MICA/B expression in macrophage foam cells infiltrating atherosclerotic plaques

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ABSTRACT

Infiltrating macrophages accumulate in fatty streak lesions and transform into foam cells, leading to the formation of atherosclerotic plaques. Inflammatory mechanisms underlying the plaque formation mediated by NKG2D-positive lymphocytes such as $CD8^+$ T cells, natural killer cells and natural killer T cells have been extensively investigated. Yet, the involvement of the NKG2D system itself remains poorly understood. Recent work in mouse models has shown that blockade of an NKG2D receptor-ligand interaction reduces plaque formation and suppresses inflammation in aortae. In this study, we conducted immunohistochemical analysis of NKG2D ligand expression in autopsy-derived aortic specimens. Foam cells expressing NKG2D ligands MICA/B were found in advanced atherosclerotic lesions accompanied by a large necrotic core or hemorrhage. Human monocyte-derived macrophages treated *in vitro* with acetylated low-density lipoproteins enhanced expression of MICA/B and scavenger receptor A, thus accounting for NKG2D ligand expression in foam cells infiltrating atherosclerotic plaques. Our results suggest that, as in mice, the NKG2D system might be involved in the development of atherosclerosis in humans.

INTRODUCTION

Infiltration of monocyte-derived macrophages (MDMs) into the arterial wall is assumed to be the initial step toward the development of atherosclerosis (Woollard and Geissmann, 2010). Infiltrating macrophages accumulate in fatty streak lesions and transform into foam cells, which then undergo necrotic or apoptotic cell death in loco, leading to the formation of atherosclerotic plaques (Sakakura et al., 2013). The class A scavenger receptor (SR-A) is a major macrophage receptor responsible for the uptake of modified low-density lipoprotein (LDL) such as oxidized LDL and acetylated LDL (AcLDL) (Goldstein et al., 1979; Kunjathoor et al., 2002) that converts macrophages into foam cells. Thus, SR-A expression is frequently observed in atherosclerotic lesions (Naito et al., 1992). Recent work has shown that exposure to high glucose *in vitro* enhances SR-A expression in human MDMs, accounting for the high incidence of atherosclerosis in diabetic patients (Fukuhara-Takaki et al., 2005). SR-A expression is also up-regulated by interferon-α, providing a possible explanation for the high risk of atherosclerosis in patients with systemic lupus erythematosus (Li et al., 2011).

Activation of lymphocytes, such as $CD8^+$ T cells, natural killer (NK) cells and natural killer T (NKT) cells, plays a crucial role in the development of atherosclerosis (Kyaw et al., 2013; Tse et al., 2013; Whitman and Ramsamy, 2006). NK cells are

atherogenic, and their production of perforin and granzyme B contributes to atherosclerosis and the expansion of necrotic cores (Selathurai et al., 2014). Thioglycolate-elicited macrophages pulsed with oxidized LDL express elevated levels of CD1d and induce interferon-γ production by NKT cells (Nakai et al., 2004). $CD8^+$ T cells also promote the development of atherosclerotic plaques by perforin- and granzyme B-mediated apoptosis of macrophages and tumor necrosis factor α-mediated inflammation (Kyaw et al., 2013).

NKG2D (natural killer group 2, member D) is a major activating receptor (Raulet, 2003) expressed constitutively on NK cells, NKT cells, $CD8^+$ T cells and γδ T cells (Eagle and Trowsdale, 2007; Kasahara and Yoshida, 2012). In humans, six ligands for NKG2D have been identified: MHC class I-related chains A and B (MICA and B) and four ULBP molecules (Bauer et al., 1999; Groh et al., 2001). NKG2D ligands are barely detectable on the surface of healthy cells and tissues, but are frequently expressed by tumor cells or infected cells (Chitadze et al., 2013; Nausch and Cerwenka, 2008). Thus, NKG2D receptor-ligand interactions play an important role in eliminating transformed or infected cells (Jonjić et al., 2008; Nausch and Cerwenka, 2008). Recent work has shown that various cellular stresses such as reactive oxygen species and DNA damage up-regulate MICA/B expression (Peraldi et al., 2009) and that dysregulated

expression of NKG2D ligands is involved in the development of inflammatory and autoimmune diseases such as rheumatoid arthritis, celiac disease and type 1 diabetes (Groh et al., 2003; Meresse et al., 2004; Ogasawara et al., 2004).

NK, NKT and $CD8^+$ T cells implicated in the development of atherosclerosis express NKG2D; however, the involvement of the NKG2D system itself is poorly understood. Recently, inhibition of NKG2D functions was shown to suppress inflammation by immune cells and reduce aortic plaque formation in mice (Xia et al., 2011). The same work also showed that endothelial cells and macrophages infiltrating atherosclerotic plaques express MICA/B in patients with type 2 diabetes (Lin et al., 2012; Xia et al., 2011).

In this study, we conducted immunohistochemical analysis of NKG2D ligand expression in autopsy-derived aortic specimens and confirmed that foam cells infiltrating atherosclerotic plaques can express MICA/B. We found that incubation with AcLDL increases SR-A expression in MDMs *in vitro* and transforms them into MICA/B+ foam cells, thus accounting for NKG2D ligand expression in foam cells infiltrating atherosclerotic plaques.

MATERIALS AND METHODS

Antibodies (Abs) and reagents. Mouse monoclonal Abs (mAbs) for human MICA/B (6D4; eBioscience, Inc., San Diego, CA), human CD68 (KP1; Dako, Glostrup, Denmark) and human macrophage SR-A (SRA-C6; TransGenic Inc., Kumamoto, Japan) were used for immunofluorescent and immunohistochemical staining. Mouse mAbs for human CD3 (F7.2.38; Dako) and CD8 (C8/144B; Nichirei Biosciences Inc., Tokyo, Japan) were used for immunohistochemical staining. Mouse mAbs for human CD80 (2D10; BioLegend, Inc., San Diego, CA) and human CD163 (RM3/1; BioLegend, Inc) were used for immunofluorescent staining. Mouse IgG2a κ isotype control (eBioscience, Inc.) was used as a negative control for staining with the mouse mAb for human MICA/B. Formalin-fixed tissue sections were subjected to immunohistochemical staining using the LSAB2 Kit/HRP (Dako). Human macrophage colony-stimulating factor (M-CSF) recombinant protein (eBioscience, Inc.) was used for generation of MDMs.

Cells. Human blood samples were obtained from healthy volunteers after informed consent had been obtained. Macrophages were differentiated from peripheral blood mononuclear cells (PBMCs) without adding growth factors. Briefly, PBMCs isolated by Ficoll gradient centrifugation from healthy donors were incubated in RPMI-1640

medium supplemented with 10% fetal bovine serum and penicillin/streptomycin for 3 h. After incubation, adhering cells were separated from non-adhering cells and then cocultured with non-adhering cells using a Cell Culture Insert (pore size; 0.4 μm, Invitrogen, Camarillo, CA). After 5 days of coculture, adherent cells in lower wells were used as MDMs. For validation of this newly developed method, MDMs were also generated by the conventional method using M-CSF (Hashimoto et al., 2014). Briefly, adhering cells were incubated with 100 ng/ml of M-CSF for 3 days and then cultured with fresh medium for another 2 days.

AcLDL uptake and immunofluorescent staining. MDMs cultured on coverslips (BioCoat™ Cellware; BD, Franklin Lakes, NJ) were treated with 10 μg/ml of Alexa 488-conjugated AcLDL (Alexa Fluor® 488 AcLDL; Invitrogen) for 1 h at 37 °C for phagocytosis uptake assays. MDMs were washed and then subjected to immunostaining. For intracellular staining, cells were treated with PBS containing 0.1% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany) for 4 min and then fixed with ice-cold 70% methanol for 4 min. Nonspecific binding was blocked with 0.05% Tween-20 in PBS containing 0.1% goat serum for 10 min. For double staining, Alexa Fluor 594-conjugated goat mAb to mouse IgG was used as a secondary Ab. Images

were acquired using an Olympus DP70 camera with its own Olympus DP controller software version 1.2.1.108 (Olympus, Tokyo, Japan).

Immunohistochemistry. A total of 10 autopsy-derived aortic specimens with sclerotic or atherosclerotic changes were employed in this study. The age of the patients ranged from 57 to 80 years, with an average age of 67.7 years. The male-to-female ratio was 1:1. All experiments using human specimens were approved by the Medical Ethics Committees of Hokkaido University Graduate School of Medicine. 3 μm-thick human aorta sections were deparaffinized and antigen retrieved by autoclaving at 121°C for 20 min. Nonspecific binding was blocked with 3% goat serum in PBS for 30 min. To prevent endogenous peroxidase activity, sections were incubated in 3% hydrogen peroxide for 10 min. Sections were incubated with primary Ab for 1 h at room temperature, then with biotinylated secondary Ab and finally with streptavidin-HRP (LSAB2 kit; Dako). After incubation, specimens were developed with diaminobenzidine (Dako) and counterstained with Mayer's hematoxylin.

RESULTS

MICA/B expression in foam cells infiltrating atherosclerotic plaques

Previous work showed that MICA/B is expressed in endothelial cells and foam cells in atherosclerotic lesions of type 2 diabetic patients (Xia et al., 2011). To examine whether MICA/B is expressed in atherosclerotic lesions in general, we performed immunohistochemical staining of MICA/B in 10 autopsy-derived aortic samples. Positive staining for MICA/B was observed in two samples with advanced atherosclerotic lesions accompanied by large necrotic cores (**Figure 1A**). Staining was observed in foam cells infiltrating thin fibrous caps or hemorrhagic lesions (**Figure 1A and B**). Only a fraction of $CD68^+$ or $SR\text{-}A^+$ foam cells showed enhanced expression of MICA/B (**Figure 1A)**. MICA/B staining was not observed in so-called stable plaques with a thick fibrous cap and no lipid core (data not shown).

Induction of MDMs without growth factors

M-CSF treatment has been often used to generate macrophages from human blood monocytes (Fleetwood et al., 2007). However, growth factors such as M-CSF and granulocyte/macrophage CSF (GM-CSF) induce distinct phenotypic changes in macrophage populations (Lacey et al., 2012). Recent work indicated that M-CSF increases SR-A expression in primary macrophages via p38 MAP kinase activation (Nikolic et al., 2011). To circumvent these problems, we attempted to induce MDMs

from PBMCs without adding growth factors. Briefly, adherent PBMCs were cocultured with autologous non-adherent PBMCs by separating the former from the latter by an insert membrane for 5 days (**Figure 2A**). To evaluate whether the adherent cells have differentiated into MDMs, we performed immunofluorescent staining with anti-CD68 Ab and DAPI. More than 99% of adherent cells displayed positive staining for CD68 (**Figure 2B**), suggesting that they have spontaneously differentiated into MDMs. To examine their phenotypes, we performed staining for CD80 (M1 profile) and CD163 (M2 profile). The MDMs were CD68+/CD80-/CD163^{low}, indicating that they are M2 macrophages (**Figure 2C, upper panels**). We generated MDMs using the conventional method employing M-CSF, an inducer of M2 macrophages (Hashimoto et al., 2014). The MDMs thus generated showed similar staining patterns (**Figure 2C, lower panels**), thus validating the newly developed method for preparing MDMs. Interestingly, coculture with heterologous non-adherent PBMCs did not efficiently induce MDMs (data not shown).

MDMs loaded with AcLDL express MICA/B

Next, we examined whether conversion of MDMs into foam cells *in vitro* by incubation with modified LDL induces MICA/B expression. When the MDMs obtained with the

protocol described above were cultured for 1 h in the presence of Alexa 488-conjugated AcLDL, they internalized AcLDL and transformed into foam cells. SR-A is a major macrophage receptor responsible for the uptake of modified LDL (Goldstein et al., 1979; Kunjathoor et al., 2002). We confirmed that SR-A expression was frequently detected in AcLDL-loaded MDMs generated by our new method (**Figure 2D**), indicating that the uptake of AcLDL enhances the phagocytic/endocytic activity of foam cells. The foam cells loaded with AcLDL also expressed MICA/B on the cell surface and in the cytoplasm (**Figure 2E**). MICA/B expression tended to increase in proportion to the extent of AcLDL uptake (**Figure 2F**). These results indicate that the uptake of modified LDL induces MICA/B expression in foam cells.

DISCUSSION

It was shown previously that MICA/B is expressed in endothelial cells and foam cells in atherosclerotic lesions in patients with type 2 diabetes (Xia et al., 2011). In the present study, we observed positive staining for MICA/B only in foam cells infiltrating advanced atherosclerotic lesions (**Figure 1**). Normal human MDMs treated with AcLDL *in vitro* transformed into foam cells and enhanced MICA/B expression (**Figure 2**). These results indicate that the uptake of modified LDL induces MICA/B expression in

foam cells. Given the observation that activation of NKG2D functions promotes atherosclerosis in mice (Xia et al., 2011), MICA/B expression in foam cells may play a similar role in the development of atherosclerosis in humans. For example, foam cells might attract NKG2D+ inflammatory cells such as $CD8^+$ T, NK and NKT cells to the atherosclerotic lesion.

Interestingly, in our limited survey (**Figure 1**), intense staining for MICA/B was observed in foam cells infiltrating thin fibrous caps and intraplaque hemorrhage, but not in those in stable plaques (data not shown), suggesting that MICA/B+ foam cells might occur preferentially in the vulnerable regions of atherosclerotic lesions.

We showed that MDMs can be induced by coculture with autologous non-adherent PBMCs without adding any growth factors (**Figure 2**). Recently, controversial issues have been raised concerning the nature of macrophages induced *in vitro* by growth factors such as M-CSF and GM-CSF (Fleetwood et al., 2007; Lacey et al., 2012). Also, recent work indicates that treatment with M-CSF up-regulates SR-A expression in macrophages (Nikolic et al., 2011). The MDMs obtained with the newly developed method might alleviate growth factor-induced, secondary effects affecting the properties of macrophages.

In conclusion, our work demonstrates that foam cells loaded with modified

LDLs express MICA/B *in vitro*, thus accounting for the observation that MICA/B+ foam cells occur in atherosclerotic lesions in human aortae.

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FIGURE LEGENDS

Figure 1. MICA/B expression in foam cells infiltrating atherosclerotic plaques with large necrotic cores. Photographs in panels A and B were taken from two different autopsy cases in which MICA/B+ foam cells were identified in atherosclerotic plaques. **(A)** Foam cells infiltrating thin fibrous caps express MICA/B**.** Low-power view (uppermost panels) and high-power view (middle and bottom panels) of atherosclerotic plaques. **(B)** Foam cells in hemorrhagic lesions in the plaque express MICA/B. Mouse IgG2a κ isotype control was used as a negative control for mouse mAb for human MICA/B.

Figure 2. MDMs loaded with AcLDL express MICA/B. (A) Adherent and non-adherent PBMCs were cocultured in RPMI-1640 medium supplemented with 10% fetal bovine serum for 5 days without adding growth factors, separating the former from the latter with a membrane cell culture insert. **(B)** CD68 expression in MDMs. MDMs were incubated in the presence of Alexa 488-conjugated AcLDL for 1 h. **(C)** The phenotypes of MDM, which were generated by coculture without growth factors (upper panels) and in the presence of M-CSF (lower panels), were compared by immunostaining for CD68, CD80 and CD163. **(D)** SR-A expression in MDM-derived

foam cells loaded with AcLDL. **(E)** MICA/B expression in AcLDL-loaded foam cells. **(F)** The levels of MICA/B expression tended to increase in proportion to the extent of AcLDL uptake. Bars: (**B and C**) 50 μm, (**D-F**) 10 μm.

Figure 1

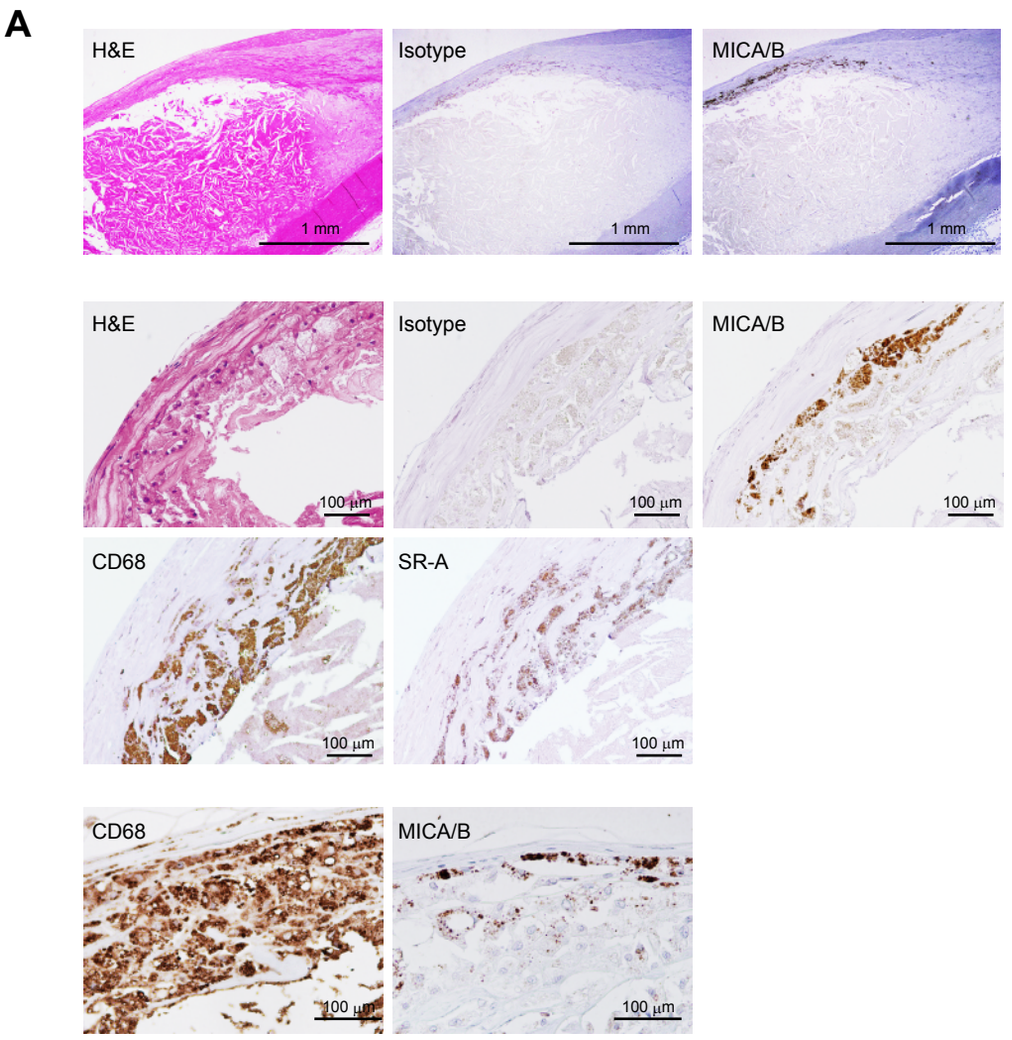
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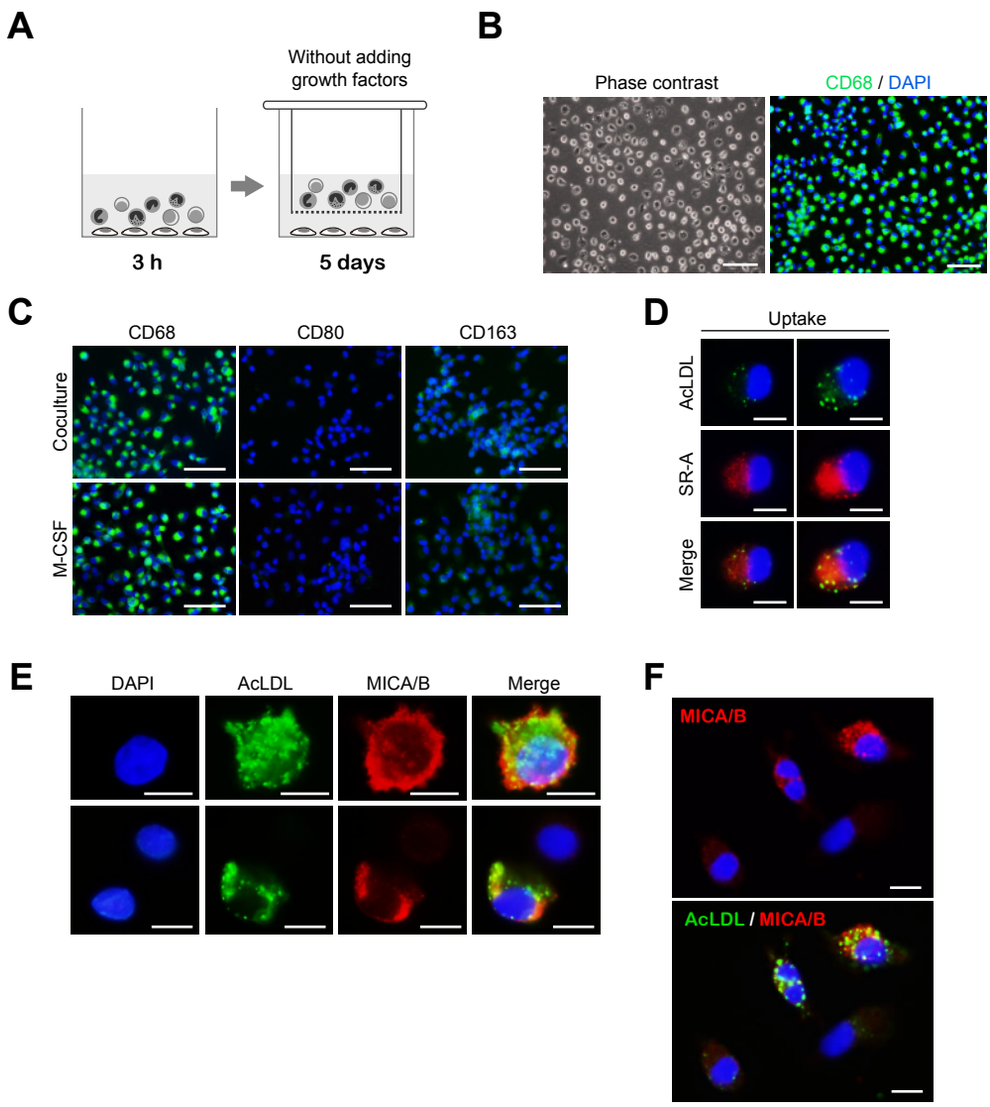
Figure 2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