Characterization of Vascular Adhesion Molecules that may Facilitate Progenitor Homing in the Post-natal Mouse Thymus

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INTRODUCTION

T cell progenitors are generated in the bone marrow and travel through the bloodstream to enter the thymus, where they develop into mature T cells. However, there is little published information regarding the molecular mechanisms by which progenitors are recruited to the post-natal thymus. Our laboratory and others have shown that T cell progenitors enter the thymus through large blood vessels near the cortico-medullary junction (Brumby and Metcalf, 1967; Brahim and Osmond, 1970; Kyewski, 1987; Dunon et al., 1997; Lind et al., 2001). This recruitment of T cell progenitors to the thymus is an active phenomenon (Foss et al., 2001; 2002); the thymus is receptive to progenitor entry for approximately 1 week, followed by a 3-week refractory period. The availability of undefined intrathyemic stromal niches is thought to regulate the status of cell entry, such that once intrathymic sites are saturated with prothymocytes, new progenitors are not able to enter until the intrathymic progenitors move on and leave the niches empty (Foss et al., 2001; 2002). Together, the data on the restricted location of cell entry, and the periodicity of this process, indicate that progenitor recruitment to the thymus is regulated both spatially and temporally.

The process of leukocyte extravasation is fairly well characterized in secondary lymphoid organs and sites of chronic inflammation (Campbell and Butcher, 2000; Worthylake and Burridge, 2001). The first steps in the process are rolling and tethering, mediated by selectins and their ligands. L-Selectin ligands, which include CD34 (Baumheter et al., 1993), GlyCAM (Mebius et al., 1993) and MadCAM (Berg et al., 1993) can be modified post-translationally with carbohydrates such as MECA79 (Hemmerich et al., 1994), which are necessary for optimal ligand binding. Subsequently, integrins are required to mediate firm adhesion, and expression of their ligands, including ICAM-1 (Springer, 1994; Salas et al., 2002) and VCAM-1 (Weber and Springer, 1998), are required for this process. Some of these, including ICAM-1 and VCAM-1 are upregulated in response to pro-inflammatory cytokines and chemokines (Osborn et al., 1989; Xu et al., 1994). In addition, Vascular Adhesion Protein-1 (VAP-1) is a member of a newly described class of adhesion molecules that differ in the expression of both an adhesion domain and a catalytic domain. VAP-1 has a known role in leukocyte extravasation (for a review see, Salmi and Jalkanen, 2001).
and is upregulated by inflammatory cytokines such as IL1 and TNFα (Arvilommi et al., 1997).

In the present manuscript, we characterize expression of some of the canonical mediators of leukocyte extravasation (described above) in thymic progenitor seeding. We show that post-capillary venules in the post-natal thymus express a number of adhesion molecules related to rolling, tethering and firm adhesion, suggesting a potential role for these in the extravasation process. Of interest, only one of these, MECA79, is regulated with respect to the periodicity of progenitor recruitment, implicating the regulation of other signaling molecules, possibly including chemokines and/or inflammatory cytokines, in regulating the periodicity of this process.

MATERIALS AND METHODS

Mice and Antibodies

C57BL/6 male mice were purchased from NCI and kept under SPF conditions at Memorial Sloan-Kettering Cancer Center. Rat anti-mouse MAdCAM-1 was a gift from Dr Klaus Ley, University of Virginia; Rat anti-mouse CD34 was a kind from Dr Steven Nimer, MSKCC; Rat anti-mouse PNAd (MECA79) was purchased from BD Pharmingen; Hamster anti-mouse ICAM-1 (Pharmingen) was a gift from Dr Ralph Steiman, Rockefeller University; Rat anti-mouse VCAM-1 (clone M/K-2.7) was generated in the investigator’s laboratory; Rat anti-mouse VAP-1 (Monoclonal antibody 7–188 against mouse VAP-1 molecule was produced in rat, detailed description will be published separately); biotin conjugated rabbit anti-rat IgG was purchased from Vector Laboratories; biotin conjugated rabbit anti-rat IgG + IgM and biotin conjugated goat anti-Armenian hamster IgG were purchased from Jackson ImmunoResearch.

Tissue Sectioning and Freezing

Thymuses were harvested from mice and lobes separated with thin forceps. Corresponding lobes from mice of 4, 5, 7 and 9 weeks of age were immediately frozen side by side in the same mold in OCT Tissue Tech reagent (VWR). Transverse frozen sections of 5 μm thickness were taken at 2, 3, 4, 5 and 6 mm depths relative to the anterior–posterior axis (the thymus is generally 7 mm from anterior to posterior). Sections were stored at −20°C in a desiccated box until use.

Immunohistochemistry

Dissicated frozen sections were fixed in ice-cold acetone for 30 min. Endogenous peroxidase activity was quenched by incubating slides for 30 min in a solution of 1 mM NaN3, 10 mM glucose, and 1 U/ml of glucose oxidase (Calbiochem). Incubation in Avidin/biotin Blocking Kit (Vector laboratories) for endogenous biotin quenching, followed by 5% Fetal Bovine Serum (FBS) incubation for blocking non-specific binding were performed before antibody incubations. All antibody incubations were performed for 30 min at room temperature. Single-color histochemical detection was performed using an avidin-peroxidase conjugate system (VectaStain Elite; Vector Laboratories), and antibody-enzyme complex was visualized with 3,3′-diaminobenzidine (DAB; Sigma-Aldrich) and 0.1% H2O2. Incubation times were carefully monitored to prevent saturation, thus favoring visualization of differences in expression levels. Sections were counterstained using Mayer’s hematoxylin (Fisher Scientific), dehydrated through graded ethanol, cleared in Hemo-De (Fisher Scientific), and cover slipped using Permount (Fisher Scientific) for examination. Photomicrographs were taken using an Olympus BX40 microscope and Pixera Studio version 1.2 software (Pixera Corporation).

RESULTS

MECA79 is Expressed in Post-capillary Venules in the Adult Mouse Thymus

When expression of MECA79, one of the carbohydrate structures that modify ligands for L-selectin, was examined by immunohistochemistry, it was found to be expressed mainly on post-capillary venules deep in the cortex or in the cortico-medullary junction (Fig. 1). Expression of MECA79 appeared to be higher in tissues from animals at 5 and 9 weeks of age (peak of progenitor recruitment), and weaker in tissue from animals at 4 and 7 weeks of age (refractory periods). Proteins modified by MECA79, such as CD34 (Suzuki et al., 1996), are not restricted to post-capillary venules at the cortico-medullary junction, but rather are expressed by all vascular elements in the mouse thymus. Together, these findings indicate that the signals that regulate both the periodicity and the location of progenitor recruitment to the thymus may involve post-translational changes in scaffold proteins, rather than alterations in adhesion molecule synthesis per se.

VCAM-1 and ICAM-1 are Expressed by both Thymic Stromal Cells and Post-capillary Venules

Figures 2 and 3 show immunohistochemical analysis of VCAM-1 and ICAM-1, respectively. VCAM-1 was found to be expressed in venules near the cortico-medullary junction, as well as in the deep cortex and medulla, and was also present on some stromal cells (Fig. 2). However, the levels of VCAM-1 on vascular cells appeared to be significantly higher than those found on stromal cells. No differences in VCAM-1 expression were found in thymic sections from animals at different ages (4–9 weeks), indicating that while VCAM-1 may play a role in facilitating progenitor homing to the thymus, it does not appear to be involved in the periodicity of this event.
Similar to the distribution of VCAM-1, ICAM-1 was expressed by blood vessels deep in the tissue, both in the cortex and in the medulla (Fig. 3). Again, there appears to be differential expression of ICAM-1 among the blood vessels present. Some of the larger ones displayed a stronger, sharper staining in the luminal aspect of the vessel (Fig. 3B, arrow), while other smaller vessels presented a more homogenous and weaker staining. Some stromal cells also expressed ICAM-1, both in the cortex and in the medulla. It is difficult to say whether the apparently higher expression by stromal cells in the medulla is truly higher than it is in the cortex (see Fig. 3B, for details), or whether the apparent difference is a consequence of the differences in cell density in each compartment. In any case, ICAM-1 is present, and its expression does not appear to vary in relation to animal age, suggesting that like VCAM-1, it may play a role in progenitor entry into the thymus, although not in the periodicity of this process.

VAP-1 Expression is Restricted to Specific Venules found near the Sites of Progenitor Homing

Analysis of VAP-1 expression showed that it was restricted to blood vessels, and more precisely, to a relatively few venules found near the corticomedullary junction (Fig. 4). VAP-1 expression was not homogenous in most cases, showing a general bias towards the cortical aspects of the vessels where it was expressed. Venules within the medullary compartment did not express VAP-1.
nor did cortical capillaries. Nonetheless, VAP-1 expression did not appear to change during peak or refractory periods of progenitor homing to the thymus. These results strongly implicate VAP-1 in the process of recruiting progenitors to the thymus, but again, VAP-1 does not appear to be related to the periodicity of entry.

MAdCAM-1 is Expressed in the Thymus, but not by Endothelial Cells

MAdCAM-1 is an adhesion molecule most frequently involved in rolling and tethering of leukocytes in the circulation. In the thymus, MAdCAM-1 is expressed, but it appears to be restricted to a relatively few cells found within the medulla proper (Fig. 5). Based on their morphology, these appear to be stromal cells, although they are clearly not present in sufficient number to include all medullary stroma. Blood vessels in the thymus are categorically negative for MAdCAM-1, even though the same concentrations of antibody readily detected MAdCAM-1 in sections of mesenteric lymph node. Thus, although MAdCAM-1 is expressed in the thymus, it does not appear to be associated with blood vessels and is therefore unlikely to be involved in any aspect of progenitor recruitment.

DISCUSSION

In the present manuscript, we show that blood vessels in the post-natal thymus express a variety of adhesion molecules that facilitate leukocyte extravasation in secondary lymphoid organs or in inflamed tissue. Only one of these, MECA79, fluctuated temporally with respect to periods of progenitor recruitment, being highest during homing peak periods. MECA79 has previously been shown to be present in the medulla of the AKR hyperplastic thymus (Michie et al., 1995). Here, we find it to be expressed by postcapillary venules at the sites where progenitors are known to enter the post-natal thymus. Since CD34, which is one of the sialomucins that can be modified by MECA79, is expressed on all thymic vasculature, this finding suggests that post-translational modification, rather than protein synthesis, may be a critical factor regulating the periodicity of progenitor recruitment.
recruitment to the thymus. This, in turn, suggests that the feedback induced by niche occupancy in the thymus (Foss et al., 2001; 2002) may activate stromal and/or endothelial cells to upregulate enzymes such as 6-O GlcNAc sulfotransferase, which are required for such post-translational modifications (Hemmerich et al., 2001). In any case, our results suggests that L-selectin ligands may play a role in progenitor recruitment to the thymus, and that post-translational modification of L-selecting ligands may be involved in regulating the periodicity of this event.

Although they did not fluctuate with respect to the status of new progenitor recruitment by the thymus, other adhesion markers were found to be expressed by thymic vascular cells as well. Both ICAM-1 and VCAM-1 were found in thymic blood vessels, including post-capillary venules at the corticomedullary junction, indicating a potential role for these molecules in leukocyte extravasation into the thymus. We also found both of these to be expressed by thymic stromal cells. We and others have previously found VCAM-1 expression by thymic stroma (Salomon et al., 1997; Prockop et al., 2002), and we have shown it to be a critical ligand for transcortical migration of intrathymic progenitors (Prockop et al., 2002). Likewise, ICAM-1 has been shown by others to be expressed on various thymocytes, including thymocytes themselves (Reiss and Engelhardt, 1999), nurse cells (Cordes et al., 1997; Oliveira-dos-Santos et al., 1997), and other stromal cells (Reiss and Engelhardt, 1999; Lucas and Germain, 2000).

While we did not observe any differences in ICAM-1 expression on vascular cells with respect to the periodicity of progenitor homing, we did observe a difference in the pattern of staining on different types of vessels (see “Results” section), although the implications of this are presently unknown.

Although they did not fluctuate with respect to periods of homing activity, the expression patterns shown by VAP-1 in the thymus are particularly interesting for other reasons. VAP-1 was restricted to only a very few venules, and all of these were near the sites of progenitor entry at the corticomedullary boundary. Furthermore, in venules that actually span the border of cortical and medullary regions, the distribution of VAP-1 was asymmetric with higher expression towards the cortical aspect of the vessel, whereas in deep cortical venules that do not contact the medulla, distribution was homogenous around the perimeter. Thus, VAP-1, which could be involved in rolling/tethering, diapedesis, or both (Salmi and Jalkanen, 2001), displays a pattern of expression that is consistent with directing cell entry specifically in the region of the peri-medullary cortex, as previously shown (Lind et al., 2001). Further, the fact that VAP-1 is only expressed on a small subset of venules in this region is consistent with the small number of cells that enter the thymus at any one time (Shortman et al., 1990). Thus, VAP-1 appears to have significant potential as a candidate regulating the specificity of progenitor homing to the post-natal thymus via the blood.

Overall, it is interesting to note that with the exception of MECA79, none of the other adhesion molecules tested here were modulated with respect to the status of new progenitor recruitment by the thymus. It is possible that fluctuations in MECA79 expression are sufficient to regulate the periodicity of this process. However, in other tissues where leukocyte recirculation does not occur constitutively, other factors, such as inflammatory cytokines, are generally required to facilitate the extravasation process (Smith et al., 1991; Vaday et al., 2001). Chemokines are also heavily implicated, both in
inflamed tissue and in tissues where leukocyte recirculation is constitutive (Bargatze and Butcher, 1993; Rossi and Zlotnik, 2000; Mackay, 2001). Since adhesion molecules expressed by thymic endothelial cells are also found on blood vessels in a variety of other tissues, it is likely that additional signals are required to impart specificity to the thymic homing process. Thus, although critical reagents are generally lacking at present, characterization of cytokine and chemokine expression in situ at the sites of progenitor entry is likely to yield important information about the recruitment process. Together with characterization of adhesion molecule expression, such as presented here, such data will provide important insights into the regulation of the critical and poorly understood process of progenitor recruitment to the thymus in the steady state.

Acknowledgements

The authors would like to thank Dr Klaus Ley, University of Virgina, for anti-MAdCAM antibody; Dr Steven Nimer, MSKCC, for anti-CD34 antibody; Dr Ralph Steinman, Rockefeller University, for anti-ICAM-1 antibody; Dr Andrew Farr, MSKCC, for the peroxidase quenching protocol. This work was supported by PHS grants AI33940 (to H.T. Petrie) and CA08748 (to MSKCC). A.P. Lepique was supported by fellowship from CNPq, 200722/01-8 PDE. Brazil.

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