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Cardiac myofibroblast induces decreased expression of major histocompatibility complex class II (Ia) on rat monocyte/macrophages

Caterina Peña[a], Renata Vargas[b], Juan Pablo Hernandez-Fonseca[b], Jesús Mosquera[b,*]

[a] Cátedra de Genética, Escuela de Bioanálisis, Facultad de Medicina, Universidad del Zulia, Maracaibo, Venezuela
[b] Instituto de Investigaciones Clínicas "Dr. Américo Negrette", Facultad de Medicina, Universidad del Zulia, Maracaibo, Venezuela

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ABSTRACT

The up-regulation of HLA antigens is important during heart inflammatory events and myofibroblasts may modulate the expression of this molecule in tissues. To test this possibility, the effect of cardiac myofibroblast:macrophage contact and the production of myofibroblast inhibitor factor(s) on the macrophage HLA (Ia) expression were studied. *Listeria monocytogenes*-elicited Ia + peritoneal macrophages (high Ia expression) were co-cultured with cardiac myofibroblasts for 3 and 7 days (myofibroblast contact). Proteosa peptone-elicited macrophages (low Ia expression) were cultured for 3 days with interferon gamma (INF-γ) and myofibroblast conditioned medium (FCM). Ia expression was analyzed by immunofluorescence and by radioimmune assay. Myofibroblast contact induced decreased expression of Ia molecule on macrophages ($p < 0.001$). This was confirmed by the radioimmune analysis in macrophage: myofibroblast co-cultures ($p < 0.001$). Double staining for Ia and CD14 showed that only CD14 positive cells (macrophages) expressed Ia molecule. FCM was capable of diminishing Ia expression induced by INF-γ on macrophages ($p < 0.001$). Decreased Ia macrophage expression induced by myofibroblasts could be important in the heart inflammation's resolution, probably involving Ia redistribution on cell: cell contact and myofibroblast inhibitor factor production.

1. Introduction

Myocarditis is an inflammatory disease caused by various microorganism infections, autoimmunity, systemic diseases, drugs, toxins and infarcted heart capable of inducing arrhythmias, cardiogenic shock and death (Park et al., 2014). During cardiac damage endogenous alarm signals referred to as damage-associated molecular patterns (DAMPs) are activated, which activates the complement cascade and stimulates toll-like receptors signalling, resulting in the activation of the nuclear factor-κB (NF-κB) system and induction of cytokines, chemokines, and adhesion molecules. The interactions between chemokines and cell adhesion molecules on endothelial cells and their receptors on leukocytes lead to the recruitment and extravasation of neutrophils, lymphocytes and mononuclear cells in the injured myocardium (Frangogiannis, 2014). The leukocyte infiltrates included mainly activated T cells (CD45RO+, CD3+) with a moderate number of cytotoxic lymphocytes (CD8+) and macrophages (CD68+) (Frustaci and Chimenti, 2015).

Approximately 40% of acute myocarditis cases resolve spontaneously (Dec et al., 1985); however, the rest of patients goes to a chronic phase due to an abnormal immune response (Miyagawa et al., 2014; Kawai and Shimada, 2014), suggesting immune modulation during myocarditis. However, the mechanisms those regulate the normal or abnormal host immune response leading either to spontaneous resolution of the inflammatory process or to immune mediated damage remain known. The up-regulation of major histocompatibility complex class II antigen (MHC II: Ia) in the myocardial tissue of patients with myocarditis may represent a marker of inflammatory events (Wojnicz et al., 2001; Pinto Ferreira et al., 2014). This molecule is essential for antigen presentation and immune response, therefore for the inflammatory processes (Pinto Ferreira et al., 2014). During the inflammatory phase of cardiac repair, resident cardiac myofibroblasts may serve as an important source of cytokines and chemokines (Shinde and Frangogiannis, 2014; Turner et al., 2009). In addition, activation of adhesive interactions between leukocytes and endothelial cells results in intense infiltration of inflammatory sites by leukocytes (Entman et al., 1991; Weinberger and Schulz, 2015). These events can induce a close interaction between cardiac myofibroblasts and infiltrating monocytes/macrophages leading to inflammatory event modulation. There is no information regarding the modulatory effect of cardiac

* Corresponding author at: Apartado Postal 23, Maracaibo 4001-A., Zulia, Venezuela.
E-mail addresses: caterinapa@hotmail.com (C. Peña), rtt_vargas@gmail.com (R. Vargas), jphernan1970@gmail.com (J.P. Hernandez-Fonseca), mosquera99ve@yahoo.com (J. Mosquera).

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myofibroblasts on the expression of the MHC II by monocyte/macrophages. Peritoneal monocyte/macrophages obtained after peritoneal stimulation, represent inflammatory and infiltrating cells in the peritoneal cavity. These cells have been used as inflammatory cells in experimental procedures (Snyder et al., 1982; Reis e Sousa and Unanue, 2014; Kurt-Jones et al., 1985). Therefore, the aim of this study was to determine the effects of rat cardiac myofibroblast on the expression of Ia molecule by rat peritoneal monocyte/macrophages. In this regard, the experiments were conducted to determine: (1) the effect of myofibroblast contact in the modulation of already expressed Ia molecule on peritoneal monocyte/macrophages; (2) the effect of soluble myofibroblast factor(s) in the Ia molecule expression induced by interferon gamma (INF-γ) on peritoneal monocyte/macrophages.

2. Materials and methods

2.1. Cardiac myofibroblast cultures

Fibroblasts were obtained from enzymatic digestion of Lewis rat pup hearts as follows: Animals were anesthetized with ether and sacrificed by decapitation using sterile scissors. Under sterile conditions hearts were isolated from 2 days old rats and minced in a balanced salt solution containing NaCl (8 g/L), KCl (0.4 g/L), $CaCl_2.2H_2O$ (1.5 mg/L), $MgCl_2.6H_2O$ (0.1 g/L), $NaHCO_3$ (0.4 g/L), dextrose (0.8 g/L) and 0.08% of collagenase type II (LS004174, Worthington Biochemical, Corp. NJ, USA). The minced hearts were incubated with the enzyme solution for 20 min at 37 °C with gently agitation. After sedimentation, the supernatant was removed and centrifuged at 1500 rpm for 10 min. The cells were washed with Hanks' balanced salt solution (HBS) 3 times and suspended in RPMI 1640 supplemented with 292 μg/ml of fresh glutamine, 50 μg/ml of penicillin, 50 μg/ml of streptomycin, 0.01 M HEPES and 10% heat inactivated fetal calf serum (10438018 Gibco, Grand Island, NY, USA). After 90 min of incubation at 37 °C, 5% of CO_2, fibroblasts were separated from cardiomyocytes by differential adhesion. The fibroblasts preferentially attach to the flask surface in this period of time. For subcultures, cells were released from flasks by trypsin treatment using 0.05% of trypsin (T1426, Sigma Chemical, Co. St. Louis, MO, USA) in HBS for 10 min at 37 °C, and then cells were washed and replanted. Fibroblasts were used for co-cultures experiments after 3–6 passages (myofibroblast); at these times, myocardial cells were not detected. Cardiac myofibroblasts were positive for vimentin and actin filament bundles (Cramer et al., 1997; Camelliti et al., 2005) when respective monoclonal antibodies were used (Vimentin M0725, Actin MA1-10046 Dako, Carpinteria, CA, USA). Cell viability was determined by trypan blue exclusion. Microscopy observation was performed using a microscopy with epifluorescence system (Axioskop, Zeiss, Göttingen, Germany). All animal studies were approved by ethical committee of Americo Negrette Clinical Investigation Institute (Zulia University, Maracaibo, Venezuela) in accordance with Arrive guidelines and the National Institutes of Health guide for the care and use of laboratory animals (NIH Publications No. 8023, revised 1978).

2.2. Macrophage harvesting and induction of Ia expression

Enriched Ia positive macrophage population was obtained from 100 to 150 g Lewis rats (Harlan Sprague-Dawley, Indianapolis, USA) by intraperitoneal injection of 3 ml of sterile PBS containing 1×10^6 live *Listeria monocytogenes* (Farr et al., 1977). After 7 days, peritoneal cells were harvested with cold HBS containing 0.01 M HEPES. After 3 washing, cells were suspended in RPMI 1640 supplemented with 292 μg/ml of fresh glutamine, 50 μg/ml of penicillin, 50 μg/ml of streptomycin, 0.01 M HEPES and 10% heat inactivated fetal calf serum (Gibco, Grand Island, NY, USA) and kept at 4 °C for co-culture assays. One aliquot of macrophage suspension was planted on round glass cover slips into 24 well plates (Costar, Cambridge MA, USA) and incubate for 4 h at 37 °C, 5% of CO_2. After washing the resulting attached cells consisted in 90–95% of Ia positive cells, as determined by indirect immunofluorescence using a mouse monoclonal antibody anti- rat Ia (YV0455-01 Accurate Chemical & Scientific Corp., Westbury, NY, USA). Its specificity has been extensively characterized (Schreiner et al., 1981; Williams et al., 1977; McMaster and Williams, 1979). It recognizes separate la determinants of the Lewis haplotype (RT 1). To determine mouse antibody on cells, a fluorescein conjugated $F(ab')_2$ rat anti-mouse IgG was used (Accurate Chemical & Scientific Corp., Westbury, NY, USA). Control labeling consisted of deleting the anti-Ia antibody and substituting the antibody by purified mouse IgG from the plasmacytoma line MOPC 195 (Bionetics Laboratory Products, Litton Bionetics Inc., Kensington, MD, USA). Microscopy observation was performed using a microscopy with epifluorescence system (Axioskop, Zeiss, Göttingen, Germany).

2.3. Radiactive iodination of monoclonal antibody

Anti-Ia monoclonal antibody (400 μg) was iodinated with 10 mCi Na ^{125}I (Amersham Corp., Arlington Heights, IL, USA) and 0.75 mg chloramine-T in 0.75 ml PBS (Greenwood et al., 1963). The reaction was stopped with 0.75 mg of sodium metabisulfite after 30 min on ice. A Sephadex G-25 column (Pharmacia Fine Chemicals) was used to separate the antibody from the unreacted ^{125}I. Ninety nine percent of the radioactivity was associated with the 10% TCA-precipitated protein. The specific activity of the antibody iodinated by this means ranged between 5 and 10 μCi/ug protein.

2.4. Experiments to determine the effect of myofibroblast contact on Ia macrophage expression

2.4.1. Immunofluorescence studies

In order to assess the Ia modulation on macrophages after contact with myofibroblast, sub confluent cell cultures on round cover slips were co-cultured with *Listeria monocytogenes*-elicited macrophages. Ia positive macrophages were cultured either onto myofibroblasts or alone on round cover slips at 5×10^5 /cell/ml/well in 24 well plates and allowed to adhere for 4 h at 37 °C, 5% of CO_2; nonattached cells were removed by washing with warm RPMI 1640 plus 0.01 M HEPES. Cultures were incubated in RPMI 1640 supplemented with 292 μg/ml of fresh glutamine, 50 μg /ml of penicillin, 50 μg/ml of streptomycin, 0.01 M HEPES and 10% heat inactivated fetal calf serum for 3 and 7 days at 37 °C, 5% of CO_2. Cell viability was determined by trypan blue exclusion. At those periods of time cells on cover slips were fixed with PBS containing 2% of paraformaldehyde and Ia expression was determined by indirect immunofluorescence as described above (2.2). Data are expressed as mean ± SD of five independent experiments.

In order to analyze the resulting Ia expression after myofibroblast contact on free cells, a second experiments were performed. Macrophage: myofibroblast co-cultures at day 3 were treated with a balanced salt solution containing NaCl (8 g/L), KCl (0.4 g/L), $CaCl_2{\cdot}2H_2O$ (1.5 mg/L), $MgCl_2{\cdot}6H_2O$ (0.1 g/L), $NaHCO_3$ (0.4 g/L), dextrose (0.8 g/L) and 0.08% of collagenase type II (Worthington Biochemical, Corp. NJ, USA) for 1 h at 37 °C. After 3 washing, double staining for Ia and CD14 expressions on detached cells was performed. In this regard, Ia expression was determined as above described and CD14 was determined using a biotin conjugated rabbit anti rat CD14 antibody (Biocompare, San Francisco, CA, USA) following by phycoerytrin conjugated streptavidin (Sigma-Aldrich, St. Louis, MO, USA). Microscopy observation was performed using a microscopy with epifluorescence system (Axioskop, Zeiss, Göttingen, Germany). Data are expressed as mean ± SD of five independent experiments.

2.4.2. Radioimmune assay studies

In addition to immunofluorescent analysis, Ia expression was also determined by a radioimmune assay. Co-cultures of macrophage: myofibroblasts were analyzed by a radioimmune assay in flexible 96-