<Original Article>

Alternatively activated M2 macrophages increase in early stages of experimental autoimmune myocarditis in Lewis rats

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Abstract : To better understand the role of macrophages in early stages of experimental autoimmune myocarditis (EAM), we compared the expression of inducible nitric oxide synthase (iNOS) and arginase-1, markers for classically activated M1 and alternatively activated M2 macrophages, respectively, in the hearts of EAM-affected and control rats. Immunohistochemical evidence revealed that both iNOS-positive and arginase 1-positive macrophages were found in EAM lesions, while some cells were co-localized with both markers. This finding suggests that the increased level of arginase-1, which is partly from M2 macrophages, contributes to the modulation of EAM, possibly through the reduction of nitric oxide in the lesion.

Keywords : arginase-1, experimental autoimmune myocarditis, inducible nitric oxide synthase, macrophage

Introduction

Experimental autoimmune myocarditis (EAM), an autoimmune inflammatory cardiac disorder, is an animal model for human giant cell myocarditis [11, 12, 26]. Although the etiologies of giant cell myocarditis and dilated cardiomyopathy are not known, an autoimmune mechanism targeting cardiac myosin has been proposed; thus, EAM is an appropriate animal model for this human cardiac dysfunction [26]. EAM is initiated by autoreactive T cells and macrophages [11, 25]. The condition is characterized by a gradual decrease in inflammatory cells through apoptosis [9, 21, 27] followed by fibrosis in the damaged cardiac tissues leading to cardiac dysfunction [28]. The role of T cells in the development of EAM has been investigated [7, 16]; however, little is known about the involvement of macrophage phenotypes.

Research during the past decade has focused on the immunopathological changes in EAM lesions [26]. These changes are characterized by T cell and macrophage infiltration in early stages and subsequent secretion of pro-inflammatory molecules, including inducible nitric oxide synthase (iNOS) [25] and osteopontin [23] and fibrotic tissue replacing inflammatory cells, causing cardiac dysfunction in the later stages of EAM [13]. Various macrophage phenotypes play important roles in the initiation and remission of autoimmune diseases, including the experimental autoimmune

encephalomyelitis (EAE) rat model [2, 24], particularly in the remission of inflammation during the peak stage of EAE, suggesting that a similar macrophage phenotype is involved in EAM.

Macrophages are highly versatile because local environmental factors shape their phenotypic and functional properties [14]. Macrophages are classified as classical M1 (iNOS positive) and alternatively activated M2 (iNOS negative) macrophages and vary according to their induction factors, cytokine production, and phagocytosis type [14, 15, 19]. Immunologically, cytokines reciprocally regulate iNOS and arginase activity in macrophages [17]. M2 macrophages are readily identified because they contain an abundance of arginase [8, 10]. The induction of arginase-1 prevents macrophages from producing nitric oxide (NO), rendering them unable to kill intracellular pathogens [22]. In addition to their immunological roles, alternatively activated macrophages may be involved in tissue remodeling because they produce several extracellular matrix proteins [6]. However, it is not known whether alternatively activated macrophages are involved in the progression of EAM.

To better understand the involvement of macrophages in the development of EAM, the present study used immunohistochemistry to detect M1 (iNOS-positive) and M2 (arginase 1-positive) macrophages in cardiac tissue during the acute phase of EAM at 2 weeks post-immunization.

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Materials and Methods

Experimental animals

The subjects were 6- to 8-week-old male Lewis rats purchased from Orient Bio (Korea). The rats were housed in plastic cages and maintained at $23 \pm 2^\circ\text{C}$ under a 12 : 12 h light-dark cycle and fed 5L79 rat formula (PMI Nutrition, USA). Food and water were provided *ad libitum*. All experimental procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals at Jeju National University.

Purification of cardiac myosin

Cardiac myosin was prepared from the ventricular muscle of human hearts according Murakami *et al.* [18]. The antigen preparation consisted primarily of a 205 kDa protein, and although it contained small amounts of several substances other than cardiac myosin, this crude antigen preparation has been used to induce EAM in rats [1, 23, 25].

Induction of experimental autoimmune myocarditis

The cardiac myosin fraction was dissolved in phosphate-buffered saline (PBS) containing 0.3 M KCl at a concentration of 2 mg/mL. The antigen solution was mixed with an equal volume of complete Freund's adjuvant (CFA) supplemented with *Mycobacterium tuberculosis* H37RA (Difco, USA) at a concentration of 5 mg/mL. The rats were immunized with 0.1 mg cardiac myosin in CFA subcutaneously administered to each hind footpad followed by intraperitoneal administration of 5 μg of pertussis toxin (Sigma-Aldrich, USA). Age- and sex- matched Lewis rats served as controls and were challenged with PBS in CFA followed by intraperitoneal administration of 5 μg pertussis toxin.

Based on the results of our previous studies [1, 23, 25], the optimal time to investigate the acutely inflamed cardiac tissue was 2 weeks post-immunization (PI). Thus, the EAM-affected rats were sacrificed under ether anesthesia on day 14 PI and the phenotype of the cardiac cells expressing iNOS, arginase 1, or both were compared between the EAM and control rats using immunohistochemistry.

Antibodies

Mouse monoclonal or rabbit polyclonal anti-arginase-1 was obtained from Santa Cruz Biotechnology (USA). Rabbit polyclonal anti-TGF- β 1 (Santa Cruz Biotechnology) for immunohistochemical staining. Rabbit polyclonal anti-iNOS was obtained from BD Biosciences (USA). Mouse monoclonal anti-rat macrophage (ED1; AbD Serotec, UK) antibody was used to identify activated macrophages.

Histopathology

Transverse sections taken through the middle of the heart specimens were sliced into two equal portions and fixed in 10% formalin for microscopic grading. The paraffin-embedded tissue was cut and stained with hematoxylin-eosin for

histological examination.

Immunohistochemical analysis

Five-micrometer-thick sections of paraffin-embedded tissue were deparaffinized, heated in citrate buffer (0.01 M, pH 6.0) in a microwave for 3 min and then treated with 0.3% hydrogen peroxide in methyl alcohol for 20 min to block endogenous peroxidase activity. After three washes in PBS, the sections were incubated with 10% normal goat serum (Vector ABC Elite Kit; Vector Laboratories, USA) and then incubated for 1 h at room temperature with primary antibody: mouse anti-arginase 1 (dilution 1 : 400), rabbit anti-iNOS (dilution 1 : 200) and rabbit anti-TGF- β 1 (dilution 1 : 200). Primary antibody was omitted as a negative control. After three washes in PBS, the sections were treated with the appropriate secondary antibody (dilution 1 : 200; Vector Laboratories) for 45 min, washed three times in PBS, and then incubated for 45 min with avidin-biotin peroxidase complex (Vector ABC Elite kit; Vector Laboratories), and prepared according to the manufacturer's instructions. The peroxidase was reacted using a diaminobenzidine substrate kit (Vector Laboratories). The sections were counterstained with hematoxylin, dehydrated, and cleared with xylene before being mounted on slides. The immunostained sections were further analysed in three different sections from three different animals. Microscopy images ($\times 20$ magnification, progres C7; Jenoptik, Germany) were automatically treated with Image J software to measure each immunoreactive intensity and the percentage (%) of positive areas relative to the total area was calculated.

Double immunofluorescence

Double-labeling to detect the two antigens was performed as previously reported [14]. Briefly, paraffin sections were incubated with 10% normal horse serum for 1 h, followed by the first primary antibody including mouse monoclonal anti-arginase-1 (1 : 100), ED1 (mouse monoclonal anti-rat macrophages, 1 : 200 in dilution), and biotinylated anti-mouse IgG (1 : 200; Vector Laboratories) for 1 h at room temperature. After washing, the sections were incubated with tetramethylrhodamine isothiocyanate-labeled streptavidin (1 : 500, Zymed; Invitrogen, USA) for 1 h at room temperature. Then the sections were washed and incubated with second primary antibodies (1 : 100 rabbit polyclonal anti-arginase 1, 1 : 100 rabbit polyclonal anti-iNOS, or 1 : 100 rabbit polyclonal anti-TGF- β 1) followed by biotinylated anti-rabbit IgG (1 : 200; Vector Laboratories) overnight at 4°C . After washing, the sections were incubated with tetramethylrhodamine isothiocyanate-labeled streptavidin (1 : 500, Zymed; Invitrogen) for 1 h at room temperature. For counterstaining, 0.001 $\mu\text{g}/\text{mL}$ 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI; Thermo Fisher Scientific, USA) in PBS was added and incubated at room temperature for 20 min. The immunofluorescence-stained specimens were examined under a fluorescence microscope (BX-51; Olympus, Japan). After photographing,

Fig. 1. Gross (A and B) and histopathological findings (C-F) of the hearts with CFA-immunized controls (A, C and E) and the hearts with EAM (B, D and F). The upper panels (A and B) exhibit the gross findings and both the middle and lower panels (C-F) show the histological changes in hematoxylin and eosin stained the heart tissue. Scale bars = 5 mm (A and B), 2 mm (C and D), 50 μ m (E and F).

the images were merged to visualize colocalization using Adobe Photoshop 7.0 software (Adobe Systems, USA).

Statistical analysis

Data are expressed as mean $\pm$ SE. The data from two groups were analyzed by Student *t* test. The *p* value < 0.05 was considered significant.

Results

Gross and histopathological changes

Fig. 1 shows representative examples of the histopathological findings in CFA-immunized control (Figs. 1A, C, and E) and EAM hearts on day 14 PI (Figs. 1B, D, and F). Gross findings included marked enlargement of the EAM heart and spotty changes in color on the surface (Fig. 1B) compared to that of the CFA-immunized control (Fig. 1A). The change in

color was the result of inflammatory cell infiltration and myocardial necrosis. Cross-sections of the heart revealed that the pericardium and ventricular myocardium of the EAM-affected heart (Fig. 1C) were thicker and paler than those of the control heart (Fig. 1D). Furthermore, massive infiltration of macrophages and T cells was observed in the pericardium and ventricular myocardium of the EAM-affected hearts (Fig. 1F), as shown in previous studies [3, 23].

Immunohistochemical analysis

No arginase 1-positive immunoreactivity was found in any area of the CFA-immunized control hearts (Fig. 2A). On day 14 PI, inflammatory cells in the EAM-affected heart showed intense immunostaining for arginase 1 (Fig. 2D). No iNOS immunostaining was found in the heart of CFA-immunized rats (Fig. 2B); however, several inflammatory cells in the EAM lesions were positive for iNOS (Fig. 2E).

Strong TGF- β 1 immunoreactivity was found only in the endothelial cells of the blood vessels in the CFA-immunized control hearts (Fig. 2C); whereas damaged cardiocytes in the myocardium of the EAM hearts were positive for TGF- β 1 on day 14 PI. Moreover, weak TGF- β 1 immunostaining was detected in a few infiltrating inflammatory cells in the EAM lesions (Fig. 2F). Semiquantitative analysis of immunoreactions using Image J has shown that the percentage area of arginase 1 immunoreactivity in the EAM lesions ($16.1 \pm 2.26\%$) was significantly higher ($p < 0.01$) compared with CFA-immunized control ($2.5 \pm 0.02\%$) (Fig. 2G), and that a similar pattern of increased dimension was also recognized in iNOS expression in the EAM lesions ($10.1 \pm 0.43\%$) compared with CFA-immunized control ($0.3 \pm 0.04\%$) ($p < 0.001$) (Fig. 2H). The significant upregulation of TGF- β 1 was also recognized in the EAM lesions ($14.9 \pm 2.21\%$) ($p < 0.05$) compared with CFA-immunized control ($8.3 \pm 1.19\%$) (Fig. 2I).

Macrophage phenotype

To verify the phenotype of the macrophages that infiltrated EAM-affected hearts, double immunofluorescence staining with ED1 was performed using arginase 1 or iNOS. Arginase 1 immunoreactivity was detected in ED1-positive macrophages (Figs. 3A-D). Similarly, iNOS immunoreactivity was detected in a few ED1-positive macrophages (Figs. 3E-H). Moreover, arginase 1 was occasionally immunopositive in iNOS-positive macrophages in EAM lesions (Figs. 3I-L), indicating that the M1 and M2 markers were colocalized in some cells. Furthermore, TGF- β 1 immunoreactivity was colocalized with arginase 1-positive macrophages (Figs. 3M-P).

Discussion

Our study is the first to find classically and alternatively activated macrophages in early inflammatory lesions in hearts affected by EAM. The distinct early appearance of M2

Fig. 2. Immunohistochemical analysis of arginase 1 (A, D), iNOS (B, E) and TGF- β 1 (C, F) in the rat hearts with CFA-immunized control (A, B and C) and with EAM on day 14 PI (D, E and F). The bar graphs (G, H and I) shows the percentage of immunopositive areas of each arginase 1, iNOS and TGF- β 1 in immunostained sections using Image J software. The percentage of immunopositive areas of each arginase 1 (G), iNOS (H), and TGF- β 1 (I) was significantly increased in the EAM affected hearts compared with those of CFA control, respectively. The data in bar graphs (G, H and I) are presented as mean $\pm$ SE. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. CFA-immunized controls. H&E. Scale bars = 25 μ m (A-F).

macrophages suggests that this phenotype plays an important role in the remission of inflammation in EAM-affected hearts.

The results of our immunofluorescence double staining experiment revealed macrophages that were positive for either iNOS or arginase 1 and some that were positive for both, suggesting the existence of three macrophage phenotypes in early stages of EAM: iNOS-positive M1, arginase 1-positive M2, and iNOS- and arginase 1-positive M1/M2, macrophages.

EAM is characterized by the loss of cardiomyocytes and infiltration of autoimmune T cells and macrophages in early stages of inflammation, followed by replacement with fibrosis in the later stage and cardiac dilatation [4, 26, 28]. To

explain the immunomodulatory factors involved in the suppression of pro-inflammatory T cells and M1 macrophages in EAM, we hypothesize that the anti-inflammatory mediators of M2 macrophages compete with pro-inflammatory factors in the early inflammatory EAM lesions.

The fibrosis observed in the EAM hearts may be explained by the association between M2 macrophages and the production of several extracellular matrix proteins [6] and TGF- β 1 in the present study. It was also postulated that Th2 cytokines including TGF- β 1, markedly enhanced in the maximum inflammatory phase of EAM, modulate the inflammation in the course of EAM in rat models [20]. Considering that TGF- β 1 signaling plays a critical role in promoting alternative macrophage activation [5], the macrophages expressing

Fig. 3. Double immunofluorescence staining of arginase 1 with ED1 (A-D), iNOS with ED1 (E-H), iNOS with arginase 1 (I-L) and TGF- β 1 with arginase 1 (M-P) in the hearts affected with EAM on day 14PI. (A-D) Arrows indicate arginase 1 (A, green) positive macrophages colocalized with ED1 (B, red) in the EAM lesions (D, merged). (E-H) A few iNOS (E, green) positive macrophages were colocalized with ED1 (F, red) in the EAM lesions (H, merged; arrows). (I-L) iNOS (I, green) was immunopositive for arginase 1 (J, red) in the same macrophages in the EAM lesions (L, merged; arrows). (M-P) TGF- β 1 immunoreactivity (M, green) was colocalized with arginase 1 (N, red) in the EAM lesions (P, merged; arrows). The sections were counterstained with DAPI (C, G, K, O). Scale bars = 50 μ m (A-P).

TGF- β 1 in our study may have been involved in the activation of M2 macrophages. The mechanisms underlying cardiac fibrosis and dilatation of the heart in the later stage of EAM are complex and warrant further study.

Collectively, these findings suggest that M2 macrophages play an important role in the progression of EAM, and that the application and/or preferential induction of M2 macrophages is an alternative therapy for cardiac autoimmune diseases.

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