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ORIGINAL PRE-CLINICAL SCIENCE

The composition of ectopic lymphoid structures suggests involvement of a local immune response in cardiac allograft vasculopathy

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BACKGROUND: Cardiac allograft vasculopathy (CAV) is a multifactorial pathology limiting the survival of cardiac transplants. The etiology of CAV is unclear, but antibody-mediated and cellular-mediated responses have been implicated. We, and others, have observed ectopic lymphoid structures (ELS) surrounding epicardial coronary arteries with CAV. The potential contribution of these ELS to CAV has not been elucidated.

METHODS: Epicardial coronary arteries were collected from 59 transplant patients at 2 centers and studied for ELS presence and composition using immunohistochemistry. The intima and ELS were isolated, and the expression of the genes involved in tertiary lymphoid organ (TLO) formation was measured by quantitative polymerase chain reaction.

RESULTS: ELS presence was related to survival after transplantation ($p = 0.013$) and histologic composition of CAV ($p < 0.001$). ELS contain B and T lymphocytes, macrophages, and antibody-producing (immunoglobulin [Ig] M and/or IgG) plasma cells. A sub-population of B lymphocytes appeared to be cluster of differentiation (CD)20^{+}CD27^{+} memory B lymphocytes. The messenger RNA expression of TLO markers (lymphotoxin-β, and chemokine [C-C motif] ligand 19 and 21) was significantly higher in ELS than in the neointimal lesions. The ELS observed in this study exhibited some TLO markers but did not exhibit the distinct areas rich in B and T lymphocytes that are normally found in classic TLOs.

CONCLUSIONS: The cellular composition of the ELS differs from the cellular infiltrate in CAV intimal lesions. The presence of memory B lymphocytes and plasma producing IgM and IgG cells suggests that ELS are related to local antibody production, potentially contributing to antibody-mediated CAV. ELS associated with coronary vessels containing CAV show features of underdeveloped TLOs; classic TLOs may not develop due to patient immunosuppression.

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Cardiac allograft vasculopathy (CAV) develops in most heart transplant recipients[1] due to a chronic immune response directed against the donor heart.[2] In the epicardial

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coronary vessels, CAV is characterized by arterial intimal thickening,[3] the etiology of which has been linked to antibody-mediated rejection.[4,5] In this study, we examined potential sites of local production of anti-donor antibodies involved in CAV.

In histologic studies, clusters of inflammatory cells have been observed in the adventitia of the vascular wall and in the adipose tissue surrounding the epicardial coronary arteries.[6] We use the term "ectopic lymphoid structures (ELS)" to describe these structures, which have not been previously characterized in coronary vessels with CAV. The presence of ELS may reflect an ongoing process of lymphoid neogenesis, leading to tertiary lymphoid organ (TLO) formation in response to the persistent allostimulation provided by the graft. TLOs have been associated with sites of chronic inflammation[7] and described in transplant settings.[8,9]

The aim of this study was to define the components of these ELS. We hypothesized that ELS would be linked in a causative manner to CAV by providing a source of activated effector lymphocytes near the affected vessels. Confirming such a link could be of clinical importance to better understand the role of local immunity in the development of CAV.

Methods

Patient population and tissue procurement

Autopsy databases from the Capital District Health Authority (Canada) and University Medical Center Utrecht (The Netherlands) were searched for patients (with or without CAV) who died after heart transplantation and from whom archival coronary artery tissue was available. For study eligibility, a sample of the proximal region of at least 1 of the major coronary arteries (left anterior descending, left circumflex, right coronary artery) was required, and 59 patients met this criterion. Tissue was obtained under approval of the respective institutional ethical organizations (Capital Health Research Ethics Board REB #CDHA-RS2011-339 and Medical Ethics Committee #12/387).

Histologic measurements and categorization

Formalin-fixed, paraffin-embedded coronary artery sections (5 μm) were stained with hematoxylin and eosin to visualize CAV and ELS. All patients were categorized using a quantitative method according to the median size and number of ELS, as described in detail in the Supplementary Methods (available on the jhltonline.org Web site). Patients with a mean ELS area of <300 μm^2 were categorized as ELS-1, and patients with a mean ELS area of >300 μm^2 were characterized as ELS-2. The histologic (H-)CAV phenotype was categorized, as previously described, by cellular density and composition of the intimal layer.[10]

Immunohistochemistry

Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Antigen retrieval was performed using ethylenediaminetetraacetic acid solution (pH 9.0) or citrate solution (pH 6.0), depending on the marker (Supplementary Table 1, available on the jhltonline.org Web site). Sections were incubated with the primary antibody diluted in serum when necessary, followed by incubation with horseradish peroxidase (HRP) or biotinylated species–specific secondary antibody (Supplementary Table 1, available on the jhltonline.org Web site).

Staining for all markers (except peripheral node addressin [pNAD]) was completed using a 2-step process; for pNAD detection, a rabbit anti-rat polyclonal antibody was used as the secondary antibody, followed by incubation with a tertiary antibody with BrightVision anti-rabbit HRP (Immunologic, Duiven, The Netherlands). Biotinylated secondary antibodies were incubated with a peroxidase avidin/biotin complex. Enzymatic activity was detected using a diaminobenzidine (DAB) solution.

For double staining of pNAD and D2-40, slides were incubated with primary antibody against D2-40, antigen retrieval was performed using citrate solution, and the 3-step process for pNAD detection, described above, was completed. All sections were counterstained with hematoxylin (diluted 1:1 with Aqua Dest for T-Bet and forkhead box P3 [FoxP3] counterstaining). For each stain, tonsil or lung tissue was used as a positive control. Species-specific isotype-matched irrelevant antibodies (immunoglobulin [Ig]G1 isotype and IgG2a isotype), or omission of the primary antibody, were used as negative controls.

Sections were analyzed using digital images (original magnification $\times$200) for markers FoxP3 and T-Bet or using light microscopy (original magnification $\times$400) for all other markers. Quantification of immunohistochemistry was performed by calculating the percentage of positively stained cells for the specific marker divided by the total number of infiltrated mononuclear cells in the field of view.

Immunofluorescence

Memory B lymphocytes were identified using mouse anti-cluster of differentiation (CD)20 and rabbit anti-CD27 antibody diluted in 5% normal goat serum (Supplementary Table 1, available on the jhltonline.org Web site). Secondary antibodies included a goat anti-mouse conjugated to Alexa 488 (Invitrogen Corp, Carlsbad, CA) and a goat anti-rabbit conjugated to Alexa 555 (Invitrogen). Hoescht 33342 (Invitrogen), at a dilution of 1:5,000, was used as a nuclear counterstain. Digital images of immunofluorescence were captured using a Zeiss Axiovert 200M microscope (Carl Zeiss, Thornwood, NY) and a Hamamatsu Orca R2 camera (Hamamatsu, Boston, MA).

Laser tissue microdissection, RNA isolation, complementary DNA synthesis, and quantitative polymerase chain reaction

High-quality samples of 10 patients with ELS were stored at –80ºC and used for messenger RNA expression analysis. To interpret the patient samples correctly, a calibrator reference sample containing lymph node and placental tissue was included. Frozen tissue sections (10 μm) were cut and placed on polyethylene-2,6-naphthalene dicarboxylate foil-mounted glass slides (PALM Microlaser Technologies, Carl Zeiss, Bernried, Germany) and stained with hematoxylin. From every patient, an area of approximately 12×10^6 μm^2 was microdissected from the coronary artery intima and ELS using the Robot-Microbeam (PALM Microlaser Technologies). Tissue pieces were collected in laser pressure catapulting microfuge tubes for RNA isolation.

Total RNA isolation was performed using the Picopure RNA isolation kit (Arcturus, Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. RNA was eluted in