Enhanced c-Fms/M-CSF Receptor Signaling and Wound-Healing Process in Bone Marrow-Derived Macrophages of Signal-Transducing Adaptor Protein-2 (STAP-2) Deficient Mice

Osamu Ikeda,a Yuichi Sekine,a Ryuta Muromoto,a Norihiko Ohbayashi,a Akihiko Yoshimura,b and Tadashi Matsuda*
a,b

a Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University; Kita-ku Kita 12 Nishi 6, Sapporo 060–0812, Japan; and b Division of Molecular and Cellular Immunology, Medical Institute of Bioregulation, Kyushu University; 3–1–1 Maidashi, Fukuoka 812–8582, Japan.

Received March 21, 2008; accepted June 30, 2008; published online July 2, 2008

Signal-transducing adaptor protein-2 (STAP-2) is a recently identified adaptor protein as a c-Fms/M-CSF receptor-interacting protein and constitutively expressed in macrophages. In our previous study, we examined the role of STAP-2 in the c-Fms/M-CSF receptor signaling using a murine macrophage tumor cells line, Raw264.7. Overexpression of STAP-2 in Raw264.7 cells markedly suppressed M-CSF-induced activation of extracellular signal regulated kinase and Akt. In addition, Raw264.7 overexpressing STAP-2 affected cell migration in wound-healing process. These results suggest that STAP-2 deficiency influences endogenous c-Fms/M-CSF receptor signaling. Here we show that loss of STAP-2 expression in knockout mouse macrophages results in marked enhancement of the c-Fms/M-CSF receptor signaling and wound-healing process. We therefore propose that STAP-2 acts as an endogenous regulator in normal macrophages functions.

Key words signal-transducing adaptor protein-2; M-CSF; c-Fms; macrophage; wound-healing

Macrophages mainly provide innate immune surveillance for every tissue in the body. Macrophages are derived from myeloid precursors in bone marrow (BM), spleen, and fetal liver. Macrophages then leave the unique environment of the BM and enter the blood, where they are exposed to a variety of cellular factors including cytokines, chemokines, hormones and immunoglobulins, which are capable of impacting their functional and phenotypic characteristics. The production of macrophages in BM is regulated by M-CSF (CSF-1), via M-CSF receptor, also known as the c-Fms proto-oncoprotein. M-CSF/c-Fms possesses the intrinsic tyrosine kinase activity that is transmitted through M-CSF binding. M-CSF induces dimerization of its receptor, leading to autophosphorylation of a number of tyrosine residues through the cytoplasmic domain of the receptor that serve as binding sites for the Src homology 2 (SH2) domain-containing proteins, such as Src-family kinases, Grb2 and phosphatidylinositol 3-kinase (PI3K). These then facilitate activation of signal transduction pathways that control macrophage proliferation, differentiation and motility. Furthermore, activation of extracellular signal regulated kinase (ERK) signaling promotes macrophage proliferation, while activation of PI3K enhances macrophage survival and migration via Akt.

Recently, we have cloned novel adaptor molecules, STAP-2 (signal-transducing adaptor protein-2) as a c-Fms interacting protein and STAP-1 as a c-Kit interacting protein. Human STAP-2 is identical to the recently cloned adaptor molecule BKS, which is a substrate of breast tumor kinase (Brk) tyrosine kinase. STAP-2 and STAP-1 contain an N-terminal Pleckstrin homology (PH) and a region distantly related to the Src homology 2 (SH2) domain (overall 33% amino acid identity). However, STAP-2 possesses a C-terminal proline-rich region and a YXXQ motif, neither of which are present in STAP-1. We previously reported that STAP-2 interacts with STAT3 through its YXXQ motif and enhances STAT3 transcriptional activity. STAP-2 also interacts with STAT5 through its PH and SH2-like domains. Our previous study also demonstrated that STAP-2 modulates TLR4-mediated NF-κB signaling in macrophages. Notably, overexpression of STAP-2 in macrophage tumor cells resulted in suppression of M-CSF-activated signaling pathways and impaired migration in wound-healing process.

In the present study, we show that bone marrow-derived macrophages from STAP-2 deficient mice exhibit enhanced c-Fms/M-CSF receptor signaling and an efficient wound-healing process. These results indicate that endogenous STAP-2 is a negative regulator of c-Fms-mediated signaling and motility in normal macrophages.

MATERIALS AND METHODS

Reagents, Antibodies, and Mice Recombinant human M-CSF was a kind gift from Morinaga Milk Industry Co., Ltd. (Tokyo, Japan). Antibody against ERK1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antibodies against Akt, phospho-Akt, and phospho-ERK were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). The generation of STAP-2-deficient mice was described previously. Mice were housed and bred in the Pharmaceutical Sciences Animal Center of Hokkaido University. All animals were maintained under pathogen-free conditions and in compliance with national and institutional guidelines. All protocols were approved by the Hokkaido University animal ethics committee.

Preparation of Macrophages, Treatment with M-CSF, Cell Growth Assay, and Flow-Cytometric Analysis To generate bone marrow-derived macrophages (BMMs), bone marrow cells from wild-type (WT) and knockout (KO) mouse were cultured in DMEM supplemented with 10% FBS and M-CSF (100 ng/ml) as described previously. The cells were collected after 3 d and continually cultured for 3 d

* To whom correspondence should be addressed. E-mail: tmatsuda@pharm.hokudai.ac.jp

© 2008 Pharmaceutical Society of Japan
for the development of BMMs. BMMs were plated in cell culture plates and incubated for 3 h prior to experiments. The numbers of BMMs after M-CSF treatment were measured using a WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay (Cell Counting Kit-8; Wako Pure Chemicals). The absorbances were measured at a test wavelength of 450 nm and a reference wavelength of 595 nm using a microplate reader (Bio-Rad, Hercules, CA, U.S.A.).

Flow-cytometric analysis of BMMs was performed as described previously.14) Mac-1 and CD16/CD32(FcγR II/III receptor) antibodies were purchased from BD Pharmingen (San Diego, CA, U.S.A.). Cells were analyzed using FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.).

Immunoblotting Western blotting assay was performed as described previously.11) The cells were harvested and lysed in lysis buffer (50 mM Tris–HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml each of aprotinin, pepstatin and leupeptin). An aliquot of total cell lysates was resolved on SDS-PAGE and transferred to PVDF transfer membrane (PerkinElmer; Boston, MA, U.S.A.). The filters were then immunoblotted with the respective antibody.

Wound-Healing Assay Wound-healing assay was performed as described previously.13,15) The assay was conducted in 6-well tissue-culture plates. BMMs were cultured as a confluent monolayer, an artificial wound was created by...
scraping with a pipette tip, and cells migrating into open space were monitored microscopically.

**Statistical Methods** The significance of differences between group means was determined by Student’s *t*-test.

**RESULTS AND DISCUSSION**

**STAP-2 Regulates M-CSF-Induced ERK and Akt Activation in BMMs** We originally identified STAP-2 as a c-Fms-interacting protein with a yeast two-hybrid system. To verify whether endogenous STAP-2 regulates c-Fms/M-CSF receptor signaling in macrophages, we used BMMs from WT or KO mouse. BMMs from STAP-2 KO mouse did not show any significant alteration of M-CSF-induced cell growth (Fig. 1A) and expression levels of cell surface makers, such as Mac1 and CD16/CD32 (Fig. 1B). We first attempted to examine the effects of loss of endogenous STAP-2 expression on M-CSF-induced tyrosine phosphorylation of c-Fms in BMMs. However, we could not observe detectable endogenous c-Fms protein in BMMs, because endogenous c-Fms expression in normal macrophages was much lower than that of tumor macrophages, Raw264.7 cells. M-CSF is known to induce recruitment of the p85 subunit of PI3K to c-Fms and Gab2, resulting in rapid activation of the p110 subunit of PI3K/Akt and Ras/ERK signaling pathways. We then tested the effects of loss of endogenous STAP-2 expression on M-CSF-induced ERK and Akt activation using activation state-specific antibodies for phosphorylated ERK (pERK) or phosphorylated Akt (pAkt) in BMMs. As shown in Figs. 1C and D, after stimulation of M-CSF, the marked enhanced activation of ERK and Akt were observed in STAP-2 KO BMMs. Therefore, endogenous STAP-2 regulates M-CSF-mediated both PI3K/Akt and Ras/ERK signaling pathways in normal macrophages.

**STAP-2 Regulates Macrophage Motility in BMMs** During the process of inflammation, macrophages are well known to migrate and extravagate into numerous tissues and play a significant role in wound-healing. We previously showed that overexpression of STAP-2 in Raw264.7 cells resulted in reduced migration in an *in vitro* wound-healing assay. We then employed an *in vitro* wound-healing assay using BMMs from WT or KO mouse. Importantly, as shown in Figs. 2A and B, enhanced wound-healing process was observed in STAP-2 KO BMMs. Therefore, endogenous STAP-2 plays an important role in the regulation of macrophage motility.

**Concluding Remarks** We here show that endogenous STAP-2 regulates c-Fms/M-CSF receptor-mediated signaling events including PI3K/Akt and Ras/ERK. Importantly, STAP-2 deficiency in macrophages resulted in increase of macrophage migration capacity. Taken together, these results indicate that STAP-2 acts as an endogenous regulator in normal macrophage functions.

M-CSF stimulates the proliferation, differentiation, motility and survival of macrophages from myeloid progenitors and also accelerates angiogenesis in *vivo*. Recent study demonstrated that M-CSF stimulates macrophages to produce vascular endothelial growth factor and matrix metalloprotease (MMP). Furthermore, M-CSF participates in tissue invasion by cancer cells and enhances metastasis of tumors. Thus, tumor-associated macrophages control tumor cell growth, angiogenesis, and extracellular matrix through c-Fms/M-CSF receptor-mediated signaling. Indeed, M-CSF blockade is demonstrated to suppress tumor growth, MMP production, and macrophage recruitment.

Therefore, our data provide the possibility that STAP-2 can be considered as a novel candidate for therapeutic drug development to regulate tumor-associated macrophages.

**Acknowledgements** This study was supported in part by Industrial Technology Research Grant Program in 2005 from New Energy and Industrial Technology Development Organization (NEDO) of Japan and Grant-in-Aid for scientific research from Ministry of Education, Culture, Sports, Science and Technology of Japan.

**REFERENCES**

9) Masahara M., Nagaoka K., Nishikawa M., Sasaki M., Yoshimura A.,...


