression by KCs preceded that of MHC class II molecules, infiltration of $CD8^+$ cells and epithelial histological changes. Tissue expression of IFN-γ mRNA and expression of IFN-γ receptor on KCs evidenced by immunohistochemistry were detected in early lesions (phase I), indicating that locally produced IFN-γ induced ICAM-1 expression by KCs. $CD8^+$ cells were bound to KCs in frozen sections of epithelial lesions, whereas no lymphocyte attachment was observed in normal KC. Adherence could be inhibited by pretreating $CD8^+$ cells with lymphocyte function-associated antigen-1 (LFA-1) antibody and/or by pretreating sections with ICAM-1 antibody. Our data suggest that in the early phase of acute oral mucosal GVHD, the induction of ICAM-1 expression on KCs leads to the migration of $CD8^+$ cells into the epithelium and that this is mediated in part by the ICAM-1/LFA-1 pathway.

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Key words: ICAM-1, GVHD, Oral mucosa, IFN-γ, Rats

Introduction

Acute graft-versus-host disease (AGVHD) is characterized by selective epithelial inflammation affecting the mucocutaneous organs, digestive tract, and the liver (Aractingi and Chosidow, 1998). Among the mucocutaneous organs, oral mucosa is one of the target tissues affected by GVHD (Woo et al., 1997). Characteristic histological findings in acute mucocutaneous GVHD include satellitosis, in which lymphocytes form clusters around dyskeratotic and/ or necrotic keratinocytes (KCs). During this process, lymphocytes, particularly $CD8^+$ cells, migrate from the perivascular interstitium into the overlying epithelial layer and induce degenerative changes in KCs. These histological changes are considered central to the concept of a target cell stage in cutaneous GVHD (Wagner and Murphy, 2005). The phenomenon has led to the proposal that determining the nature of lymphocyte-KC interactions will aid in understanding the pathogenesis of acute mucocutaneous GVHD.

Specific molecular changes must occur in KCs to allow lymphocyte migration into the surface epithelial layer. For example, previous studies have reported that KCs express elevated levels of MHC class II molecules in mucocutaneous GVHD (Lampert et al., 1981; Sviland et al., 1988; Singer et al., 1989), in advance of lymphocytic infiltration of the epithelium (Sloane et al., 1988). Although MHC class II expression may be interpreted as a sign of immune response to KCs, direct evidence is lacking that these molecule mediate the interactions between KCs and lymphocytes. Intercellular adhesion molecule-1 (ICAM-1; CD54) also appears to play an

important role in this process because KCs express ICAM-1 *in vivo* only in the vicinity of dermal or epidermal lymphocytic infiltrates, and several studies have reported a strong correlation between ICAM-1 expression by KCs and the clinical severity of skin disease (Lisby et al., 1989; Singer et al., 1989; Vejlsgaard et al., 1989). Although KC expression of ICAM-1 has been detected in skin biopsies of patients with AGVHD, it remains controversial as to whether ICAM-1 expression by KCs is of any value in terms of early diagnosis of cutaneous GVHD (Norton and Sloane, 1991, 1994).

Epidermal KCs are a major target of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α). IFN-γ and, to a lesser degree, TNF-α stimulate the expression of ICAM-1 and MHC class II molecules by KCs both *in vitro* and *in vivo* (Griffiths et al., 1989; Barker et al., 1990; Albanesi et al., 1998). Moreover, ICAM-1 plays a critical role in T-lymphocyte adhesion to KCs since it is a ligand for ß2-integrin lymphocyte function-associated antigen-1 (LFA-1; CD11a/CD18) (Dustin et al., 1988). Although the secretion of IFN-γ and the resulting induction of the ICAM-1/ LFA-1 pathway likely induce the migration of lymphocytes into the surface epithelium of the oral mucosa during the development of acute oral mucosal GVHD, conclusive evidence for this mechanism does not exist. Our approach to this question has been to focus on ICAM-1 expression by KCs and its interaction with LFA-1 in acute oral mucosal GVHD. The study had three goals: (1) to determine ICAM-1 expression by KCs prior to epithelial infiltration of $CD8^+$ cells, (2) to investigate the relationship between ICAM-1 expression by KCs and the tissue expression of IFN-γ mRNA, and (3) to determine whether KC-lymphocyte adhesion occurs in oral lesions and, if so, to determine whether ICAM-1 mediates binding of $CD8^+$ cells to KCs. The results indicate that during the development of acute oral mucosal GVHD in rats, the induction of ICAM-1 expression by KCs leads to the migration of $CD8^+$ cells into the epithelium, mediated in part by the ICAM-1/LFA-1pathway.

Materials and methods

Animals

Inbred adult female rats (250 - 350 g), Lewis (LEW, $RT1^l$) and LEW x Brown Norway F1 hybrid (LBNF1, $RTl^{l/n}$), were purchased from Kyudo Co. (Saga, Japan). Animal studies were conducted in accordance with protocols approved by the Animal Care and Use Committee of Fukuoka Dental College.

Induction of AGVHD

Spleens removed from the LEW rats were dissected in Hanks' solution, forced through a stainless steel sieve, and the resulting cell suspension was filtered through a nylon mesh (Cell Strainers; BD Biosciences, CA, USA). The cells were washed three times in Hanks' solution and resuspended at a concentration of 10^8 cells /ml in RPMI-1640 medium containing 10% fetal calf serum. Cell viability was determined by trypan blue exclusion analysis. AGVHD was induced by intraperitoneal injection of $3x10^8$ LEW spleen cells (3 ml) into $LBNF_1$ rats (n=63). Untreated $LBNF_1$ rats (n=21) and $LBNF_1$ rats (n=42) injected with an equal number of syngeneic LBNF1 splenocytes were used as controls. All rats were weighed daily and carefully observed for clinical signs of disease.

Assessment of AGVHD

Clinical assessment of AGVHD was determined by weight loss and the development of cutaneous or mucosal erythema, especially on the ears, nasal mucosa, foot-pads, and lips. Both symptoms appeared on day 10 post-injection and became severe thereafter. Spleen weights were determined at autopsy to help support the subsequent immunological assessment of AGVHD. After day 10, the experimental rats showed remarkable splenomegaly due to over expression of AGVHD-related immune responses (Grant et al. 1989). All control animals survived and appeared healthy.

Tissue preparation

Whole tongues were excised 1-21 days post-injection from three animals from each treatment group. Normal or control tongues were collected from the untreated or the syngeneic group. Half of the tongue specimens were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin. Paraffin sections (4 μm) were then stained with hematoxylin and eosin (HE) to help visualize histopathological changes. The other specimens were immediately frozen in liquid nitrogen, and serial frozen sections were used for immunostaining, extraction of total RNA, *in situ* hybridization (ISH), and *in vitro* adhesion assays.

Immunohistochemistry

Acetone-fixed frozen sections were first incubated with normal rabbit serum to reduce nonspecific binding and then reacted with one of the following monoclonal antibodies (mAbs): anti-CD54 (ICAM-1) mAb (1:100 dilution, 1A29), anti-CD11a (LFA-1α) mAb (1:50 dilution, WT.1), anti-CD8 mAb (1:100 dilution, OX8), anti-class II mAb (1:100 dilution, OX6), anti-CD5 mAb (1:50 dilution, OX19) (all purchased from Cedarlane Lab., Ontario, Canada), and anti-IFN-γ receptor ß mAb (1:100 dilution, 9.PL.3; Abcam, Tokyo, Japan). Sections were then incubated with alkaline phosphatase-conjugated anti-mouse antibody (1:150 dilution;

DakoCytomation, Tokyo, Japan). Immune complexes were visualized using 5-bromo-4-chloro-3-indolyl phosphate/ nitro blue tetrazolium chloride solution (BCIP/NBT solution; DakoCytomation). As a control, sections were treated with normal mouse IgG instead of the first set of antibodies.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from serial frozen sections by acid guanidinium thiocyanate-phenol-chloroform extraction using an ISOGEN kit (Nippon Gene, Toyama, Japan).

RT-PCR was performed using the Access RT-PCR System (Promega Corp., Tokyo, Japan) according to the manufacturer's instructions. The primer sets were as follows: (1) glyceraldehyde-3-phosphate dehydrogenase (G3PDH), 5'-TGAAGGTCGGTGTCAACGATTTGGC-3' and 5'-CATGTAGGCCATGAGGTCCACCAC-3' (983-bp product); (2) IFN-γ, 5'-ATCTGGAGGAAC TGGCAAAAGGACG-3' and 5'-CCTTAGGCTAGA TTCTGGTGACAGC-3' (288-bp product). Thirty-five cycles were carried out in the thermal cycler. The amplified products were analyzed in 1.8 % agarose gel and visualized by ethidium bromide staining.

In situ hybridization (ISH) dectection of IFN-γ mRNA

ISH for IFN-γ mRNA was performed using a degoxigenin (DIG)–labeled oligonucleotide probe. The nucleotide sequences of the probes were as follows: (1) sense (control), 5'-CCTCTTGGATATCTGGAGGA ACTGGCAAAAGGACGGTAACACGAAAATAC-3', and (2) anti-sense, 5'-GTATTTTCGTGTTACCGT CCTTTTGCCAGTTCCTCCAGATATCCAAGAGG-3'. Frozen serial sections were first treated with 0.2 M HCl to remove endogenous alkaline phosphatase activity, incubated with proteinase K (20 μg /ml; Sigma, St. Louis, MO, USA) in PBS, fixed with 4% paraformaldehyde in PBS, and incubated with 0.1 M triethanolamine containing 0.25 % acetic anhydride. Sections were then prehybridized in 50% formamide containing dextran sulfate (Sigma), ssDNA (Sigma), and tRNA (Sigma) in PBS for 1h at 50°C and then hybridized with the DIG-labeled probe (100 ng /ml) at 50°C overnight. Sections were then incubated with anti-DIG-conjugated alkaline phosphatase (1:1,000 dilution; Roche Diagnostics, Germany) for 90 min at room temperature and the resulting products were incubated with BCIP/NBT solution for 10 min at room temperature.

In vitro adhesion assay

Stamper-Woodruff-type frozen-section assays were performed as described previously (Stamper and Woodruff 1976). Lymphocytes were teased from the superficial and deep cervical lymph nodes of $LBNF_1$ rats exhibiting splenomegaly, and suspended in RPMI-1640 medium at 4°C. The cells were dispersed by rapid, in-out pipetting, and the cell clumps were removed by passage through a nylon mesh (Cell Strainers; BD Biosciences). The resultant single-cell suspension was washed three times in the same medium and resuspended at a concentration of 3 x 10^7 mononuclear cells/ml. $CD8^+$ cells from the suspension were isolated by magnetic bead purification using the Miltenyl CD8a microbeads according to the manufacturer's protocol (Miltenyi Biotec, Tokyo, Japan). Thereafter, aliquots containing $2.5x10^5$ $CD8^+$ cells in 200 μl of RPMI-1640 medium were added to freshly cut (8 μm) frozen sections of the tongue obtained from rats in the control and AGVHD groups. The sections were agitated on a rotary shaker (60 rpm) for 60 min at room temperature. Next, the cell suspension was carefully decanted, and cells adhering to sections were fixed with 2.5 % glutaraldehyde in PBS for 5 min. Slides were then washed in PBS and stained with 0.5% toluidine blue. The number of adherent $CD8^+$ cells was determined by light microscopy examination of fields at 200x magnification (each high-power field represented approximately 400 μm of oral epithelium). The number of $CD8^+$ cells attached directly over KCs (but not in the cornified layer) were counted.

Several blocking experiments were performed: (a) $CD8^+$ cells were preincubated with 10 μg/ml of OX19 or WT.1 mAb for 30 min at room temperature, before the entire reaction mixture was transferred onto frozen sections of phase III tongues; (b) frozen sections of phase III tongues were incubated with OX 6 or 1A29 mAb for 30 min at room temperature following a brief wash with PBS, and were added to the lymphocytes; and (c) the two approaches were used simultaneously, i.e., $CD8^+$ cells were treated with WT.1 mAb and the tissue sections were treated with 1A29 mAb. The results are presented as the percentage of binding relative to control lymphocytes and tissue sections exposed to buffer alone.

Statistical analysis

The values shown represent the means ± standard error of means (SEM). Probability (p) values were calculated using Student's t-test.

Results

Expression of ICAM-1 precedes intraepithelial infiltration of $CD8^+$ cells in the oral mucosa

First we examined whether ICAM-1 expression could be detected in the epithelium during the development of AGVHD. We classified the oral mucosal lesions diagnostic for AGVHD as three consecutive temporal phases based on epithelial binding patterns of ICAM-1 (Fig. 1, Table 1).

Untreated control tongues

In untreated LBNF1 rats, ICAM-1 expression was restricted to endothelial cells and perivascular cells of blood vessels (Fig. 1a). MHC class II antigens were expressed by epithelial Langerhans cells, lamina propria dendritic cells, and endothelial cells (Fig. 1e). Neither ICAM-1 nor MHC class II antigens were detected on epithelial KCs. Only a few $CD8^+$ cells were observed in the lamina propria and muscle tissue (Fig. 1i). No histological changes were seen with HE staining (Fig. 1m). The results were the same for tissue sections taken from experimental rats 5 days after injection.

Phase I (days 6-8)

This phase consisted of specimens taken from AGVHD rats 6-8 days post-injection and is characterized by ICAM-1 expression by KCs before histological or histochemical signs of AGVHD of the oral mucosa. ICAM-1 was uniformly expressed in the basal cell layer of the tongue (Fig. 1b). In contrast, the level of MHC class II expression, numbers of infiltrating $CD8^+$ cells, and the HE staining pattern closely resembled that of the controls (Fig. 1f,j,o).

Phase II (days 9-11)

In specimens taken from AGVHD rats 9-11 days post-injection, ICAM-1 expression could now be detected and extended from the parabasal to the lower spinous layers of the epithelium (Fig. 1c). MHC class II expression by KCs appeared in the basal layer of the epithelium, paralleling basal ICAM-1 expression in phase I (Fig. 1g). A few infiltrating $CD8^+$ cells were observed in the lamina propria beneath the surface epithelium (Fig. 1k). Small infiltratres of round cells in the upper lamina propria were revealed by HE staining,

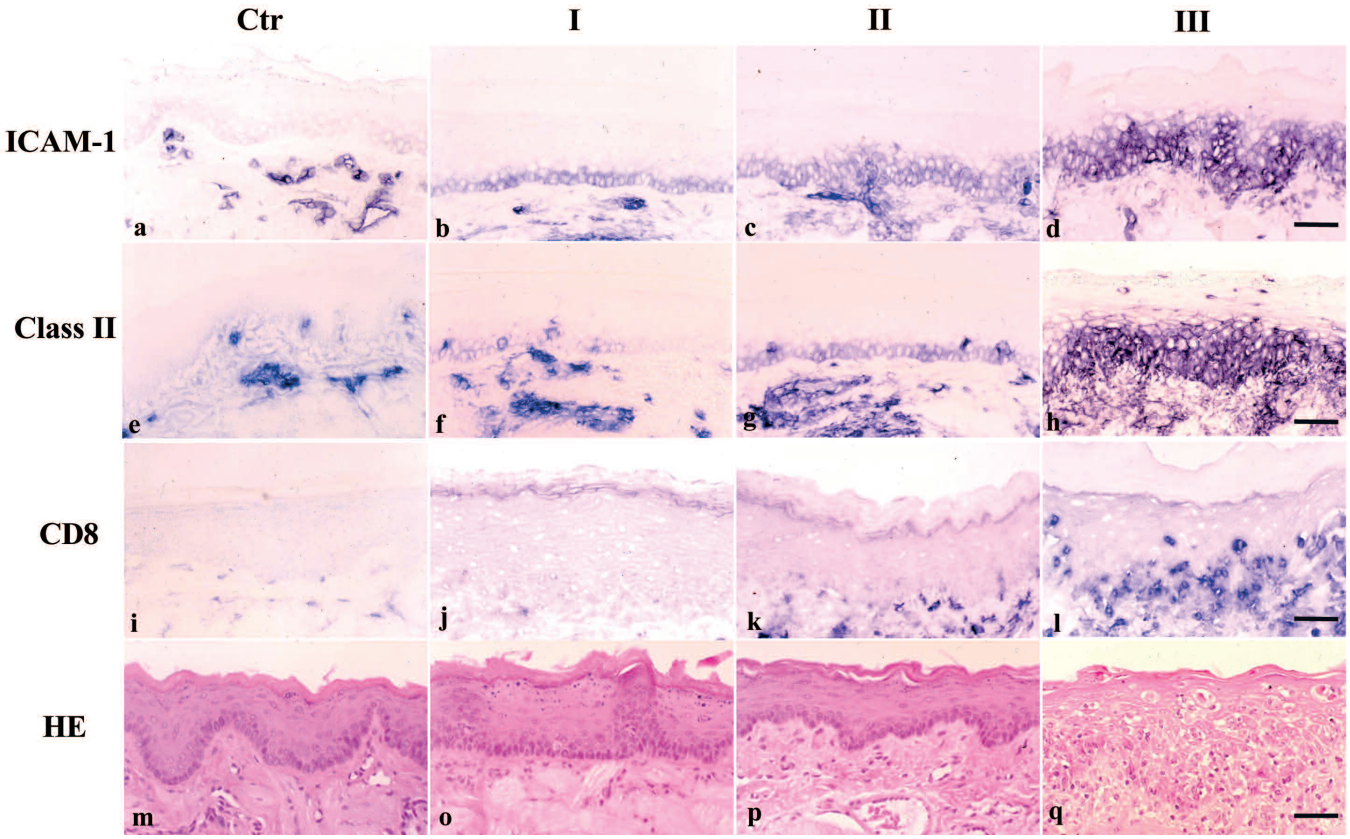

Fig. 1. Immunohistopathological staging of AGVHD by analyzing ICAM-1 expression. **a-d.** In tongue specimens from control rats, ICAM-1 is expressed only in the endothelial cells **(a)**. Epithelial ICAM-1 expression changes from being distributed in the basal **(b)** and parabasal **(c)** layers only to extend through the pan-epithelial region except for the cornified layers **(d)** during phase III disease. **e-h.** MHC class II antigen is detected in the dendritic cells of both the oral epithelium and lamina propria in control **(e)** and phase I **(f)** specimens.MHC class II expression is observed in the basal cell layer of the oral epithelium in phase II specimens **(g)**. In phase III specimens, all epithelial cells, except for the cornified cells, reacted with MHC class II (h). **i-l.** Only a few $CD8^+$ cells are found in the control **(i)** and phase I **(j)** specimens. Phase II AGVHD is associated with subepithelial infiltration of $CD8^+$ cells **(k)**. Extensive intraepithelial infiltration of $CD8^+$ cells is observed in phase III specimens(l). **m-q.** No obvious changes are observed in HE- stained tongue sections in control (**m**), phase I (**o**) or phase II (**p**) specimens. Epithelial degeneration is observed in phase III specimens **(q)**. Abbreviations: Ctr, control; I, phase I; II, phase II; III, phase III. Bar: 100 μm.

but no histological changes could be detected in the epithelium (Fig. 1p).

Phase III (day 12 and later)

Epithelial degeneration occurred during the third phase, which started after day 12, and was accompanied by ICAM-1 and MHC class II expression extending to the upper spinous layers of the epithelium (Fig. 1d,h). The number of $CD8^+$ cells increased in the lamina propria of the tongue (Fig. 1l). Furthermore, these cells tended to infiltrate the surface epithelium. HE staining revealed lesions typical of acute mucocutaneous GVHD, indicating eosinophilic cytoplasmic degeneration and necrosis of epithelial KCs (the so-called satellitosis) resulting from intraepithelial infiltration of lymphocytes (Fig. 1q).

IFN-γ mRNA and IFN-γ receptor expression in acute oral mucosal GVHD

IFN-γ induces ICAM-1 and MHC class II expression by KCs (Griffiths et al., 1989; Barker et al., 1990; Albanesi et al., 1998). We therefore examined tissue expression of IFN-γ mRNA in the tongue during each phase of the study. RT-PCR analyses showed that expression of IFN-γ mRNA was negligible in the tongue of untreated controls, but increased during phases I to III in treated rats (Fig. 2A). All tongue specimens were positive for G3PDH. To examine the distribution of IFN-γ mRNA expressing cells, ISH was performed using serial frozen sections of tongues with ICAM-1 expression on KCs taken from rats with AGVHD. IFN-γ mRNA was detected in mononuclear cells collected from the tongue of rats with AGVHD in phase I. $CD8^+$cells seldom infiltrated into the lamina propria (Fig. 2Ba). Cells with positive signals also infiltrated the surface epithelium. Tongue specimens from untreated controls lacked detectable expression of IFN-γ mRNA cells (data not shown). We also investigated whether KCs on tongues taken from rats with AGVHD expressed IFN-γ receptor. The IFN-γ receptor was immunolocalized to KCs residing in the basal and parabasal layers of the epithelium (phase I; Fig. 2Bb).

Binding of $CD8^+$ cells to the oral epithelium in AGVHD

Adhesion of $CD8^+$ cells to the oral epithelium in tongue tissues taken from rats in various phases of AGVHD was found to increase in comparision to controls as determined by Stamper-Woodruff binding assay (SWBA). This increase coincided with the progression of oral lesions (Table 2). In the oral epithelium of rats in the control group, adhesion of $CD8^+$ cells was minimal. The oral epithelium in phase I

Table 1. Three phases of AGVHD in the oral mucosa. Summary of the most striking changes in epithelial expression by ICAM-1 and MHC class II, infiltration by $CD8^+$ cells, and histology.

	ICAM-1	Class II	$CD8^+$ cells	Histology
Ctr	absent	absent	a few cells	intact
Phase I	basal	almost absent	unchanged	unchanged
Phase II	basal to parabasal	basal	increased	almost unchanged
Phase III	to upper spinous	to upper spinous	strongly increased	epithelial destruction

Ctr, control

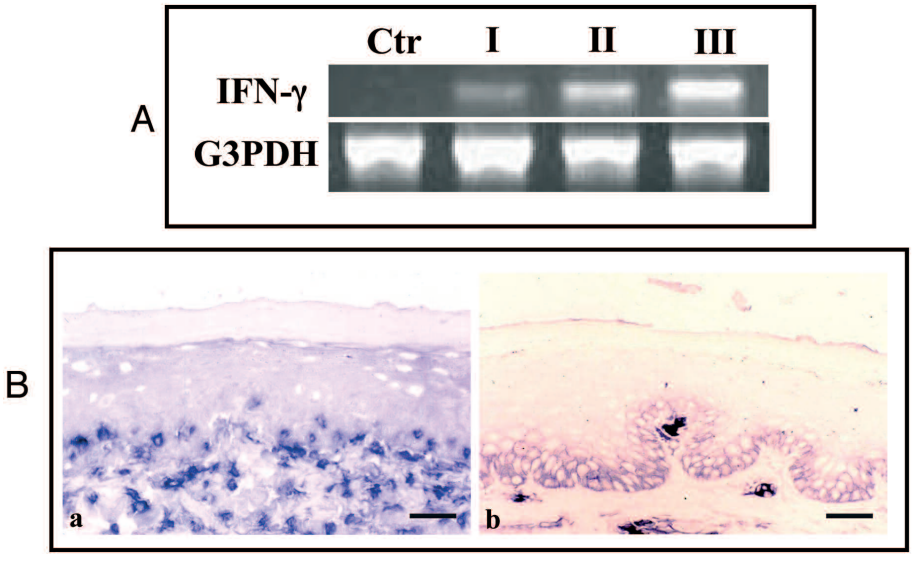

Fig. 2. Tissue expression of IFN-γ mRNA and IFN-γ receptor protein in acute oral mucosal GVHD. **A.** IFN-γ and G3PDH PCR amplification products. Oral lesions from phase I - III AGVHD specimens express IFN-γ mRNA.G3PDH mRNA, as an internal control, is also expressed in all lanes. Ctr, control; I, phase I; II, phase II; III, phase III. **B. a:** Detection of IFN-γ mRNA-expressing cells using an antisense DIG-labeled probe in tongue sections taken from rats with phase I AGVHD. Positive cells can be seen infiltrating both the epithelium and the lamina propria beneath the surface epithelium. **b:** Immunohistochemical detection of the IFN-γ receptor in tongue specimens taken from rats with phase I AGVHD.Signals can be observed in the basal and suprabasal layers of the epithelium. Bar: 100 μm.

specimens bound more lymphocytes than normal epithelium. The binding numbers of lymphocytes in phase II and III increased approximately 64- and 133-fold, respectively, compared to controls (P<0.001).

Figure 3 displays a representative binding assay of $CD8^+$ cells to the oral epithelium in tongue specimens taken from rats with AGVHD. The tongue specimens from phase I rats exhibited binding of lymphocytes mainly in the basal and parabasal layers of the oral epithelium (Fig. 3a). As the lesions developed, the lymphocyte binding extended into the epithelial layers. In phase III specimens, lymphocytes bound all KCs, except in oral epithelial cornified cells (Fig. 3b).

Table 2. Number of lymphocytes bound/mm on the oral epithelium of AGVHD.

Phases	Mean±SEM
Ctr	0.7±0.2
Phase I	9.6±0.9*
Phase II	44.5±3.6*
Phase III	93.2±3.8*

Ctr, tongue tissue from normal LBNF1 rats; SEM, standard error of the means. *P-value was calculated according to Student's t-test; P<0.001, analysis of data for phases I-III versus control value.

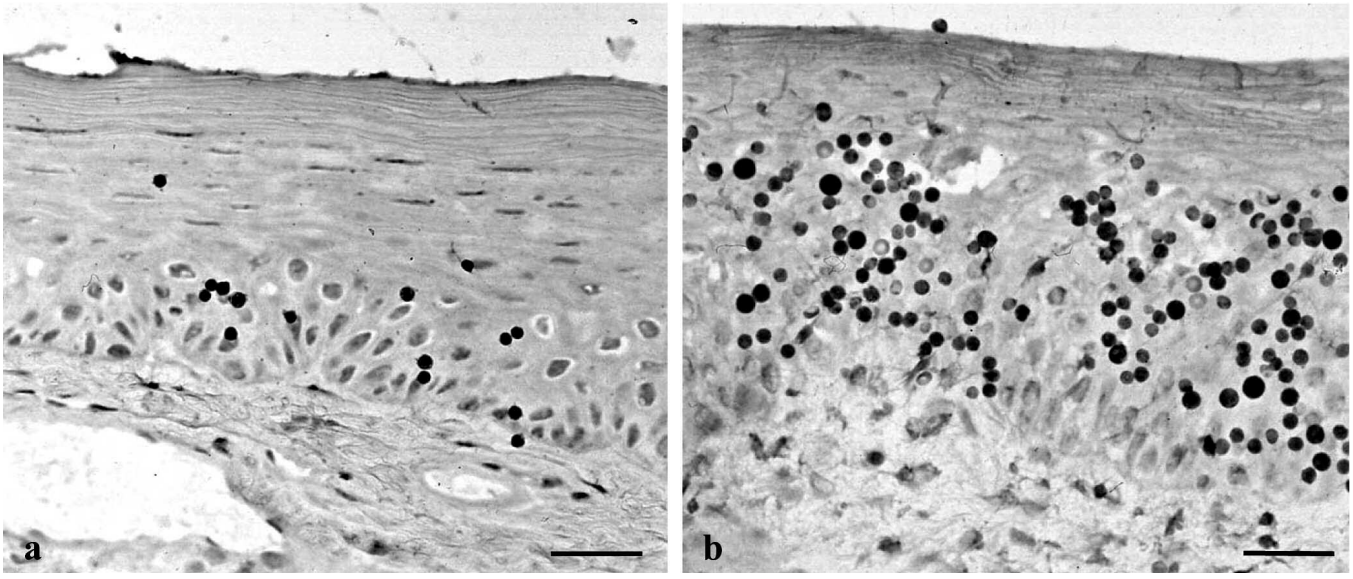

Fig. 3. Adhesion of $CD8^+$ cells to epithelial KCs in specimens from rats with AGVHD analyzed using SWBA. **a.** Adherent $CD8^+$ cells (round, back dots) are mainly located in the basal and parabasal layers of the oral epithelium in phase I specimens. **b.** Elevated $CD8^+$ cell adhesion to the oral epithelium in phase III specimens can be observed. $CD8^+$ cells adhered to all KCs except for the cornified cells. Bar: 100 μm.

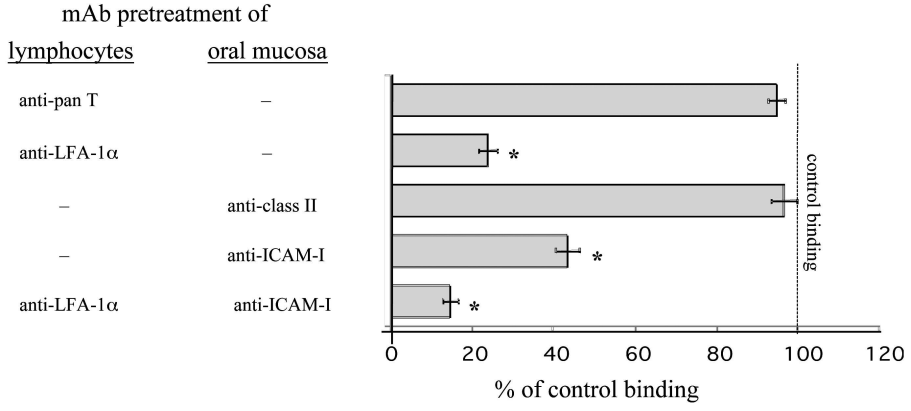

Fig. 4. Effect of mAbs on $CD8^+$ cell adhesion to the epithelium in AGVHD. Binding of $CD8^+$cells was significantly reduced when the cells were pretreated with LFA-1 mAb, as well as when epithelia were pretreated with anti-ICAM-1 mAb. * P-value was calculated by Student's t test: P<0.001 for either anti-pan T-treated $CD8^+$ cells or anti-class II-treated oral mucosa.

Anti-LFA-1 and anti-ICAM-1 mAbs inhibit adhesion of CD8⁺ cells to KCs in tongue specimens from rats with AGVHD

To provide evidence indicating a direct role of the ICAM-1/ LFA-1 pathway in the binding of $CD8^+$ cells to epithelial KCs, we performed SWBA using $CD8^+$ cells and/or epithelia taken from rats with AGVHD. Pretreatment of lymphocytes with anti-OX19 mAb had no effect, whereas the anti-LFA-1 mAb (WT.1) decreased lymphocyte adhesion by 24% of the control value (Fig. 4). When the tissue sections prepared from the oral mucosa of phase III AGVHD rats were incubated with anti-ICAM-1 mAb, lymphocyte adhesion to epithelial KCs was 44% of the control value (Fig. 4). In contrast, pretreatment of the tissue sections with anti-MHC class II mAb had no effect on lymphocyte binding. When the two approaches were applied simultaneously, i.e., when the lymphocytes were treated with anti-LFA-1 antibody and the tissue sections were treated with anti-ICAM-1 antibody, lymphocyte adhesion was reduced to less than 20% of the control value (Fig. 4).

Discussion

Here we use the haploidentical allogeneic F_1 hybrid rat model to elucidate the role of ICAM-1 expression by KCs in AGVHD. Although this model does not directly represent clinical hematopoietic cell transplantation in humans, it nevertheless provides a convenient and genetically well-defined system in order to gain insights into the mechanisms underlying immune-mediated host epithelial tissue damage by studying the *in vivo* interactions between KCs and lymphocytes (Thomas et al., 1996). We present three lines of evidence to support the conclusion that ICAM-1 expression by KCs plays a role in the migration of $CD8^+$ cells into the epithelium during the development of AGVHD. First, immunohistochemical approaches confirmed that ICAM-1 expression by KCs was one of the earliest events to occur during lesion development. Second, RT-PCR and ISH results indicated that ICAM-1 expression by KCs was induced by local IFN-γ release. Third, $CD8^+$ cell infiltration of the mucosal surface epithelium was mediated by the ICAM-1/LFA-1 adhesive pathway as revealed by using SWBA.

The immunohistochemical results presented here indicate that specific molecular changes in KCs occur in the early stage of acute oral mucosal GVHD. Both ICAM-1 and MHC class II expression by KCs precedes epithelial degeneration accompanied by intraepithelial infiltration of $CD8^+$ cells (confirming the presence of AGVHD-related lesions). Previous studies have demonstrated that the expression of both ICAM-1 and MHC class II molecules is induced in the epidermis of the skin during the development of AGVHD (Lampert et al., 1981; Sloane et al., 1988; Sviland et al., 1988; Singer et al., 1989; Norton and Sloane, 1991, 1994). The data presented here support the proposal made by Norton and Sloane (1994) that immunohistochemical assays for MHC class II and ICAM-1 could be helpful in diagnosing early GVHD. In this regard, we made the intriguing observation that ICAM-1 expression by KCs preceded MHC class II antigen expression. Basal cells harvested during phase I uniformly expressed ICAM-1but not MHC class II molecules. MHC class II expression occurred uniformly in phase II basal cells during the time when ICAM-1 expression by KCs extended to the parabasal region. These findings are consistent with parallel observations that ICAM-1 expression was strongly induced in cultured KCs after treatment with IFN-γ for 24 hours. In contrast, MHC class II molecule expression could not be detected. Identical results were reported by Dustin et al. (1988). That is, KCs treated with IFN-γ for 48- and 72-h strongly expressed both ICAM-1 and MHC class II molecules. We report here that the increase in ICAM-1 expression by KCs correlated with the onset of MHC class II expression by KCs and was accompanied by $CD8^+$ cell infiltration in both the upper lamina propria and the surface epithelium of the oral mucosa. Finally, typical histological changes indicating satellitosis appeared in phase III specimens when both ICAM-1 and MHC class II expression spread to the upper spinous layers of the epithelium. These findings suggest that ICAM-1 expression by KCs may trigger the onset of epithelial destruction by AGVHD-related immune responses in the oral mucosa.

RT-PCR and ISH studies showed that ICAM-1 expression by KCs coincided with tissue expression of IFN-γ mRNA, suggesting that local release of IFN-γ induces ICAM-1 expression. Previous RT-PCR analyses of human AGVHD show differences regarding the levels of IFN-γ mRNA expression. One group reported that skin specimens obtained from AGVHD patients were positive for IFN-γ mRNA (Roy et al., 1995). A second group failed to detect IFN-γ mRNA in skin biopsy specimens from patients with AGVHD (Trinchieri et al., 1984). This discrepancy may be explained by the limitations of performing PCR with templates derived from small biopsy specimens and/or by the timing of the biopsy. Various skin disorders (including AGVHD) associated with lymphocytic infiltration of the epidermis are believed to progress as the result of IFN-γ-induced ICAM-1 expression by KCs (Lisby et al., 1989; Singer et al., 1989; Norton and Sloane, 1994). Based on our detection of IFN-γ mRNA, it is reasonable to suggest that the induction of ICAM-1 expression by KCs is due to the presence of IFN-γ in oral lesions.

By immunohistochemistry, phase I specimens were found to express IFN-γ mRNA, along with ICAM-1 antigens in the absence of either $CD8^+$ or $CD4^+$ cells (data not shown). These findings suggest that cells other than T lymphocytes may be associated with IFN-γ synthesis, even though alloreactive T cells are known to participate in the secretion of this cytokine (Trinchieri et al., 1984; Klein et al., 1985; Fong and Mosmann, 1990). Our ISH results reported here do not identify the type(s)

of cells expressing IFN-γ mRNA. We speculate that natural killer (NK) cells (Trinchieri et al., 1984) are a likely source. In fact, previous studies identified NK cells as possible effector cells that infiltrate the skin of mice with AGVHD (Guillen et al., 1986; Ferrara et al., 1989). Our preliminary studies similarly show that cells morphologically resembling NK cells and bearing a NK marker can be found in the oral mucosa of rats with AGVHD prior to $CD8^+$ cell infiltration (data not shown). Future work will address the origin of cells bearing IFN-γ mRNA, and how these cells interact with KCs during the development of AGVHD.

Immunohistochemical detection of the IFN-γ receptor reported here provides indirect evidence that the local release of IFN-γ at the onset of AGVHD impacts KCs. This suggestion is supported by results showing that KC expression of the IFN-γ receptor correlates with infiltration of the epithelium by cells expressing IFN-γ mRNA. Furthermore, previous studies have demonstrated that the IFN-γ receptor can be expressed by KCs in some skin diseases (Nickoloff, 1987; Fransson et al., 1995; Farrell et al., 2006).

The concurrence of $CD8^+$ cell-adhesion to the epithelium and ICAM-I expression by KCs were demonstrated using the SWBA. Thus, the binding of $CD8^+$ cells on the epithelium during AGVHD increases along with ICAM-1 expression and correlates with disease progression. These results are consistent with *in vivo* observations of the ability of KCs expressing ICAM-1 to adhere to $CD8^+$ cells during AGVHD. Similar interactions have been reported previously (Nickoloff et al., 1987, 1988; Nickoloff and Griffiths, 1989). These studies demonstrate that tetradecanoylphorbol acetate-activated peripheral blood mononuclear leukocytes could bind to KCs expressing ICAM-I in either normal skin or in cultured IFN-γ treated KCs. This suggests that LFA-1 may play an in vivo role as an ICAM-1 receptor during the development of AGVHD. Furthermore, the results of mAb blocking experiments supported the idea that ICAM-1/ LFA-1 interactions account for the binding of $CD8^+$ cells. Thus, anti-ICAM-1, anti-LFA-1, or anti-ICAM-1 together with anti-LFA-1, blocked all $CD8^+$ cell-binding compared to mAbs recognizing unrelated molecules or to controls. These findings are identical to those of a previous study in which LFA-1 mAb blocked binding of activated mononuclear leukocytes to KCs treated with IFN-γ (Nickoloff and Mitra, 1988).

In conclusion, these results provide additional support for the role of ICAM-1, LFA-1, and IFN-γ, during the development of AGVHD by inducing migration of $CD8^+$ cells into the oral epithelium where they adhere to KCs. Regulation of KC ICAM-1 expression by IFN-γ is undoubtedly a key step in the onset of the disease. Therapeutic targeting of cytokine pathways that inhibit ICAM-1 induction may prove useful for blocking initiation of oral lesions related to AGVHD. Inhibition of ICAM-1/LFA-1 binding by treatment with cognate mAbs may also prevent oral mucosal lesions of AGVHD. The latter approach has already been applied to the mouse GVHD model (Blazar et al., 1995). These studies, as well as our data reported here, strongly indicate that specific ICAM-1/LFA-1 antagonists may interrupt development of acute oral mucosal GVHD. We are actively involved in a search for such molecules.

Acknowledgements. This study was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (#11671876, #13671977 and #1659190 to J.O.).

Conflicts of interest. The authors declare that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Accepted December 27, 2010