Recruitment of Immune Cells Across Atrial Endocardium in Human Atrial Fibrillation

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Background: Although clinical studies have suggested a link between inflammation markers and atrial fibrillation (AF), it is still unclear whether local immunologic responses actually exist in human atria during AF.

Methods and Results: To address this point, human left appendages were obtained from 16 patients who underwent cardiac surgery (5 with sinus rhythm (SR) and 11 with AF) and subjected to immunohistochemical analysis. In all the AF specimens, adhesion and migration of CD45-reactive cells were consistently observed predominantly in the atrial endo- and subendomyocardium and more prominently than in SR. Most of them were immunologically active CD68-positive macrophages, whereas CD3-positive T cells infiltrated to a lesser extent. Scavenger-receptor A staining revealed maturation of macrophages not in the endocardium but in the midmyocardium, a gradient from endo- to midmyocardium. In the endocardium, along with adhesion molecules (intracellular adhesion molecule-1 and vascular cell adhesion molecule-1), a chemotactic protein-1, which facilitates the recruitment, was more abundantly expressed in AF than in SR. Cytokines including transforming growth factor-β and interleukin-6 were frequently expressed by these macrophages.

Conclusions: These observations collectively imply active adhesion and recruitment of macrophages across the endocardium in human fibrillating atria, thereby supporting the concept of local immunologic inflammatory responses around the atrial endocardium of AF. (Circ J 2010; 74: 262–270)

Key Words: Atrial fibrillation; Endocardium; Inflammation
Immune Cells in AF

There were no significant differences in age and LAD between SR and AF, although they were both tended to be greater in AF than in SR. AF, atrial fibrillation; HD, heart disease; EF, ejection fraction; LAD, left atrial dimension; SR, sinus rhythm; A, ACE or ARB inhibitors; B, β-blockers; C, calcium-channel blockers; L, loop diuretics; N, nitrates; W, warfarin; AD, antiarrhythmic drug.

Table 1. Patient Characteristics

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<th>Sex</th>
<th>Age</th>
<th>AF history (months)</th>
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<th>EF (%)</th>
<th>LAD (mm)</th>
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<td>180</td>
<td>MR/AR/TR</td>
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<td>Total</td>
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<td>63.6±7.0</td>
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Histology and Immunohistochemistry
Blocks of tissues were optimal cutting temperature compound-embedded and immediately frozen in liquid nitrogen after resection. Frozen cryostat sections (8-μm thick) were cut, air-dried, fixed in acetone and then evaluated with standard protocols for staining with H&E and Masson’s trichrome. Immunostaining was carried out in sequential sections by Dako EnVision+ Systems (Dako) with primary antibodies listed in Table 2.

Infiltration of immune cells in the atrium was examined by light microscopy with immunostained images at a magnification of 100× captured with a digital camera (Nikon). Using Image Pro-plus software (Mediacybernetics, Carlsbad, CA, USA), the percentage of positive staining representing immunoreactivity was recorded and corrected by the total section areas. The mean value was obtained from 10 blindly selected different fields for each patient.

Immunofluorescence labeling for microscopy was carried out by treatment with Alexa Fluor 488 or 568-conjugated goat anti-rabbit antibodies or goat anti-mouse antibodies (Molecular Probes, 1:300 dilution). Immunofluorescence-labeled samples were examined with a Pascal Zeiss laser scanning microscope. The green channel had an excitation of 488 nm and an emission of 525 nm. The red channel had an excitation of 594 nm and an emission 620 nm. A lack of any cross-talk between the channels was established. Control

Table 2. Antibodies Used in This Study

<table>
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<tr>
<th>Antibody</th>
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<td>CD45</td>
<td>422721</td>
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<td>HLA-DRβ</td>
<td>ab20184</td>
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<td>CD3</td>
<td>413591</td>
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<td>CD8</td>
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<td>ICAM-1 (CD54)</td>
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<td>VCAM-1 (CD106)</td>
<td>BBA5</td>
<td>R&amp;D Systems</td>
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<td>IL-6</td>
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<td>TGF-β</td>
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IL, interleukin.
experiments carried out by incubation with secondary antibodies only did not show positive staining under the same experimental conditions.

**ELISA Assay**
Snap frozen blocks of tissues were homogenized in TE buffer and centrifuged. The aqueous extracts were subjected to a specific ELISA assay. All of the tissue samples were run in parallel for total protein content (Pierce, Rockford, IL, USA), and the results were standardized by expressing values as ng or pg of a particular protein per mg total protein.

**Statistical Analysis**
Values were expressed as mean±SD and compared using the unpaired t-test. Statistical significance was set at a P-value of <0.05.

**Results**
**Light Microscopy of Human Atria With SR and AF**
Light microscopic analysis showed a loss of contractile elements in some atrial myocytes and interstitial fibrosis to a varying degree in all specimens (Figures 1A, D). Patients with AF showed a greater extent of sarcomere loss and fibrosis than those with SR (fibrotic area: 9.9±2.2 vs 4.0±0.8 %, P<0.05). In addition, the intercellular space was more hyper-expanded in AF than in SR, as shown in Figure 1. These observations were all consistent with previous studies.23–25

**Imune Cells in Human Fibrillating Atria**
Immunohistochemistry revealed the inflammatory infiltration of immune cells, leukocytes positive for CD45, in human atria with AF in all specimens examined (Figure 1E), while it was also observed in the atria with SR to a lesser extent (Figure 1B). Their distribution was proved inhomogeneous, and the endo- and subendomyocardium of the atrium were more subjected to their infiltration than the midmyocardium (Figures 1B, E). Sequential sections suggested that the distribution of these CD45-positive leucocytes resembled that of cells positive for the macrophage-specific CD68 antibody (Figures 1C, F). To quantify the infiltration of immune cells, the acquired images for CD45 and CD68 antibodies were...
analyzed, and the results revealed that the infiltration of leucocytes and macrophages resembled each other in their distribution (more prominent in the endo-/subendocardium than in the midmyocardium and were also significantly greater in AF than in SR (Figures 2A, B).

Recruitment of Immune Cells Across Atrial Endocardium
Magnified images of the specimens identified the adhesion of many monocytes/macrophages to the atrial endocardium and showed their staged infiltration at several layers of the endocardium (Figure 1J). And, interestingly, HLA-DRβ staining showed that some of the apparent endocardial cells were also positive for this antibody, suggesting that they were immunologically active leukocytes (arrow in Figure 1K). These findings were more evident in AF (Figure 2C) and could be well explained by the fact that immunologically active monocytes/macrophages attached to and migrated into the atrium across the endocardium more in AF than in SR.

Immunofluorescence images supported this hypothesis, showing that most of the CD45-positive cells were positive also for CD68 (Figure 3A) and that the CD68-positive macrophages were positive also for the HLA-DRβ antibody (Figure 3B). Scavenger-receptor A (MSR) is known to be one of the maturation markers of macrophages. The antibody labeling of the specimens showed another piece of evidence for the recruitment of monocytes/macrophages across the atrial endocardium. The receptor labeling was remarkably inhomogeneous in the atrium and scarce in the endo- and subendocardium, suggesting a site-specific existence of immature and mature macrophages (Figures 1I, L). In the midmyocardium of the atrium, almost all macrophages were positive for scavenger-receptor A (matured macrophages, Figure 3C). In contrast, in the endo- and subendocardium, many macrophages did not express scavenger-receptor A (immature macrophages, Figure 3D), implying that immature macrophages migrated from the endocardium to the midmyocardium through the processes of maturation. The inhomogeneous distribution of MSR-positive cells was also confirmed quantitatively using immunohistochemical images stained with scavenger-receptor A (Figure 2D). The receptor-positive macrophages were observed much more in the midmyocardium than in the endo- and subendocardium, and more in AF than in SR.

Innate and Acquired Immune Responses in Human Fibrillating Atria
To identify other cell types infiltrating the atrium, CD3 and
CD8 staining was carried out and showed that CD3-positive T cells also infiltrated into the atrium (Figure 3E), although the degree was much smaller than that of the macrophages. This finding suggests that not only do innate immune responses operate in human atria with AF, but acquired immune responses also, at least in part, operate in human atria with AF. The infiltration was more evident in AF than SR, as was with that of macrophages. Some of the CD3-positive T cells were also positive for CD8 antibody (Figure 3E).
Figure 4. Immunostaining of human atrial samples with (A, D) intracellular adhesion molecule-1 (ICAM-1), (B, E) vascular adhesion molecule-1 (VCAM-1) and (C, F) monocyte chemotactic protein-1 (MCP-1) antibodies. The upper and lower panels show pictures from patients with sinus rhythm (SR) and atrial fibrillation (AF), respectively. (A, D) ICAM-1 was observed in the vasculatures and atrial endocardium. While the signals were similar in the vasculatures in SR and AF, endocardial expression was more evident in AF. (B, E) VCAM-1 expression was observed more prominently in the vasculature and endocardium in AF. (C, F) MCP-1 was predominantly expressed in the atrial endocardium both in SR and AF.

Figure 5. Content of (A) intracellular adhesion molecule-1 (ICAM-1), (B) vascular adhesion molecule-1 (VCAM-1) and (C) monocyte chemotactic protein-1 (MCP-1) in tissue homogenates by the ELISA assay. Although ICAM-1 and VCAM-1 contents were not significantly different between SR and AF, MCP-1 content was significantly greater in AF than in SR (P<0.05).
Adhesion and Chemotactic Molecules Expressed in Atrial Endocardium

In the vasculatures, recruitment of immune cells to the vessel wall across an endothelium requires activation of the endothelium, including expression of adhesion and chemotactic molecules. We sought to examine whether similar processes occurred in the endocardium of human atria. Immunohistochemical analysis showed that ICAM-1 expression was observed not only in the vasculature of the atrium, but also in the atrial endocardium (Figures 4A, D). Moreover, VCAM-1 expression was more abundant in the endocardium than in the atrial vessels in AF (Figure 4E), although its expression was scarce in SR. These observations would suggest that activation of the endocardium interacts actively with immature macrophages, leading to dominant localization of immature macrophages in the subendocardium. However, quantitative analysis using an ELISA assay with tissue homogenates could not detect any significant differences in the expression between SR and AF (Figures 5A, B) and it is partly because these adhesion molecules were expressed both in the vasculature and the atrial endocardium of the specimens.

After adhesion of immune cells, their transmigration into the endothelial layer is known to be governed by chemotactic proteins, the most characterized of which is monocyte chemotactic protein-1 (MCP-1). MCP-1 was more abundantly expressed in the atrial endocardium than in the vasculature both in SR and AF (Figures 4C, F). Comparing its expression between them showed that AF was more associated with the abundant expression of MCP-1 than SR, which was also confirmed by the ELISA assay with tissue homogenates (Figure 5C).

Expression of Transforming Growth Factor (TGF)-β and IL-6 in Atria With AF

In human atherosclerosis, many cytokines secreted from immune cells are known to be involved in lesion formation or thrombus formation. Therefore, we sought to determine whether the prominent recruitment of immune cells in atria with AF is associated with the local expression of cytokines including TGF-β and IL-6. Figures 5A, B shows the location of macrophages and expression of TGF-β and IL-6 by immunofluorescence images, respectively. These figures clearly showed that some of the macrophages that migrated into the atria highly expressed these cytokines. While TGF-β positive macrophages were detected in only 1 of 5 patients with SR, they were detected in 7 of 11 patients with AF. Similarly, IL-6 positive macrophages were detected in only 1 patient with SR, and they were significantly more detected in the 9 patients with AF (both P<0.05).

Discussion

The major findings of the present study were as follows: (1) In human left atrial appendages with AF, immune cells infiltrated atria predominantly in the endo- and subendocardium, more evidently in AF than in SR; (2) the cells were mostly composed of immunologically active monocytes/macro- phages in addition to a smaller number of CD3+ T cells; (3) the atrial endocardium expressed abundant ICAM-1, VCAM-1 and MCP-1, possibly providing feasible states for adhesion and transmigration of the immune cells; (4) in accordance with these alterations of the atrial endocardium, many immature macrophages were observed attached to the surface of, as well as buried in the layers of, the atrial endocardium,
Immune Cells in AF

with their maturation occurring in the midmyocardium; and (5) the migrated macrophages expressed TGF-β or IL-6 in atria more frequently in AF. These observations, all taken together, suggest that recruitment of immune cells, mostly monocytes/macrophages, occurred predominantly across the atrial endocardium during AF.

AF and Inflammation
There is mounting clinical evidence to support the influence of inflammation in the pathogenesis of AF. Numerous studies have demonstrated increases in serum or plasma inflammation markers in AF patients. Serum concentration of hs-CRP, one of the most sensitive markers for inflammation, has been known to be higher in AF patients than in controls and also to be higher in persistent AF than in paroxysmal AF. Levels of serum IL-6 concentrations, a cytokine that is produced by immune cells and endothelial cells, are also noted to be increased in AF patients compared with healthy participants. Although the precise mechanism for the increased circulating markers is uncertain, the results might reflect active participation of local inflammatory responses during AF and/or systemic inflammation caused by patient comorbidities.

Irrespective of these results, there have been few histological studies that investigate the association between AF and inflammation, which would provide direct evidence for the link. Results of atrial biopsies from patients with AF have demonstrated inflammatory infiltrates and oxidative damage within the atrial tissue. Other studies have also demonstrated that inflammatory CD45-positive cells infiltrated the right and left atrium with AF, as is consistent with the present results. In fact, both in SR and AF, the percentage area of CD45-positive staining in the endo/subendocardium in the patients of the present study was almost identical to that reported recently. These previous studies have emphasized the role of occult myocarditis in AF, and therefore data is limited about the distribution or cell types of the leukocytes in atria.

In the present study, we examined whether active and local inflammatory responses occur in human atria with or without AF, using immunohistochemical analysis. Our observations not only supported the presence of immunologically active immune cells in human atria with AF, but further identified the immune cell types and their distribution. The immune cells were mostly composed of active monocytes/macrophages, which were abundantly attached to the surface of the endocardium and thereafter apparently transmigrated into the myocardium. Moreover, the infiltration of the immune cells seemed to be affected by AF duration in the AF group, suggesting that the inflammation occurs slowly and progressively as the rhythm disorder continues. The gradient distribution of the maturation marker (scavenger receptor A) supported the recruitment of macrophages across the atrial endocardium. This novel mode of recruitment in AF requires more attention because the finding implies 2 different sources of immune cells. The finding also suggests that recruitment of immune cells in AF is attributed to oxidant stress produced by AF itself. Until now, atrial endocardial dysfunction has been believed to contribute to thrombus formation.

Furthermore, the present study advances the role of endocardial dysfunction in AF. In addition to providing feasible states for thrombus formation, the atrial endocardium of human AF facilitates the recruitment of macrophages into the atrium by expressing adhesion molecules and chemotactic proteins. Two immunoglobulin-like adhesion molecules, ICAM-1 and VCAM-1, which are abundantly expressed in the endocardium, cause a firm adhesion by the interaction between adhesion molecules and the surface of immune cells. Thereafter, abundant MCP-1 in the endocardium guides the adherent immune cells across the endocardium.

Roles of Immune Cell Infiltration
Proinflammatory macrophages are well known to contribute importantly to the progression of various cardiovascular diseases. They include atherosclerosis, myocardial Infarction and congestive heart failure. In atherosclerosis, activated macrophages transmigrate across the endothelium and participate critically in every stage of lesion formation by secreting matrix-degrading enzymes, prothrombotic molecules and also a variety of proinflammatory cytokines.

It would be reasonable to consider that similar mechanisms by immune cells could also operate in AF progression because the degree of the recruitment of immune cells increased in AF with the increment of MCP-1, TGF-β and IL-6 expression in fibrillating atria. These cytokines are well known to affect the contractility and electrical stability of myocardocytes inhomogeneously and to induce fibroblast activation leading to deposits of extracellular matrix fibrosis. These effects provide substrates for reentrant arrhythmias. In contrast, stretching of the myocardium is reported to induce activation of the endocardium, which initiates and promotes inflammation processes. Therefore, inflammation facilitates AF occurrence and atrial stretching by AF induces atrial inflammation (a vicious cycle between AF and inflammation). However, whether the local inflammation is a cause or a result of AF could not be determined and should be investigated in future studies. Even though the inflammation may be a consequence of AF in combination with underlying heart disease, it might subsequently perpetuate AF by triggering inflammatory cascade with secreted cytokines.

Study Limitations
A potential limitation of the present study is the lack of information regarding patients without structural heart diseases because of ethical issues, and our data focused on the differences between SR with a history of paroxysmal AF and persistent AF patients undergoing surgery. Therefore, it may be argued that surgical intervention or the valvular disease itself might induce local inflammation in the atrium. However, the different infiltration pattern of immune cells between SR and AF could not be explained only by these factors, provided that no comment can be made about the impact of immune cells in other patient populations with AF. Second, only left atrial appendage samples were available; therefore, our present findings may not represent other areas of the atrium. However, a recent study has demonstrated a good correlation in the number of inflammatory cells between the right and left atrial myocardium. Also, from a clinical point of view, thrombus formation in the left atrial appendage is more common and more important than other atrial tissues and, therefore, our findings in the left atrial append-
age are significant. Lastly, the number of patients examined was relatively small. Although limited for these reasons, the present study, by demonstrating the recruitment of immune cells, mostly active macrophages, across the atrial endocardium with abundant chemotactic protein in human AF, provides an aid for constructing an idea that AF is associated with chronic inflammation disorders. However, the time sequence and interrelationships among inflammation and AF perpetuation remain to be determined by future studies.

References