Allograft inflammatory factor-1 (AIF-1) is crucial for the survival and pro-inflammatory activity of macrophages

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Keywords: allograft inflammatory factor-1, macrophages

Abstract

Our previous studies revealed that macrophages played an important role in linking injury, inflammatory and immune response in small-for-size liver transplantation. However, the molecular basis that promoted macrophage activation was not clear. In the present study, we explored the potential role of allograft inflammatory factor-1 (AIF-1) in mediating the survival and pro-inflammatory activity of macrophages in a macrophage cell line. First, the expression of AIF-1 was investigated with the stimulation of pro-inflammatory cytokines and anti-inflammatory treatment. Second, the level of inducible nitric oxide synthase (iNOS) and the survival and migration activity of macrophages were determined with the alterations of AIF-1 expression. Finally, a potential molecule that regulated AIF-1 expression was identified by the proteomic approach. The macrophage cell line expressed a certain level of endogenous AIF-1, which could be enhanced by pro-inflammatory cytokines IL-1β or tumor necrosis factor-α and suppressed by anti-inflammatory drug sodium salicylate. AIF-1 augmentation induced by AIF-1/PCDNA3.1(+) transfection enhanced the levels of iNOS and monocyte chemoattractant protein-1, and promoted the cell migration. On the other hand, suppression of AIF-1 expression by AIF-1/short interference RNA (siRNA) inhibited iNOS production, induced macrophage cell apoptosis and blocked the cell migration. Using two-dimensional electrophoresis, a disintegrin and metalloproteinase domain 3 (ADAM3) was identified after AIF-1/siRNA transfection. Transfection of ADAM3/PCDNA3.1(+) up-regulated the expression of AIF-1 and iNOS, whereas suppression of ADAM3 expression down-regulated AIF-1 and iNOS expression. In conclusion, AIF-1 played an important role in the survival and pro-inflammatory activity of macrophages, and ADAM3 might be an upstream molecule that regulated AIF-1 expression.

Introduction

The monocyte/macrophage system has been shown to be responsible for a variety of diseases, including autoimmune disease (1), tissue injury (2) and acute and chronic allograft rejections (3, 4). Our previous studies revealed that early activation of macrophages played an important role in mediating injury, inflammation and cellular rejection in small-for-size liver transplantation (5, 6), and the angiogenesis processes during small-for-size graft regeneration might facilitate proliferation and migration activities of the macrophages (7). However, the molecular basis that mediates macrophage activities is still largely unclear.

Allograft inflammatory factor-1 (AIF-1) was first identified by Utans et al. (8) in 1995 in rat cardiac allografts with chronic rejection, in which inflammatory reaction played a key role, and Autieri et al. (9) further demonstrated that expression of AIF-1 was correlated with the severity of cardiac rejection in clinical heart transplantation. Enhancement of AIF-1 expression was found to be able to augment production of IL-6, -10 and -12 in a mouse macrophage cell line (10). In addition, AIF-1 was identified to be a Rac1-activating protein that promoted vascular smooth muscle cell migration (11). However, the direct relationship between AIF-1 expression and macrophage activities during the acute phase injury and inflammation is not fully understood. In addition, the molecular basis that regulates AIF-1 expression also remains to be determined.
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Therefore, in an in vitro system, we tried to explore the expression of AIF-1 under the inflammatory circumstance, and the impact of AIF-1 alterations on macrophage activities and molecular changes in these cells. In addition, we attempted to identify any potential upstream molecule that regulates AIF-1 expression.

Methods

Cell lines

Macrophage cell line CRL-2192 was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in F-12K medium with 15% fetal bovine serum (FBS) and 1% penicillin–streptomycin (Life Technologies, Carlsbad, CA, USA) at 37°C in a humidified atmosphere of 5% CO₂ in air. Unstained cells were used as a negative control.

Transfection and short interference RNA

Full-length AIF-1 and a disintegrin and metalloproteinase domain 3 (ADAM3) were cloned into PCDNA3.1(+)(+) vector by T4 DNA ligase (Life Technologies). Primer sequences for rat AIF-1 were sense, 5’ CGGGATCCGAGCTATGAGCCAGCACGCAAG 3’ and anti-sense, 5’ GGAATTCCGAGCTATGAGCCAGCACGCAAG 3’. Primer sequences for rat ADAM3 were sense, 5’ CAGGATCTTCTCTCTGCTTGCTCTT 3’ and anti-sense, 5’ GAAATCCGGAGAAAGAGATGGACTGG 3’. BamHI and EcoRI sites (underlined sequences) were inserted into the primer sequences for cloning purpose. AIF-1/PCDNA3.1(+) and ADAM3/PCDNA3.1(+) were then transfected into the macrophage cell line by FuGENE 6 transfection reagent (Roche, Basel, Switzerland) and cultured for 24 h. The three pairs of AIF-1/short interference RNAs (siRNAs) for AIF-1 targeted on the sites of 1323 (S1), 1271 (S2) and 1135 (S3), respectively, in rat AIF-1 gene, with each sequence lengthening 21 bp. The two pairs of ADAM3/siRNAs targeted on the sites of 253 (siAD1) and 1373 (siAD2), respectively, in the rat ADAM3 gene, with each sequence lengthening 21 bp. The siRNAs were transfected into the macrophage cell line by jetSI siRNA delivery reagent (Qbiogene, Irvine, CA, USA), and incubated for 24 h. The siRNA targeting on luciferase gene was used as a negative control, and the same amount of siRNA delivery reagent only was added to the cell line to determine its possible toxicity in apoptosis. The AIF-1/PCDNA3.1(+) and AIF-1/siRNAs were also co-transfected into the macrophage cell line by FuGENE 6 transfection reagent, and maintained for 24 h.

Flow cytometry

After a 24 h incubation, the cells were labeled with FITC-conjugated Annexin V antibody (BD Biosciences PharMingen, San Diego, CA, USA), and detected in a FACS Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). Unstained cells were used as a negative control.

Cell migration assay

After receiving the above treatment for 24 h, cells were harvested and suspended in 50 μl F-12K medium with 1.5% FBS, and inoculated on the surface of a polycarbonate filter of a 96-well microchemotaxis chamber (1 × 10⁵ cells per well) (Neuroprobe, Bethesda, MD, USA). In the lower chamber, 299 μl 1.5% FBS–F-12K medium was applied into each well. The cells were then cultured at 37°C in a humidified atmosphere of 5% CO₂ in air for 4 h before they were counted under microscope. Five areas were randomly chosen in each well and the number of cells was expressed as mean ± standard deviation.

Reverse transcription–PCR and western blot

After a 24-h culture, macrophages were harvested, and reverse transcription–PCR and western blot were performed. Total RNA extraction, first-strand DNA synthesis and PCR reaction were performed according to the methods previously described (5). Primer sequences for rat inducible nitric oxide synthase (iNOS) were sense, 5’ CTGGGACAGGCGCCCTACGTG 3’, and anti-sense, 5’ GAAAGACCACCGCAAGATG 3’, and for rat ADAM3 were sense, 5’ GACTTACGAGTTAACCTTCAAGG 3’, and anti-sense, 5’ TAACATCAGCTCATCTGAAGCTTG 3’. Total protein was extracted from the cells and the protein levels were determined by standard western blot analysis using 12% SDS-PAGE gel. Antibodies were purchased from Santa Cruz Biotechnology (polyclonal rabbit anti-rat iNOS antibody), Upstate, Waltham, MA, USA [polyclonal rabbit anti-rat monocyte chemotactant protein-1 (MCP-1) and polyclonal rabbit anti-rat caspase 3 antibodies] and Cell Signaling Technology, Inc., Beverley, MA, USA (polyclonal rabbit anti-rat Bax antibody). The rabbit anti-rat AIF-1 polyclonal antibody was kindly provided by Michael Autieri (12).

In addition, culture medium was also collected at the time of cell harvesting for detection of nitric oxide (NO).

Measurement of NO levels in culture medium

After transfection with AIF-1/PCDNA3.1(+) and being cultured for 24 h, the culture medium was collected, and the production of NO by the macrophage cell line was detected using Nitric Oxide Assay Kit (Calbiochem, San Diego, CA, USA). Fifty microliters of sample was added into each well of a 96-well plate. All the procedures were performed according to the instruction of the Kit.

Two-dimensional electrophoresis, in-gel trypsin digestion and mass spectrometry

After transfection and incubation for 24 h, the macrophage cells (1 × 10⁵) were harvested. Proteins were extracted by homogenizing in 0.1 ml extraction buffer containing 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, 0.2% (w/v) carrier ampholytes (pH 3–10) and 2 mM tributyl phosphine. Thirty micrograms of protein was applied to two-dimensional (2-D) electrophoresis according to our previous protocol (13). An up-regulated protein (compared with non-transfected or siRNA-targeting luciferase gene), with molecular weight ~80 kDa, in AIF-1/siRNA transfected cells was excised from the gel, and digested by trypsin. The digested peptides were analyzed.
using a Voyage-De STR matrix-assisted laser desorption ionization with time of flight (MALDI-TOF) mass spectrometer (Applied Biosystems, Foster City, CA, USA) and the peptide mass spectra were generated. Peptide mass profiles produced by MALDI-TOF were then analyzed using MS-FIT (http://prospector.ucsf.edu). The protein was identified with high confidence based on high scores and sequence coverage by comparing its peptide masses with the theoretical masses derived from the sequences displayed in SWISS-PROT and the National Center for Biotechnology Information data banks.

**Statistical analysis**
Comparisons of Annexin V-positive cells and the number of migrated cells between different treatment groups were performed using one-way analysis of variance (GraphPad, San Diego, CA, USA). P-value <0.05 was considered as statistically significant.

**Results**

**Pro-inflammatory cytokines induced up-regulation of AIF-1 in macrophages**
The macrophage cell line expressed a certain level of endogenous AIF-1. With the stimulation of pro-inflammatory cytokines IL-1β or TNF-α, an increased expression of AIF-1 was detected. Administration of sodium salicylate down-regulated the endogenous level of AIF-1, and also reversed the up-regulation of AIF-1 which was induced by either IL-1β or TNF-α (Fig. 1).

The levels of iNOS and NO production in macrophage cell line altered with the expression of AIF-1
After transfection with AIF-1/PCDNA3.1(+) (A), an enhanced expression of AIF-1 was detected in the macrophage cell line, whereas AIF-1/siRNA (both S2 and S3) down-regulated the level of AIF-1 in both lower cell number (5 x 10⁵) and higher cell number (1 x 10⁶), whereas transfection of siRNA-targeting luciferase gene did not alter the level of AIF-1. Simultaneously, the mRNA and protein levels of iNOS displayed a similar pattern with AIF-1 expression. Co-transfection of AIF-1/siRNA with AIF-1/PCDNA3.1(+) could obviously reverse the up-regulated protein levels of AIF-1 and iNOS induced by AIF-1/PCDNA3.1(+) transfection (Fig. 2A).

The macrophage cell line produced a certain level of NO under normal culture condition. With the transfection of AIF-1/PCDNA3.1(+) (A), an increased level of NO was detected in the culture medium, whereas a reduction of NO was detected with the transfection of AIF-1/siRNA. Co-transfection of AIF-1/PCDNA3.1(+) and AIF-1/siRNA reversed the increase of NO production induced by AIF-1/PCDNA3.1(+) (A), but the level was still higher than that without transfection (Fig. 2B).

**Fig. 1.** Pro-inflammatory cytokines stimulated AIF-1 expression, whereas anti-inflammatory treatment decreased the expression of AIF-1. The macrophage cell line was treated with pro-inflammatory cytokines IL-1β and TNF-α or anti-inflammatory drug sodium salicylate for 12 h. The expression of AIF-1 was determined by western blot. NT, no treatment; S, sodium salicylate.

**Fig. 2.** The expression of AIF-1 and iNOS and production of NO were altered with AIF-1/PCDNA3.1(+) or siRNA (AIF-1/siRNA) transfection. (A) The macrophage cell line was transfected with AIF-1/PCDNA3.1(+) or AIF-1/siRNA or AIF-1/PCDNA3.1(+) and AIF-1/siRNA. The expression of AIF-1 and iNOS was detected by reverse transcription–PCR and western blot. (B) Culture medium was collected at the time of cell harvest, and the NO level in the culture medium was detected using Nitric Oxide Assay Kit. NT: only transfection reagent was added; V: vector; A: AIF-1/PCDNA3.1(+) (A); S1, S2 and S3: siRNA for the regions 1, 2 and 3, respectively, of the rat AIF-1 gene; AS2 and AS3: AIF-1/PCDNA3.1(+) and AIF-1/siRNA co-transfection.
AIF-1 suppression induced apoptosis of macrophages

With the normal culture condition, a small number of macrophage cells were undergoing apoptosis. The siRNA delivery reagent only did not alter the number of apoptotic cells. In addition, transfection of AIF-1/PCDNA3.1(+) or siRNA-targeting luciferase gene; S2 and S3, siRNA for the regions 2 and 3, respectively, of the rat AIF-1 gene; AS2 and AS3, AIF-1/PCDNA3.1(+) and AIF-1/siRNA co-transfection. *P < 0.05, compared with transfection reagent only; **P < 0.05, compared with S2 or S3 transfection.

AIF-1 up-regulation promoted macrophage cell migration

By cell migration assay, it was found that AIF-1/PCDNA3.1(+) transfection could induce a significantly increased number of migrated cells, whereas the migration behavior was partially suppressed when AIF-1/siRNA (both S2 and S3) was co-transfected with AIF-1/PCDNA3.1(+) (Fig. 4A). With the increase of migrated cells, an augmentation of MCP-1 expression was detected after AIF-1/PCDNA3.1(+) transfection, whereas blockade of the expression of AIF-1 could down-regulate the level of MCP-1 (Fig. 4B).

ADAM3 regulated AIF-1 expression

By 2-D electrophoresis, 10 proteins were found up-regulated with AIF-1/siRNA transfection. Among these 10 molecules, 4 demonstrated a down-regulation in the AIF-1/PCDNA3.1(+) transfected cells. Therefore, we considered that these molecules might be closely related to AIF-1 expression. By mass spectrometry and after searching in the online database, a protein with molecular weight ~80 kDa was identified as ADAM3. By using specific primers, the mRNA level of ADAM3 was confirmed to be higher in the AIF-1/siRNA transfected cells (Fig. 5). Transfection of ADAM3/PCDNA3.1(+) and suppression of ADAM3 by ADAM3/siRNA up-regulated and reduced the mRNA level of this molecule, respectively, and at the same time, altered the levels of AIF-1 and iNOS accordingly (Fig. 6).

Discussion

Our previous studies have demonstrated the potential role of macrophages in linking injury, inflammation and acute rejection in small-for-size liver transplantation (5, 6). However, the initiation of the early macrophage activation in the small-for-size liver grafts remains unclear. Our unpublished data revealed that AIF-1 was up-regulated as early as 1 h after reperfusion in these small-for-size liver grafts, indicating the potential role of AIF-1 in mediating macrophage activity during acute phase injury and inflammation. Therefore, we further investigated the relationship between AIF-1 expression and pro-inflammatory activity of macrophages in the present study.

It was reported that pro-inflammatory cytokine IFN-γ induced AIF-1 expression in macrophages (14). Our study revealed that pro-inflammatory cytokines IL-1β and TNF-α...
could also induce AIF-1 up-regulation, and non-steroid anti-
inflammatory drug sodium salicylate could decrease the
expression of AIF-1, and suppress the up-regulation of AIF-1
stimulated by pro-inflammatory cytokines, indicating a close
relationship between inflammatory status and AIF-1 expres-
sion. In addition, an enhanced expression of AIF-1 by AIF-1/
PCDNA3.1(+) transfection stimulated an up-regulation of
iNOS in the macrophage cell line, and a reduced expression
of iNOS was induced by AIF-1 suppression, providing a direct
evidence that AIF-1 achieved its pro-inflammatory properties
in an iNOS-dependent manner.

Corresponding to iNOS expression, the production of NO
was also regulated by AIF-1/PCDNA3.1(+) and AIF-1/siRNA
transfection. However, the role of NO on cell viability and
apoptosis was quite variable, depending largely on the level of
NO produced (15, 16). In our unpublished experiments, we
found an increased number of apoptotic cells during the
course of stable clone selection after AIF-1/PCDNA3.1(+) transfection, indicating that high levels of AIF-1 and NO might
be toxic to the cells. However, further study is needed to
explore this phenomenon.

The present study revealed, for the first time, that AIF-1
expression was crucial for macrophage cell survival, as AIF-1/
siRNA transfection induced a significantly increased number
of apoptotic cells in the macrophage cell line, whereas AIF-1/
PCDNA3.1(+) and AIF-1/siRNA co-transfection protected

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**Fig. 5.** Identification of ADAM3 from AIF-1/siRNA transfected cells. After AIF-1/siRNA transfection, the cells were harvested and subjected to 2-D electrophoresis. A protein with molecular weight ~80 kDa, which was up-regulated after transfection, was isolated from the gel and identified by mass spectrometry. This protein turned out to be ADAM3. The augmentation of ADAM3 mRNA was confirmed by reverse transcription–PCR. NT, only transfection reagent was added; SL, siRNA-targeting luciferase gene; S2, siRNA for the region 2 of the rat AIF-1 gene.

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those macrophages from undergoing apoptosis. AIF-1/siRNA transfection stimulated up-regulation of pro-apoptotic molecules, Bax and caspase 3, whose over-expression was associated with cytochrome c release (17) and a marker of the end stage of the apoptotic pathway (18), whereas AIF-1/PCDNA3.1(+) and AIF-1/siRNA co-transfection reversed the increased expression of these two molecules, indicating that the alterations of Bax and caspase 3 played an important role in AIF-1-related cell death and survival. Similar to its ability to promote vascular cell migration, AIF-1 was also involved in the migration activity of macrophages, probably through augmenting the expression of MCP-1, as MCP-1 was well documented to be a chemotactic molecule that promoted macrophage migration (19, 20).

Although the pro-inflammatory characteristics of AIF-1 were documented by some studies (21, 22), few reports have explored the molecules that directly regulated AIF-1 expression. Members of the ADAM family of proteolytic enzymes play an essential role in signaling many single transmembrane-bound proteins, ranging from cell-surface receptors to growth factors and cytokines (23–25), and it was reported that a metalloproteinase disintegrin could regulate cell migration in Caenorhabditis elegans (26). Therefore, targeting on the ADAM family is a new therapeutic strategy for the treatment of a variety of diseases (27, 28). Using the proteomic technique, we identified one of the ADAM members, ADAM3, as a potential upstream molecule that regulated AIF-1 expression as stimulating the expression of ADAM3 could induce an increased expression of AIF-1 and iNOS in the macrophages, whereas suppressing the expression of ADAM3 could significantly down-regulate the levels of AIF-1 and iNOS in these cells. However, suppression of AIF-1 expression induced up-regulation of ADAM3, probably due to a feedback regulation between these two molecules. On the other hand, blocking of the ADAM3 expression neither affected the proliferation properties of the macrophages nor obviously increased the number of apoptotic cells (data not shown), implying that ADAM3 mediated the pro-inflammatory activities of macrophages in a cell proliferation-independent manner and ADAM3 might affect multiple apoptosis-related molecules. However, further studies are needed to explore the mechanism.

In conclusion, AIF-1 expression was related to the inflammatory status of macrophages. AIF-1 expression played an important role in mediating the survival, migration and pro-inflammatory properties of macrophages. ADAM3 was identified as a potential upstream molecule that regulated AIF-1 expression.

Acknowledgements
The study was supported by the grant from the Sun Chieh Yeh Research Foundation for Hepatobiliary and Pancreatic Surgery of the University of Hong Kong. The rabbit anti-rat AIF-1 polyclonal antibody used in this project was kindly provided by Michael Autieri, Department of Physiology, Cardiovascular Research Centre, Temple University School of Medicine, Philadelphia, PA, USA.

Abbreviations
ADAM3 a disintegrin and metalloproteinase domain 3
AIF-1 allograft inflammatory factor-1
References


