Allograft inflammatory factor-1 augments macrophage phagocytotic activity and accelerates the progression of atherosclerosis in ApoE^{-/-} mice

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Abstract. Allograft inflammatory factor (AIF)-1, originally cloned from a rat heart allograft under chronic rejection, is induced in various inflammatory conditions including atherosclerosis. Using mouse AIF-1 transfected macrophages and AIF-1 transgenic (AIF-1[Tg]) mice, we analyzed the influence of AIF-1 overexpression on macrophage phagocytosis and the development of atherosclerosis. The AIF-1 transfectants showed significantly increased phagocytosis of latex beads and *E. coli* BioParticles as well as incorporation of acetylated low-density lipoprotein (LDL) compared to those of vector controls. Concordant results were obtained with elicited peritoneal exudate cells from AIF-1[Tg] mice. When AIF-1[Tg] mice were crossbred with apolipoprotein E knockout mice (ApoE^{-/-}), these AIF-1[Tg]ApoE^{-/-} mice developed significantly increased atherosclerotic lesions compared to ApoE^{-/-} mice. These results suggest that enhanced AIF-1 expression leads to augmented incorporation of degenerated LDL by macrophages and promotes development of atherosclerotic vasculopathy.

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Introduction

Allograft inflammatory factor (AIF)-1, a cytokine-inducible calcium-binding protein, is expressed in infiltrating macrophages in rat heterotopic cardiac allografts (1,2). AIF-1 is expressed in cells of a monocyte/macrophage lineage and augmented by interferon (IFN)-γ, suggesting a novel molecular involvement in allogeneic responses. AIF-1 expression has also been demonstrated in macrophages and microglial cells during experimental autoimmune inflammation (3,4) and cerebral infarction (5), as well as in devascularized skeletal muscle tissues (6), vascular smooth muscle cells (VSMC) at the site of injury with balloon angioplasty (7,8) and activated T cells (9). Thus, AIF-1 may play a pivotal role not only in immune responses to allo-antigens but also in various host responses to inflammatory stimuli. Furthermore, AIF-1 or AIF-1-like genes have been cloned from a wide range of organisms, from sponges to humans (10-13). The nucleotide sequences in the sponge and vertebrates have 70% homology. Interestingly, sponge AIF-1 was also expressed in the rejection zone where different species of sponges were forced to fuse (10).

We previously cloned mouse AIF-1 cDNA and established AIF-1 transfectants and AIF-1 transgenic (AIF-1[Tg]) mice to analyze the effects of AIF-1 overexpression (14,15). AIF-1 overexpression modulates the capability of macrophages to produce various cytokines, which might influence subsequent immune responses. Recent studies also showed that AIF-1 is important in the survival and pro-inflammatory activity of macrophages (16). Inhibition of AIF-1 expression by small interfering (si) RNA reduced macrophage migration and proliferation (17). Moreover, the findings that oxidized low-density lipoprotein (LDL) induces AIF-1 expression (17) and that AIF-1 expression regulates the proliferation of VSMC (8), suggest that AIF-1 is critically involved in the pathogenesis of atherosclerosis, a disease related to inflammation (18).

In the present study, we first analyzed the phagocytotic activity of particles and the incorporation of acetyl-LDL using

AIF-1 transfectants and peritoneal exudate cells (PEC) from AIF-1^{Tg} mice. Next, in order to examine the potential pathophysiological role of AIF-1 on the development of atherosclerosis, we analyzed and quantified atherosclerotic lesions in AIF-1^{Tg} mice crossbred with apolipoprotein E-deficient mice (ApoE$^{-/-}$) and compared the lesion size of these AIF-1^{Tg}ApoE$^{-/-}$ mice to that of ApoE$^{-/-}$ mice (19). We will demonstrate the pro-atherogenic property of AIF-1 overexpression and discuss the mechanism underlying augmented atherogenesis.

Materials and methods

Mice. AIF-1^{Tg} mice were generated with mouse AIF-1 cDNA construct under the human CD11b promoter as previously described (15), and backcrossed to C57BL/6 (B6; Japan SLC, Hamamatsu, Japan) for more than 9 generations. Non-transgenic litter mates (NTgm) were used as controls. In some experiments, AIF-1^{Tg} mice were crossbred with ApoE$^{-/-}$ (B6 background; The Jackson Laboratory, Bar Habor, ME) (19) to generate AIF-1^{Tg}ApoE$^{-/-}$ mice. Mice were maintained under specific pathogen-free conditions. All experiments were performed with a normal chow diet. The animal care and experimental procedures conformed to the regulations of Hokkaido University Animal Care and Use Committee.

Blood chemistry. Amounts of total cholesterol (T-Cho), triglyceride (TG), high density lipoprotein cholesterol (HDL-Cho) and fasting blood glucose (FBG) were determined with colorimetric assay kits (Kyowa Medex, Tokyo, and Serotekku, Sapporo, Japan) according to the manufacturer's protocol.

Cells and cell culture. Mouse AIF-1 transfectants with a mouse macrophage cell line, RAW 264.7 (American Type Culture Collection, Rockville, MD) as parent (wild-type), were established as previously described (14). Stable transfectants were established as G418 (Life Technologies, Gaithersberg, MD)-resistant colonies and each clone was examined for AIF-1 expression by immunoblot analysis. The cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol.

Resident PEC were prepared by washing the peritoneal cavity of AIF-1^{Tg} mice or NTgm at 8-10 weeks of age with phosphate-buffered saline (PBS) and cells were collected without stimulation. In some experiments, 4% (w/v) thioglycolate broth (2 ml) was intraperitoneally injected and the elicited PEC were collected 4 days later (20).

Phagocytotic assay. Wild-type RAW 264.7, three lines of AIF-1 transfectants with different AIF-1 levels, vector control and PEC obtained from NTgm or AIF-1^{Tg} mice were subjected to phagocytotic assay as previously described (20). In brief, the cells (1x10^6/tube) were incubated with 10X FITC-microbeads (f=2.00 μm; Polysciences, Warrington, PA) at 37˚C for 2 h. After the reaction, the cells were extensively washed three times with cold FACS buffer (0.1% bovine serum albumin (BSA), 0.1% NaN$_3$ in PBS) containing 1.5 mM ethylenediaminetetraacetic acid (EDTA). The incorporation of microbeads was analyzed by FACScalibur (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

Data were analyzed with CellQuest software (Becton Dickinson). For analysis of the incorporation of *E. coli* BioParticles (Molecular Probes, Eugene, OR) (21), PEC were prepared in 96-well plates at 0.5x10^6 cells/well. Incorporation of *E. coli* BioParticles was assessed by Vybrant Phagocytosis Assay Kit (Molecular Probes) according to the manufacturer's protocol using the fluorescent spectrophotometer LS50B (PerkinElmer Japan, Yokohama, Japan).

Incorporation of acetylated LDL (AcLDL). Cells were incubated for the indicated time with 10 μg/ml of 1,1-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine perchlorate-acetylated LDL (DiI-AcLDL; Biomedical Technologies, Stoughton, MA) at 37˚C (22). After incubation, the cells were washed with cold FACS buffer and analyzed on FACScalibur with CellQuest software. Incorporation was estimated with the mean fluorescence intensity (MFI) of the incubated cells. In some experiments, Alexa Fluor 488-AcLDL (Molecular Probes) was used at 10 μg/ml. After 30 min, cells were washed and stained with Alexa Fluor 633-Concanavalin A (ConA) (Molecular Probes), fixed in 4% paraformaldehyde and subjected to confocal microscopy to confirm intracytoplasmic incorporation of LDL, but without adherence of Alexa Fluor 488-AcLDL to the cell membrane.

Histological and quantitative analyses of atherosclerotic lesion areas. Atherosclerotic lesions were analyzed as previously described (22). In brief, the basal portion of the heart and proximal aortic root were excised, embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and frozen in liquid nitrogen. Eight serial cryosections of 10-μm thickness at 80-μm intervals throughout the aortic sinus were stained with oil red O (Sigma, St. Louis, MO) and hematoxylin. The lesion images were captured with an Olympus BX50 microscope (Tokyo, Japan) equipped with a Fujix HC-300Z/OL digital camera (Fujifilm, Kanagawa, Japan) and Photograb-300 SH-3 software (Fujifilm). Captured images were further analyzed by a computerized image analysis system (Scion Image software, Scion, Frederick, MD).

Immunohistology. Aortae were excised from ApoE$^{-/-}$ or AIF-1^{Tg}ApoE$^{-/-}$ mice, snap-frozen in OCT compound and sectioned at ~5-6 μm as described elsewhere (23). The sections were fixed in acetone for 10 min and endogenous peroxidase activity was blocked with PBS containing 0.1% NaN$_3$ and 0.3% hydrogen peroxide. The AIF-1 expression in atherosclerotic lesions was detected using rabbit anti-Iba1 polyclonal antibody (Wako Pure Chemical, Tokyo, Japan) [AIF-1 is also known as Iba1 (ionized calcium binding adaptor molecule 1)]. The primary antibody was detected using the biotinylated F(ab')$_2$ fragment of swine anti-rabbit immunoglobulin followed by streptavidin-horseradish peroxidase (both from Dako Japan, Kyoto, Japan). Development was performed with the Vectastain kit (Vector Labs, Burlingame, CA) according to the manufacturer's protocol. Hematoxylin and eosin, Elastica-Masson, and oil red O staining were performed with formaldehyde-fixed heart samples as described elsewhere (22) by Sapporo General Pathology Laboratory Co., Ltd (Sapporo, Japan).

Immunoblot analysis. The cells (1x10⁶) were lysed with buffer containing 1% Nonidet-P40, 10 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM EDTA and 1 mM orthovanadate supplemented with protease inhibitor mixture (Complete™; Roche Diagnostics). The lysates were centrifuged at 17,000 x g for 10 min, followed by separation on a 15% SDS-polyacrylamide gel, and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Membranes were blocked in 5% non-fat dry milk in PBS and then incubated with an anti-AIF-1 monoclonal antibody (14). After washing, primary antibody was detected using horseradish peroxidase-conjugated rabbit anti-rat immuno-globulin antibody and visualized with a chemiluminescence detection kit (ECL, Amersham).

Statistical analysis. Results were expressed as mean ± SE and analyzed by one-way ANOVA. Multiple comparisons were performed with the Mann-Whitney U test (Stat View; Abacus Concepts, Berkeley, CA). Values with $p < 0.05$ were considered statistically significant.

Results

Increased incorporation of FITC-beads in AIF-1 transfectants. In our previous study (14,15), we reported that AIF-1 over-expression modulates macrophage functions. To examine whether the phagocytotic activity of macrophages is also modulated by AIF-1 overexpression, AIF-1 transfectants and the vector control were examined for incorporation of FITC-labeled latex beads. The amount of AIF-1 protein expressed in the transfectants was confirmed in increasing order as follows: wild-type (WT) RAW 264.7 cells = mock transfectants <#182 <#24 = #203 as previously reported (14). The fraction of cells that incorporated FITC-beads at 2 h of incubation was demonstrated in the FL-1 histogram as sharp peaks with a factor of integer multiplication (Fig. 1A). The fraction (gated with a line) was markedly greater in all transfectants than in WT or vector control (mock). When the proportion of the fraction with incorporated beads was compared with that of the mock group, all AIF-1 trans-fectants incorporated more than three times as many FITC-microbeads (Fig. 1B). The proportion appeared to be associated with the level of AIF-1 expression (WT = mock transfectants <#182 <#24 = #203).

Increased particle incorporation in PEC from AIF-1^Tg mice. Next, we examined whether the enhancement of phagocytosis was also observed in macrophages from AIF-1^Tg mice. First, the expression level of AIF-1 was compared between PEC from NTgm and AIF-1^Tg mice. AIF-1 expression was evident even in resident PEC from AIF-1^Tg mice (representative result of two mice is shown in Fig. 2A), whereas no AIF-1 expression was detected in resident PEC of NTgm (data not shown). AIF-1 expression was further augmented in PEC from AIF-1^Tg mice 4 days after injection of thioglycolate broth (elicited PEC) (Fig. 2A). Only negligible bands of AIF-1 were seen in the elicited PEC from NTgm. AIF-1 expression may be under the control of not only the CD11b promoter but also exogenous stimuli such as elicitation by thioglycolate broth in AIF-1^Tg mice.
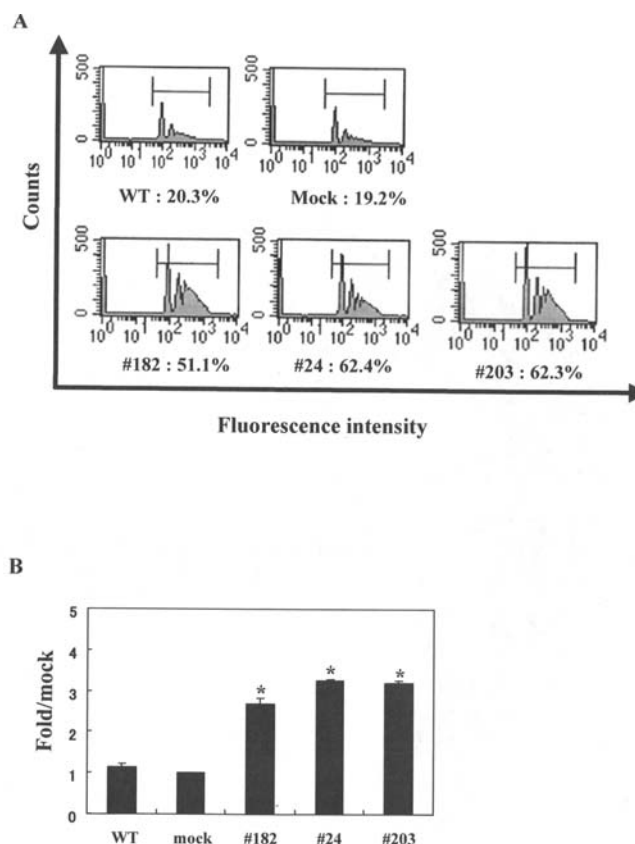

Figure 1. Influence of AIF-1 overexpression on phagocytotic activity. RAW 264.7 (WT), a vector control (mock) and three AIF-1 transfectants (#182, #24, #203) were cultured in triplicate. Each cell group was incubated with 10 times more FITC-microbeads (f=2.00 µm) at 37˚C for 2 h. After the reaction, cells were extensively washed three times with cold PBS containing 1.5 mM EDTA and the incorporation of microbeads was analyzed with FACScan. (A) A representative FACS profile of each cell group taking up FITC-microbeads from three independently performed experiments. Numbers under each panel represent the percentage of cells that ingested more than one microbead. (B) Bar graphs show the phagocytotic activity ratios (phagocytosed cell fractions in WT and three lines of transfectants/phagocytosed cell fractions in mock transfectants). Each bar represents mean ± SE (n=3). A significant difference from mock was denoted with *$p < 0.05$.

We then examined the incorporation of FITC-microbeads using the elicited PEC. When the incorporation was sequentially compared between AIF-1^Tg and NTgm PEC, greater phagocytosis was demonstrated in AIF-1^Tg PEC than in NTgm PEC at 15 (data not shown) and 30 min after culture (Fig. 2B). Thereafter, the proportions of phago-cytosed cells increased in both AIF-1Tg mice and NTgm, and no significant difference in these proportions was observed between AIF-1^Tg PEC and NTgm PEC as shown by data at 180 min (Fig. 2B). These findings suggest that the AIF-1 overexpression promoted phagocytosis of relatively large size microbeads by elicited PEC at an early phase in AIF-1^Tg mice. The longer contact between the microbeads and the PEC may have masked the difference in phagocytotic activity.

To examine phagocytotic activity of biological particles smaller than latex beads, the incorporation of *E. coli* BioParticles was evaluated in elicited PEC. Phagocytosis of *E. coli*, detected as an increase of fluorescent intensity was
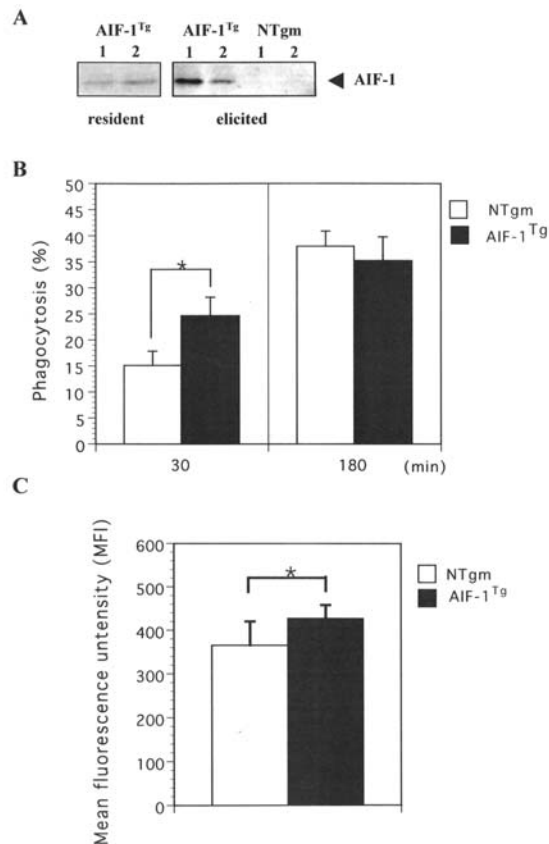
Figure 2. AIF-1 expression and phagocytotic activity of PEC from AIF-1^{Tg} and NTgm. (A) AIF-1 expression in PEC from AIF-1^{Tg} before stimulation with thioglycolate (left panel) and in thioglycolate-elicited PEC from AIF-1^{Tg} and NTgm (right panel). A representative Western blot from two mice in each group is shown. (B) Incorporation of FITC-microbeads by thioglycolate-elicited PEC from AIF-1^{Tg} and NTgm. PEC and microbeads were co-cultured for the indicated time. A significant difference from NTgm was denoted with *p<0.05. (C) Incorporation of *E. coli* BioParticles by thioglycolate-elicited PEC from AIF-1^{Tg} and NTgm. Incorporation of *E. coli* BioParticles was measured 120 min after incubation with a fluorescent spectrophotometer as described in Materials and methods. A significant difference from NTgm was denoted with *p<0.05.

still significantly vigorous at 120 min after incubation in AIF-1^{Tg} PEC compared with NTgm PEC (AIF-1^{Tg}: 427±54, n=6 vs NTgm: 366±36, n=6, p<0.05) (Fig. 2C). This finding is basically consistent with that of larger particles, although the effect of the AIF-1 transgene was evident at a later time of incubation.

Increased acetyl-LDL incorporation by the AIF-1 transfectants and elicited PEC from AIF-1^{Tg} mice. We next examined whether the overexpression of AIF-1 also induced enhanced incorporation of modified LDL in AIF-1 transfectants. As shown in Fig. 3A, the incorporation of DiI-AcLDL was significantly enhanced in the #203 cells, the transfectants with the highest AIF-1 expression, compared with that of the vector control cells 30, 60 and 120 min after culture. The #203 cells exhibited significantly greater incorporation of DiI-AcLDL than mock-transfected cells at any of the time points analyzed (Fig. 3B).

When mock and AIF-1-transfected cells (#203) were incubated with Alexa Fluor 488-AcLDL and Alexa Fluor 633-Con A and analyzed with a confocal laser scanning
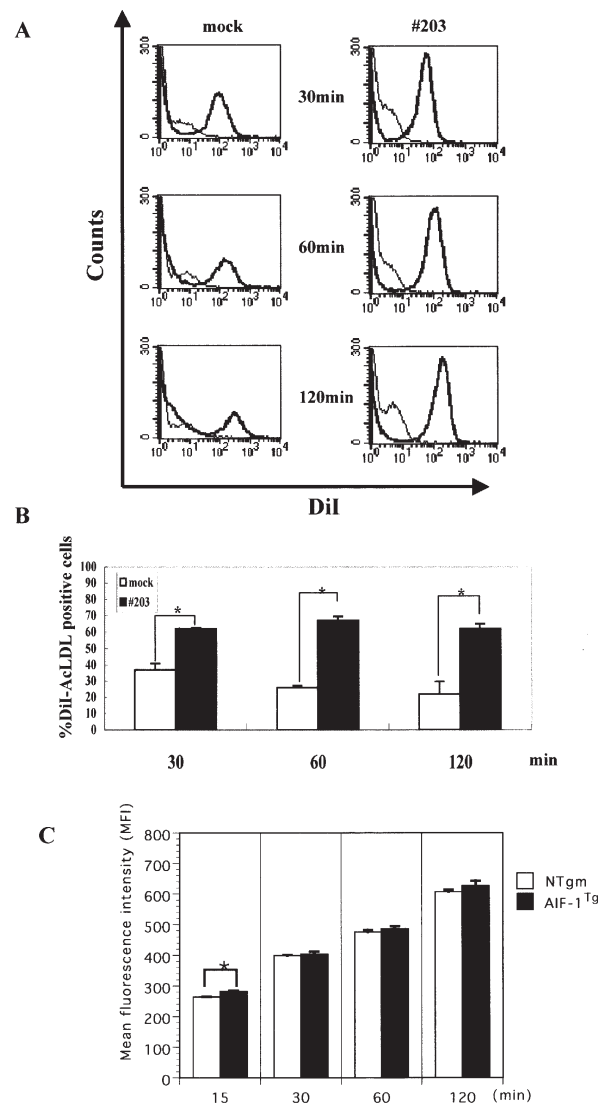

Figure 3. Influence of AIF-1 overexpression on incorporation of DiI-AcLDL by AIF-1 transfectants and PEC from AIF-1^{Tg} mice. A vector control (mock) and AIF-1 transfectants (#203), or thioglycolate-elicited PEC from NTgm or AIF-1^{Tg} were incubated with DiI-AcLDL for the indicated time in triplicate. The incorporation of AcLDL was quantitated with flow cytometry as described in Materials and methods. (A) FACS profiles of the incorporation of DiI-AcLDL by AIF-1 transfectants (thick line) or mock cells (thin line) *in vitro* after 30, 60 and 120 min of culture. A representative profile is shown. (B) The mean proportion of DiI-AcLDL-positive cells in #203 and mock-transfected cells. A significant difference from mock transfected cells was denoted with *p<0.05. (C) Mean fluorescence intensity (MFI) of DiI-AcLDL-incorporated PEC (thioglycolate-elicited) from AIF-1^{Tg} mice or NTgm at the indicated time assessed by flow cytometry. A significant difference from NTgm was denoted with *p<0.05.

microscope, green signals of AcLDL did not co-localize with red stains with ConA that binds to the cell surface, suggesting that AcLDL was indeed incorporated into the cells (data not shown). When AcLDL incorporation by elicited PEC was compared between AIF-1^{Tg} mice and NTgm, a slight increase was noted in AIF-1^{Tg} PEC at all time points (15-120 min) tested (Fig. 3C), even though statistical significance was only demonstrated at 15 min of incubation.

Increased lesion areas of atherosclerosis in AIF-1^{Tg}ApoE^{-/-} mice. The above results suggested that AIF-1 overexpression

Table I. Characteristics of AIF-1^{Tg}ApoE$^{-/-}$ mice and ApoE$^{-/-}$ mice.

	AIF-1^{Tg} ApoE$^{-/-}$ (n=11)	ApoE$^{-/-}$ (n=12)	p
Body weight (g)	18.7±1.4	18.0±1.0	0.27
T-Cho (mg/dl)	714±107	693±183	0.79
TG (mg/dl)	443±79	405±62	0.28
HDL-Cho (mg/dl)	10±9	5±4	0.20
FBG (mg/dl)	80±22	79±29	0.73

in cells of a monocyte/macrophage lineage may modulate the development of atherosclerosis by enhancing incorporation of degenerated LDL. However, our AIF-1^{Tg} mice developed marginal atherosclerotic lesions even with an atherogenic diet (data not shown). Thus, to directly examine the effect of AIF-1 overexpression on the development of atherosclerosis, we established AIF-1^{Tg}ApoE$^{-/-}$ mice and compared the lesion areas with those of ApoE$^{-/-}$ mice (all B6 background).

Normal chow was given to AIF-1^{Tg}ApoE$^{-/-}$ mice and ApoE$^{-/-}$ mice for 15 weeks and atherosclerotic lesions were analyzed by staining serial sections of the aortic sinus with oil red O (22). No significant differences were noted in the body weight, T-Cho, TG, HDL-Cho and FBG value between AIF-1^{Tg}ApoE$^{-/-}$ and ApoE$^{-/-}$ mice (Table I). Thus, AIF-1 over-expression exerted no significant influence on the metabolic parameters in these mice.

In contrast, prominent atherosclerotic lesions were demonstrated with hematoxylin and eosin staining (Fig. 4A), Elastica-Masson staining (Fig. 4C), and oil red O staining (Fig. 4E) in AIF-1^{Tg}ApoE$^{-/-}$ mice compared to ApoE$^{-/-}$ mice (Fig. 4B, D and F). The average lesion area was significantly larger in AIF-1^{Tg}ApoE$^{-/-}$ mice (281,893±19,846 μm^2, n=21) than in ApoE$^{-/-}$ mice (235,386 ± 20,439 $\mu m2$, n=18) (Fig. 4G, p<0.05). Then, these atherosclerotic lesions were immuno-histochemically examined to determine if AIF-1 was expressed *in situ*. Although weak signals of endogenous AIF-1 were expressed by the cells in the atherosclerotic plaques of the ApoE$^{-/-}$ mice (Fig. 4I), considerably more prominent AIF-1 signals were detected in the lesions of the AIF-1^{Tg}ApoE$^{-/-}$ mice (Fig. 4H). These findings suggest that AIF-1 overexpression is related to the promotion of atherosclerotic lesion development.

Discussion

In the present study, we analyzed the influences of AIF-1 overexpression on the development of atherosclerosis. We demonstrated that AIF-1 overexpression enhanced phagocytosis of two kinds of particles and the uptake of degenerated LDL by macrophages. These functional alterations of macrophages appeared to result in aggravation of the atherosclerotic lesion areas.

We demonstrated that FITC-microbeads were vigorously incorporated by AIF-1 transfectants, in proportion to the expression level of AIF-1 in the transfectants. Elicited PEC from AIF-1^{Tg} mice also showed increased incorporation of
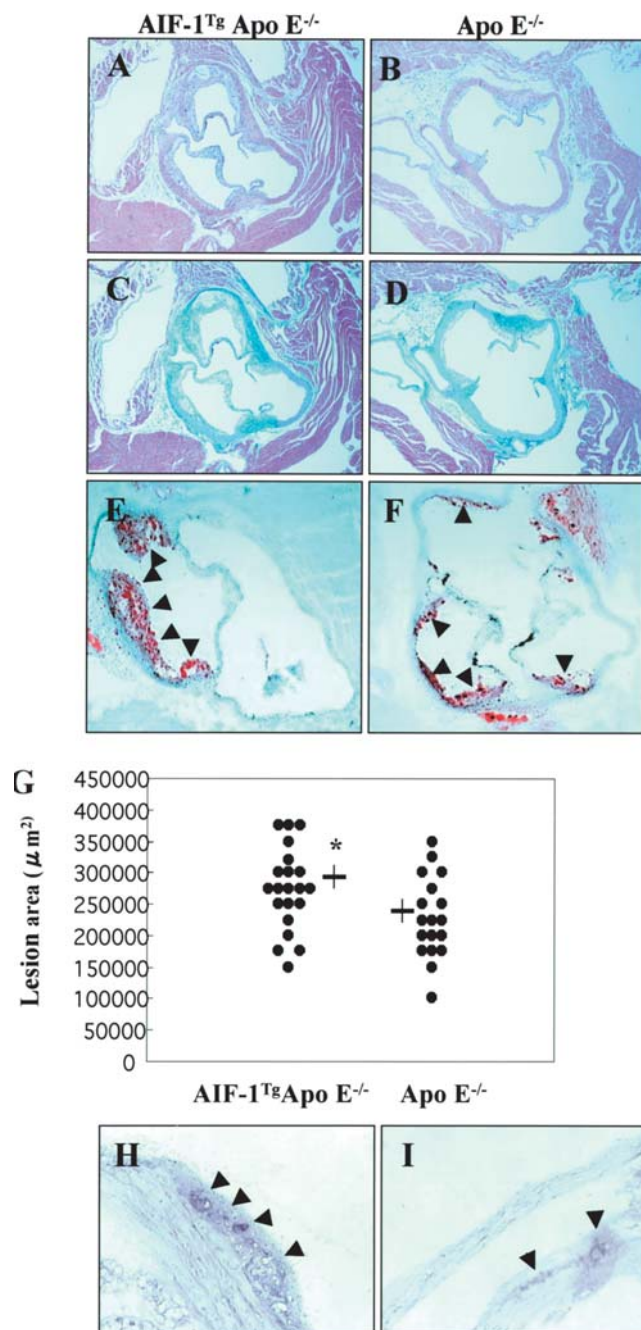

Figure 4. Histological and quantitative analyses of the atherosclerotic lesions in AIF-1^{Tg}ApoE$^{-/-}$ or ApoE$^{-/-}$ mice. (A,B) Hematoxylin and eosin staining of the atherosclerotic lesion of the aortic sinus from AIF-1^{Tg}ApoE$^{-/-}$ (A) or ApoE$^{-/-}$ (B) mice (original magnification x40). (C, D) Elastica-Masson staining of the atherosclerotic lesions of the aortic sinus from AIF-1^{Tg}ApoE$^{-/-}$ (C) or ApoE$^{-/-}$ (D) mice (original magnification x40). (E, F) Representative oil red O staining of the atherosclerotic lesions from the aortic sinus of AIF-1^{Tg}ApoE$^{-/-}$ (E) or ApoE$^{-/-}$ (F) mice (x40, arrow heads indicate atherosclerotic lesions). (G) Quantification of the atherosclerotic lesion area in AIF-1^{Tg}ApoE$^{-/-}$ and ApoE$^{-/-}$ mice. Each circle represents the lesion area of a mouse. The horizontal bar represents the mean lesion area and the vertical bar represents SE. A significant difference from ApoE$^{-/-}$ mice was denoted with *p<0.05. (H, I) Immunohistological detection of AIF-1 expression in atherosclerotic lesions in thoracic aortae from AIF-1^{Tg}ApoE$^{-/-}$ (H) and ApoE$^{-/-}$ (I) mice. Longitudinal sections of thoracic aortae were prepared from frozen tissue and stained with anti-Iba1 rabbit polyclonal antibody as described in Materials and methods. (x100, arrow heads indicate positive staining).

microbeads at an earlier time point compared to that in PEC from NTgm. After reaching a plateau, however, no difference in the amount of phagocytosed beads was seen between AIF-1[Tg] PEC and NTgm PEC. The difference in the capability of phagocytosis that was seen between AIF-1 transfectants and PEC from AIF-1[Tg] mice was presumably attributable to the amount of expressed AIF-1 in the macrophages of these two systems. Alternatively, the systemic and long-lasting expression of AIF-1 molecules in cells under the CD11b promoter during ontogenesis might modulate functions of AIF-1 in AIF-1[Tg] mice. Quantification of the AIF-1 protein level and various macrophage functions in AIF-1 transfectants and macrophages from different lymphoid tissues of AIF-1[Tg] mice is now underway in our laboratory.

AIF-1 is also called Iba1(24), MRF-1 (microglia response factor-1) (25), and daintain (12). In addition, AIF-1 includes various splice variants (26). Ohsawa *et al* revealed that Iba1 is involved in membrane ruffling and phagocytosis (27). Dominant-negative type Iba1 suppressed the phagocytic cup formation in microglial cell line, MG5 (27). It has also been demonstrated that Iba1 exhibits actin-cross-linking activity through binding to fimbrin and enhances its actin-bundling activity (28,29). These reports seem to be consistent with our present finding that overexpression of AIF-1 enhanced phagocytosis by macrophages.

The AIF-1 transfectants also showed significantly enhanced incorporation of AcLDL. PEC from AIF-1[Tg] mice also exhibited slightly augmented incorporation of AcLDL at an early period of incubation. Since the level of AcLDL uptake by macrophages is directly related to susceptibility to atherosclerosis in mice (30), these results suggest that AIF-1 is involved in the augmented development of atherosclerotic vasculopathy. In addition, the survival and migration of macrophages were affected by AIF-1 expression (14,15), which might alter and modulate the development of atherosclerotic disease.

Autieri's group (17) demonstrated that oxidized LDL induces AIF-1 expression. AIF-1 expression regulates the proliferation of VSMC (8,31). Thus, AIF-1 appears to be involved in the pathogenesis of atherosclerosis through not only macrophage activation but also vascular regeneration in the lesion and we were able to demonstrate that AIF-1[Tg]ApoE^{-/-} mice exhibited significantly increased areas of atherosclerotic lesions as compared to those in ApoE^{-/-} mice. Prominent expression of AIF-1 was seen in the lesions of AIF-1[Tg]ApoE^{-/-} mice, but not in those of ApoE^{-/-} mice.

However, the exacerbation of lesion development in the AIF-1[Tg]ApoE^{-/-} mice was relatively modest compared to that in the ApoE^{-/-} mice. This may be attributed to the prominent atherosclerosis development in ApoE^{-/-} mice compared to the wild-type B6 mice. Another possible explanation may be that AIF-1 overexpression biases the immune response from Th1 to Th2 as we have demonstrated in *in vitro* experiments (14) and in the trinitrobenzene sulphonate-induced colitis model in mice (15). Since atherosclerosis is aggravated in the Th1 condition, the Th2-biased immune deviation (elevation of IL-6 and IL-10 levels) may exert regulatory influences on atherogenesis in the AIF-1[Tg] environment (32-36). However, AIF-1 transfectants also produce IL-12, a pro-atherogenic factor, upon stimulation with LPS (14). The balance between these cytokine productions with opposite functions may result in a slightly aggravated atherogenic lesion in AIF-1[Tg]ApoE^{-/-} mice compared to that in ApoE^{-/-} mice.

AIF-1 may be a novel pro-atherogenic factor in certain conditions. Thus, proper regulation of AIF-1 may provide new therapeutics that prevent the progression of atherosclerosis. Further investigation of AIF-1 properties including the manipulation of AIF-1 expression and functions, i.e. suppression by RNA interference or antibody-mediated neutralization, or vector-mediated expression, needs to be pursued.

Acknowledgements

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