Immunology Letters 135 (2011) 144–150

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/immlet

Allograft inflammatory factor-1 is overexpressed and induces fibroblast chemotaxis in the skin of sclerodermatous GVHD in a murine model

Aihiro Yamamoto[a], Eishi Ashihara[b], Yoko Nakagawa[b], Hiroshi Obayashi[c], Mitsuhiro Ohta[d], Hirokazu Hara[e], Tetsuo Adachi[e], Takahiro Seno[a], Masatoshi Kadoya[a], Masahide Hamaguchi[a], Hidetaka Ishino[a], Masataka Kohno[a], Taira Maekawa[b], Yutaka Kawahito[a,*]

[a] Department of Inflammation and Immunology, Graduate School of Medical Science, Kyoto Prefectural University of Medicine, 465, Kajii-cho, Kawaramachi-Hirokoji, Kamigyo-ku, Kyoto 602-8566, Japan
[b] Department of Transfusion Medicine and Cell Therapy, Kyoto University Hospital, 54 Kawahara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan
[c] Institute of Bio-Response Informatics, 22-15, Yousai, Momoyama-cho, Fushimi-ku, Kyoto 612-8016, Japan
[d] Department of Medical Biochemistry, Kobe Pharmaceutical University, 4-19-1, Motoyama-kita-machi, Higashinada-ku, Kobe 658-8558, Japan
[e] Department of Clinical Pharmaceutics, Gifu Pharmaceutical University, 1-25-4, Daigakunishi, Gifu 501-1196, Japan

ARTICLE INFO

Article history:
Received 19 August 2010
Received in revised form 23 October 2010
Accepted 24 October 2010
Available online 30 October 2010

Keywords:
Allograft inflammatory factor-1 (AIF-1)
GVHD
Scleroderma
Migration
Fibroblast

ABSTRACT

Allograft inflammatory factor (AIF)-1 has been identified in chronic rejection of rat cardiac allografts and is thought to be involved in the immune response. We previously showed that AIF-1 was strongly expressed in synovial tissues in rheumatoid arthritis and that rAIF-1 increased the IL-6 production of synoviocytes and peripheral blood mononuclear cells. Recently, the expression of AIF-1 has been reported in systemic sclerosis (SSc) tissues, whose clinical features and histopathology are similar to those of chronic graft-vs-host disease (GVHD). To clarify the pathogenic mechanism of fibrosis, we examined the expression and function of AIF in sclerodermatous (Scl) GVHD mice. We demonstrated that immunoreactive AIF-1 and IL-6 were significantly expressed in infiltrating mononuclear cells and fibroblasts in thickened skin of Scl GVHD mice compared with control. The immunohistochemical findings were confirmed by Western blot analysis. Wound healing assay also revealed that rAIF-1 increased the migration of normal human dermal fibroblasts (NHDF) directly, but cell growth assay did not show that rAIF-1 increased the proliferation of them. These findings suggest that AIF-1, which can induce the migration of fibroblasts and the production of IL-6 in affected skin tissues, is an important molecule promoting fibrosis in GVHD. Although the biological function of AIF-1 has not been completely elucidated, AIF-1 can induce IL-6 secretion on mononuclear cells and fibroblast chemotaxis. AIF-1 may accordingly provide an attractive new target for antifibrotic therapy in SSc as well as Scl GVHD.

1. Introduction

Allograft inflammatory factor (AIF)-1 is an IFN-γ-inducible, Ca^{2+}-binding EF-hand protein that is encoded within the HLA class III genomic region in the direct vicinity of TNF-α [1,2]. AIF-1 was originally identified and cloned from rat cardiac allografts undergoing chronic rejection [3]. AIF-1 expression has been documented in various human tissues and cells such as macrophage cell lines [4], peritoneal macrophages, spleen, peripheral blood leukocytes, and thymus [5], although its detailed physiological functions remain unclear. We previously proved that AIF-1 is strongly expressed in infiltrating mononuclear cells and synovial fibroblasts in rheumatoid arthritis (RA) compared with osteoarthritis (OA). In addition, AIF-1 induced the proliferation of cultured synovial cells in a dose-dependent manner and increased the IL-6 production of synoviocytes and peripheral blood mononuclear cells (PBMCs) [6]. Moreover, the expression of AIF-1 has been reported in systemic sclerosis (SSc) tissues [7]. The frequency of the AIF-1 rs2269475 TT genotype that results in a tryptophan to arginine amino acid substitution is significantly high in the patients with RA and SSc, and is associated with an increased risk of their development [8–10].

SSc is a chronic autoimmune disease characterized by fibrosis of skin and major organs such as lung, heart, gastrointestinal tract and widespread blood vessels. Progressive substitution of tissue structure by collagen-rich extra cellular matrix induces functional impairment of affected organs. The etiology is probably associated

Abbreviations: AIF-1, allograft inflammatory factor-1; BMT, bone marrow transplantation; GVHD, graft-vs-host disease; NHDF, normal human dermal fibroblasts; PDGF, platelet-derived growth factor; RA, rheumatoid arthritis; SSc, systemic sclerosis; Scl, sclerodermatous.

* Corresponding author. Tel.: +81 75 251 5505; fax: +81 75 252 3721.
E-mail address: kawahity@koto.kpu-m.ac.jp (Y. Kawahito).

0165-2478/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.imlet.2010.10.015

with environmental and occupational exposure to organic solvents [11], genetic background, vascular damage, disorders of autoimmunity, collagen metabolism, cell growth factor and cytokines like connective tissue growth factor (CTGF) and TGF-β [12,13]. These factors are intricately intertwined and induce multiple clinical manifestations, but have not been elucidated in detail yet. In cells and tissues of various diseases including SSc, AIF-1 is expressed in T cells [14,15], macrophages, and endothelial cells [16,17] and may promote the expression of adhesion molecules that mediate specific homing into affected tissues [7]. Another recent study in vitro indicated that AIF-1 enhances the activation of T cells, which increase chemotaxis and induces a profibrotic phenotype [15,14], but the T cells are forcedly transfected with the vector expressing AIF-1. Thus, AIF-1 is thought to play a fundamental role in several cell types involved in chronic immunological inflammatory processes.

SSc resembles graft-vs-host disease (GVHD) both in its clinical features and histopathology [18]. For example, chronic GVHD has SSc like clinical features such as a skin fibrosis. Investigation of the fibrosing process in GVHD may help to elucidate the pathogenesis of fibrosis in SSc as well. The occurrence and extent of tissue fibrosis are thought to be influenced by minor histocompatibility mismatches and radiation exposure before transplant. These factors cause various immune responses of graft failure in bone marrow transplantation [19,20], but the detailed mechanism of fibrosis is still unclear. The phenotypic features in Scl GVHD get expressed about 3 weeks after transplant and are characterized by loss of dermal fat, thickening of skin, infiltration of numerous mononuclear cells and fibroblasts, and acceleration of collagen synthesis [19,21]. Clarification of the immunological mechanisms underlying skin fibrosis in GVHD will also shed light on the pathogenesis of SSc. However, no reports are available on the involvement of AIF-1 in GVHD. Furthermore, the pathophysiologic significance of AIF-1 in skin fibrosis has not been elucidated. This prompted us to examine the expression of AIF-1 and its function in fibrosis by using this Scl GVHD model [19] that recapitulates important features of SSc.

2. Materials and methods

2.1. Bone marrow transplantation

Six to 8-week-old male B10.D2 mice (H-2^d, Oriental Bio Service) were used as donors and 6–8-week-old female BALB/c mice (H-2^d, SRL) as recipients, for bone marrow transplantation to Scl GVHD with a standard method using spleen cells as the source of mature T cells. BALB/c mice were irradiated with 7.5 Gy. About 6 h later, recipient mice were injected via the tail vein with male donor bone marrow (4×10^6/mouse) and spleen cells (1×10^7/mouse) suspended in PBS. The control group consisted of female BALB/c recipient mice that received male B10.D2 bone marrow cells, namely a T cell depleted-bone marrow transplantation (TCD-BMT) group. Transplanted mice were maintained in sterile cages and supplied with autoclaved food. We performed the experiments five times, with five animals per group (Scl GVHD or TCD-BMT) examined in each experiment. Approval for these studies was obtained from the Committee on Animal Research of the Kyoto University Faculty of Medicine.

2.2. Collection of tissue and preparation of normal human dermal fibroblasts

Allogeneic BMT mice had significantly lower body weights than TCD-BMT mice from day 10 after BMT and extensive thickened skin (data not shown). Five transplanted animals per group were sacrificed by cervical dislocation on day 21 after BMT. Day 21 after BMT was chosen because it is the earliest time point when Scl GVHD reliably develops in mice receiving allogeneic BMT. Skin was depilated and harvested for immunostaining and Western blotting. Normal human dermal fibroblasts (NHDF) were obtained from Sanko Junyaku Co., Ltd [22,23] and cultured in fibroblast basal medium with human fibroblast growth factor-B (1 μg/ml), insulin (5 mg/ml), 0.1% gentamicin/amphotericin-B, and 10% fetal bovine serum in a humidified incubator at 37 °C in the presence of 5% CO_2. We actually used NHDF after three or four passages in cell culture.

2.3. Peptide synthesis and preparation of anti-human AIF-$1_{53–71}$ and AIF-$1_{113–129}$ Abs

Two synthetic peptides, which corresponded to residues 53–71 and 113–129 of human AIF-1 (AIF-$1_{53–71}$ and AIF-$1_{113–129}$, respectively) as deduced from the nucleotide sequence of the human AIF-1 gene, were obtained with an additional cysteine residue at the N terminus (Biologica). Following purification by reversed phase HPLC, the synthetic peptide (purity >90%) was coupled to keyhole limpet hemocyanin with N-(ε-maleimidocaproyloxy) succinimide (Sigma–Aldrich). The carrier-conjugated peptide was then emulsified with Freund's complete adjuvant (Difco Laboratories) and injected s.c. (0.5 mg/injection) into rabbits. The rabbits were immunized six times at 10-day intervals. Blood samples were collected 10 days after the last injection, and the specific Ab in the sera was purified using an AIF-1 peptide-coupled cyanogen bromide-activated Sepharose affinity column. The Abs reacted with protein from abdominal adipose tissue and PBMC that is identical to the molecular size of purified recombinant human AIF-1.

2.4. Expression of rAIF-1 and preparation of anti-rAIF-1 Ab

Human AIF-1 cDNA was amplified from human peripheral lymphocyte cDNA (BD Clontech, California, USA) using PCR. The forward and reverse primers were 5′ -GTG GAT CCA TGA GCC AAA CCA GGG ATT T-3′ (containing *Bam*HI site) and 5′-CAC TCG AGT CAG ATA GGG CTT TCT TGG CT-3′ (containing *Xho*I site), respectively. To express AIF-1 as a GST fusion protein, the DNA fragment obtained was inserted in the *Bam*HI/*Xho*I sites of pGEX-4 (Amersham Biosciences) in frame. The fusion protein was purified with a GST purification system (Amersham Biosciences) and affinity chromatography with anti-AIF-$1_{113–129}$ Ab. To investigate the effect of AIF-1 on the cell proliferation and cytokine induction, rAIF-1 was treated with detoxi-gel endotoxin removing gel (Pierce). Endotoxin detection was performed using *Limulus* amoebocyte lysate analysis (Wako Pure Chemical), and treated AIF protein was confirmed to contain <0.1 ng/μg of endotoxin. AIF protein we synthesized was named AIF-5 according to a new nomenclature of the AIF family of proteins [24]. This AIF splice variants IRT-1, G1, BART-1 are encoded in the same region of the BAT2 gene on chromosome 6 [5]. Anti-rAIF-1 antiserum was raised in a similar manner as anti-synthetic peptide Ab by injecting 50 mg of human rAIF-1 into a rabbit. The human rAIF-1 Ab IgG fraction was prepared by chromatography on a human rAIF-1-coupled cyanogen bromide-activated Sepharose affinity column and biotinylated with 5-(N-succinimidyloxycarbonyl)pentyl D-biotinamide (Dojindo Chemical).

2.5. Immunohistochemical analysis of AIF-1 expression in skin

Immunohistochemical staining was performed using the avidin–biotin peroxidase complex system. Skin tissue specimens were preserved in 10% buffered formalin and embedded in paraf-