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Immune suppressor factor confers stromal cell line with enhanced supporting activity for hematopoietic stem cells

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Abstract

Immune suppressor factor (ISF) is a subunit of the vacuolar ATPase proton pump. We earlier identified a short form of ISF (ShIF) as a stroma-derived factor that supports cytokine-independent growth of mutant Ba/F3 cells. Here, we report that ISF/ShIF supports self-renewal and expansion of primary hematopoietic stem cells (HSCs). Co-culture of murine bone marrow cells with a stromal cell line overexpressing ISF or ShIF (MS10/ISF or MS10/ShIF) not only enhanced their colony-forming activity and the numbers of long-term culture initiating cells, but also maintained the competitive repopulating activity of HSC. This stem cell supporting activity depended on the proton-transfer function of ISF/ShIF. Gene expression analysis of ISF/ShIF-transfected cell lines revealed down-regulation of secreted frizzled-related protein-1 and tissue inhibitor of metalloproteinase-3, and the restoration of their expressions in MS10/ISF cells partially reversed its enhanced LTC-IC supporting activity to a normal level. These results suggest that ISF/ShIF confers stromal cells with enhanced supporting activities for HSCs by modulating Wnt-activity and the extracellular matrix.

Keywords: Immune suppressor factor; ISF; ShIF; Hematopoietic stem cell; HSC; Stromal cell; Co-culture

Self-renewal, proliferation, and differentiation of hematopoietic stem cells (HSCs) are governed by a variety of environmental cues mainly supplied by the stem cell niche, a microenvironment where HSCs reside in the bone marrow [1]. Such cues comprise signals from various cytokines, growth factors, and cell surface molecules that are expressed in the stem cell niche. However, the precise nature of the signaling networks between HSC and stem cell niche is complex and poorly understood.

It has been widely accepted that the direct contact between HSC and bone marrow stroma is critical for maintaining HSC in a immature state, since attempts to maintain or expand HSCs in vitro by a defined combination of cytokines have been largely unsuccessful [2] and it could only be achieved by co-cultivation on bone marrow stromal cells [3]. Along this line, many researchers have tried to identify cell surface molecules of stromal cells that are essential for HSC-support [4]. We earlier reported the short form of immune suppressor factor (ISF), ShIF, as a stroma-derived factor that is capable of supporting a mutant subline of Ba/F3 cells which became stroma-dependent by chemical mutagenesis [5]. ISF was first cloned as a

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suppressor of the T-cell response [6], but later proved to be the subunit of vacuolar ATPase proton pumps, an energy-dependent proton transport complex critical for a wide variety of biological functions in vivo [7]. We found that a stromal cell line overexpressing ShIF could support interleukin-3 (IL-3)-independent growth of mutant Ba/F3 and suggested the possibility that ShIF could also support the growth of primary hematopoietic cells [5].

Vacuolar H^+ ATPases (V-ATPases) are multi-subunit proton pumps that are highly conserved throughout organisms and are critical for acidification of various intracellular compartments such as lysosomes, endosomes, secretory vesicles, and clathrin-coated vesicles [8]. Intracellularly localized V-ATPases are essential for receptor-mediated endocytosis, intracellular targeting of lysosomal enzymes, and protein processing and degradation. In addition to intracellular compartments, V-ATPases also localize in the plasma membrane of certain type of cells such as renal cells, osteoclasts, and macrophages, where they are critical for secretion of proton ions, bone resorption, and control of cytoplasmic pH. Vacuolar ATPase is composed of two functional sectors, V_1 and V_0. V_1 sector, which is composed of eight different subunits, contains catalytic sites for ATP. V_0 sector is an integral complex that forms a pore for transporting proton ions. V_0 sector consists of five different subunits (a, c, c′, c″, and d) and subunit 'a' is the product of four isoforms, a1–a4. ISF corresponds to the a2 isoform and is expressed ubiquitously in various tissues including heart, brain, liver, and kidney. Although the physiological roles of a3 and a4 subunits are evident in osteoclasts [9] and renal intercalated cells [10], respectively, the role of the a2 subunit has remained unknown.

Here, we demonstrate that ISF or ShIF can enhance supportive capacity of stromal cells for primary HSC in vitro. Bone marrow stromal cell lines overexpressing ISF or ShIF showed an enhanced supportive capacity for HSC by clonogenic assays, long-term culture-initiating cell (LTC-IC), and long-term reconstitution assays. Proton transport activity of ISF/ShIF was essential for these effects and decreased expression of secreted frizzled-related protein (SFRP)-1 and tissue inhibitor of metalloproteinase-3 (TIMP-3) seemed to be the critical downstream target of ISF. These findings should lead to develop novel strategies for HSC expansion using stromal cells in vitro.

Materials and methods

Cell lines and culture. MS10, PA6, OP9, AGM-S3, and PLAT-E cells were maintained in αMEM/10% fetal bovine serum (FBS, Sigma), DMEM (F-12)/10% FBS, αMEM/20% FBS, αMEM/10% FBS, and DMEM/10% FBS, respectively. MS10 and PA6 cells expressing ISF or ShIF are described previously [5]. MS10 cells expressing ISF and TIMP-3 or SFRP-1 were established by infecting MS10/ISF cells with pMXs-IG/TIMP-3 or SFRP-1 retrovirus and sorting GFP-positive cells by FACS. All mediums were supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin, and 2 mM L-glutamine.

Flow cytomertry. Antibodies used in flow cytometry were anti-Sca-1-FITC, anti-c-Kit-PE, anti-Gr-1-PE, anti-CD11b-PE, anti-B220-PE, anti-CD3-PE, and anti-CD45.1-FITC, and all were purchased from Pharmingen. Depletion of lineage marker-positive cells was done using MACS Lineage depletion kit (Miltenyi Biotec) according to the manufacturer's protocol.

Co-culture of bone marrow cells and hematopoietic stem cell/progenitor assay. Bone marrow cells were harvested from 8- to 12-week-old C57BL/6 mice by flushing out of femurs and tibias. MS10 or PA6 cells expressing ISF or ShIF were seeded onto a 24-well dish and subconfluent layers were mitotically inactivated by 1500 rads of irradiation. Bone marrow cells (1×10^4) or 100 c-$Kit^+Sca\text{-}1^+lineage^-$ (KSL) cells were plated in a well and cultured for 7 days in αMEM/10% FBS without cytokines. Cells were harvested and subjected to colony assays using MethoCult3434 (Stem Cell Technologies) or bone marrow reconstitution assays, as described below. In the case of bulk LTC-IC assay, cells were co-cultured for 5 weeks and subjected to colony assays using MethoCult3434. For bone marrow reconstitution assay, we isolated KSL cells from C57BL/6-Ly5.1 mice (Sankyo Laboratory) by FACS sorting using FACS Vantage (Beckton–Dickinson) and performed co-cultivation on stromal cells for 7 days. Cells were collected by trypsinization and transplanted into lethally irradiated (950 rads) recipient mice (C57BL/6-Ly5.2) with 2×10^5 of bone marrow cells (Ly5.2) as a competitor. Contribution of Ly5.1 cells for peripheral blood was analyzed after 5, 10, and 20 weeks of transplantation by FACS. All animal experiments were reviewed and approved by Institutional Review Board.

Cobblestone-like area forming cell (CAFC) assay. Bone marrow cells (1×10^4/well) were plated on mitotically inactivated MS10 cells expressing ISF or ShIF in 24-well plates. Three to four days later, numbers of cobblestone-like area were counted under a microscope.

cDNA, plasmids, and retrovirus production. Murine TIMP-3 and SFRP-1 cDNA was obtained by PCR with *Pfu* polymerase (Stratagene) using bone marrow cDNA as a template. Amplified cDNAs were subcloned into pMXs-IG [11]. Integrity of the amplified sequence was confirmed by DNA sequencing. Retrovirus was produced as described [12]. Briefly, retrovirus vector was transiently transfected into PLAT-E cells using Fugene (Roche diagnostics), and retrovirus supernatant was collected after 48 h of transfection. Retrovirus infection was carried out in the presence of 10 μg/ml of Polybrene (Sigma).

Gene expression profiling by microarray. Total RNA was extracted from MS10 cells expressing vector alone, ISF or ShIF by Trizol (Invitrogen). cRNA was generated and hybridized against the Affymetrix HG_U95Av2 oligonucleotide arrays according to the manufacturer's protocol. The arrays were scanned using a Hewlett–Packard confocal laser scanner and analyzed using MicroArray Suite 5.0 (Affymetrix), GeneSpring 4.0 (Silicon Genetics, Redwood City, CA).

Northern blot. Total RNA was extracted from 1×10^7 cells by TRIzol (Gibco-Invitrogen) according to the manufacturer's protocol. The RNA samples (20 μg/lane) were separated on a formaldehyde-denaturing 1.0% agarose gel and transferred onto Hybond N+ membrane (Amersham). Full-length cDNA of ISF, TIMP-3, and SFRP-1 was used as probes. All probes were labeled using a Rediprime kit (Amersham). Hybridizations with ^{32}P-labeled probes were carried out in ExpressHyb buffer (Clontech) according to the manufacturer's protocol. The membranes were washed in 2× SSC, 0.1% SDS washing buffer for 30 min at room temperature with several buffer changes, followed by washing twice in 0.1× SSC, 0.1% SDS for 15 min at 42 °C. The membranes were exposed on XAR films (KODAK) at −80 °C for 1–5 days.

RT-PCR. Total RNA was reverse-transcribed by Superscript II reverse transcriptase (Gibco-Invitrogen) using a random hexamer. The amount of cDNA was normalized by GAPDH. Semi-quantitative RT-PCR was run for 25–35 cycles by Ex *Taq* polymerase (Takara) using normalized cDNAs as templates. Appropriate PCR cycles were chosen to obtain linear phase of amplification. The primers used were: GAPDH forward 5′-ACC ACA GTC CAT GCC ATC AC-3′, reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′; SFRP-1 forward 5′-CTA CGT GAG CTT CCA GTC CG-3′, reverse 5′-TGG AGG ACA CAC GGT TGT AC-3′; and TIMP-3 forward 5′-TAC CAT GAC TCC CTG GCT TG-3′, reverse 5′-TGC AAT TGC AAC CCA GGT GG-3′.