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Ron expression in the primary culture of rat hepatocytes

Takashi Kato[1,2]☼

1. Department of Pathology, Faculty of Medicine, Kindai University, Osaka, Japan
2. Department of Pharmacology, Faculty of Medicine, Kindai University, Osaka, Japan

☼**Address correspondence to (Present address):**
Dr. Takashi KATO
Urologic Oncology Branch, Center for Cancer,
Research Building 10, Rm 1-5940,
Center Drive, MSC 1107, Bethesda, MD,
20892-1107, USA
E-mail: takashi0920k@gmail.com
Tel; 1-301-451-8116

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ABSTRACT

Macrophage stimulating protein (MSP), identified to HGF-liked protein (HLP), was discovered as a serum protein promoting macrophage motility. MSP activation is an important physiological response to tissue injury, and mediates its biological activities including the anti-inflammation through a receptor tyrosine kinase, RON. In general, resident peritoneal macrophages express Ron. In this study, I detected the Ron expression in the primary culture of rat hepatocytes, and phosphorylation of tyrosine 1238/1239 in Ron by adding recombinant MSP protein. I next examined the mitogenic activity of MSP in hepatocytes, but MSP could not

sufficiently induce DNA synthesis. Actually, MSP insufficiently promoted the phosphorylation of ERK and Akt. Moreover, HGF unregulated the Ron expression in the hepatocytes, and therefore, might regulate the MSP-RON signaling. HGF might induce MSP-Ron signaling through increasing Ron expression.

Key Words: MSP, Ron, hepatocytes, proliferation, kupffer cells

1. INTRODUCTION

Macrophage stimulating protein (MSP) was discovered as serum protein promoting mouse macrophage motility. Based on the amino acid sequence homology and the biological activity, HGF-liked protein (HLP) was identical to MSP (Shimamoto et al., 1993). MSP is a heterodimeric protein consisting of a disulfide-linked 53-kDa α-chain and 25-kDa β-chain, and has a 45% amino acid sequence similarity to HGF (Han et al., 1991, Bazerra et al., 1993). Its activation is not observed in freshly prepared from human serum, thus the activation by serum convertases is an important physiological response to tissue injury. MSP activation is regulated in tissue homeostasis and in disease pathologies, such as inflammation and cancer progression. MSP mediates its biological activities through a receptor tyrosine kinase, RON (recepteurd'originenantais) / STK (stem cell-derived tyrosine kinase) (Kato, 2016, Wang et al., 1994). RON belongs to a family of receptor tyrosine kinases that includes Met, and MSP-induced dimerization leads to its autophosphorylation and kinase activation. Ron is expressed on resident peritoneal macrophages, but not on exudate peritoneal macrophages or mononuclear phagocytes from the bone marrow, peripheral blood, spleen, or alveoli (Iwama et al., 1995). In addition to the stimulatory activities of MSP include the induction of macrophage spreading, migration and phagocytosis, MSP-RON has the anti-inflammatory activity via suppression of NF-κB signaling (Morrison et al., 2002). MSP is a crucial regulator of inflammation in multiple animal disease models of the liver, kidney, lung, gut and other organs (Stuart et al., 2011, McDowell et al., 2002, Goyatte et al., 2008, Rampino et al., 2007). In addition to stromal cells, some recently report that the MSP-Ron signaling functions on the epithelial cells (Glasbey et al., 2015, Lee et al., 2013, Kulkarni et al., 2014). However, it is yet disclosed what roles and on what cells MSP-Ron signaling plays in liver. The objective of this study is to investigate whether this signaling effects on hepatocytes or kupffer cells, and has the sufficient mitogenic activity. Although HGF-Met signaling is well-known to promote the mitogenesis of hepatocytes, it is not well-known what roles MSP-Ron signaling plays in liver. Here, I investigated whether hepatocytes or kupffer cells express Ron in liver, and MSP-Ron signaling have the mitogenesis activity as HGF does. Moreover, I examined whether HGF could up-regulate Ron expression in hepatocytes.

2. MATERIALS AND METHODS

2.1. Reagents

Recombinant human HGF was purified from the medium of CHO cells transfected with human HGF cDNA (Nakamura et al., 1989, Ishiki et al., 1992). Recombinant MSP was purchased from Peprotech (Rocky Hill, NJ, USA). The following reagents were used as the primary antibodies: anti-c-Met (Santa Cruz Biotechnology, Dallas, TX, USA), anti-MR (ab64693, Abcam, Cambridge, UK), and anti-β-actin (Sigma, St. Louis, MO, USA). Anti-rat TDO antiserum were used for immunoblot (Nakamura et al., 1989, Niimi et al., 1983).

2.2. Isolation of rat hepatocytes

Male Sprague–Dawley (SD) rats were purchased from Japan SLC (Shizuoka, Japan). Hepatocytes were prepared from 8–15 week old SD rats by in situ perfusion of the liver with collagenase (Wako, Osaka, Japan), as reported (Nakamura et al., 1984, Machine et al., 2006).The cells were cultured on plastic dishes precoated with Cellmatrix type I-A collagen (Nitta gelatin, Osaka, Japan) at a cell density of 3 x 10^4/cm^2 in DMEM (Nacalai) supplemented with 5% fetal bovine serum. After 3–4 hrs incubation, the medium was replaced with the serum-free DMEM.

2.3. [^{3}H]-thymidine incorporation assay

MSP and HGF were added to cultures of hepatocytes. The culture was sustained for 20 h, and then pulse-labeled with 2.5 lCi/ml of [^{3}H]-thymidine for 8 h. The cells were washed twice with cold PBS and immersed in 10% trichloroacetic acid. After solubilization in 1 N NaOH, radioactivity of [^{3}H]-thymidine incorporated into DNA was measured using a b-counter (Nakamura et al., 1984, Machine et al., 2006).

2.4. Western blot analysis

Cells were lysed on ice with lysis buffer [50 mMTris-HCl (pH 7.5), 150 mMNaCl, 1% Triton X-100, 5 mM EDTA, protease inhibitor cocktail, 2mM Na_3VO_4 and 50 mMNaF]. The lysates were immunoblotted as described previously (Kato et al., 2012).

2.5. Real-time RT-PCR

Total RNA was extracted from cells using Trizol (Thermo Fisher, Waltham, MA, USA). The quantitative RT-PCR was performed as described previously (Kato et al., 2012). Expression was normalized to GAPDH mRNA levels. The Taqman probe for rat *Ron* mRNA (Rn01446673-m1, Thermo Fisher) was used.

2.6. Statistical analyses

The significance of differences was evaluated by Student's-t test. Values are expressed as the mean +/- standard deviation, and *P*-values < 0.05 were considered significant.

3. RESULTS

3.1. Primary hepatocytes express Ron protein

I at first tested whether hepatocytes or kupffer cells expressed Ron. To confirm the hepatocytes or kupffer cells, I performed the immunoblot assay of TDO for hepatocyte marker and mannose receptor (MR) for kupffer cells (Nakamura et al., 1987, Ohnishi et al., 2012), and detected these proteins, respectively (Figure 1A). Ron was expressed in the primary culture of rat hepatocytes (Figure 1A). Next, to investigate whether MSP can activate the Ron receptor, recombinant MSP was added into the cultured hepatocytes. Through the immunoblot assay for phosphorylation of tyrosine1238/1239 in the Ron, I found that recombinant MSP protein phosphorylated Ron (Figure 1B).

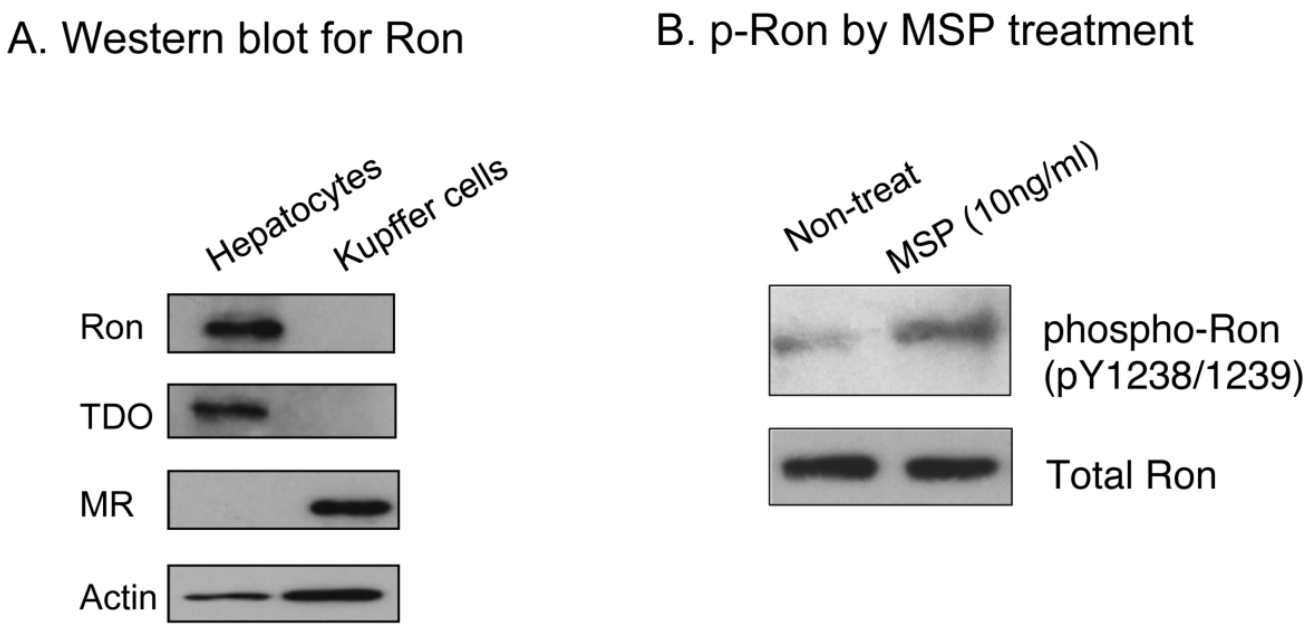

Figure 1

Ron expression in primary culture of hepatocytes. (A) Immunoblotting for Ron in the primary culture of hepatocytes and kupffer cells. (B) Tyrosine phosphorylation of Ron protein in the primary culture of hepatocytes treated with 10 ng/ml of recombinant MSP for 15 min.

3.2. MSP-Ron signaling has less activity of mitogenesis

Although the addition at a concentration of 2-50 ng/ml, MSP could not sufficiently induce DNA synthesis: the level of incorporated [^{3}H]-thymidine was less than 1 ng/ml of HGF protein (Figure 2A). Hence, MSP might effects much less on mitogenic actions.

Moreover, it is generally known that MEK-ERK and PI3K-Akt activation are essential for cell proliferation (Murata et al., 2014, Gao et al., 2015). Therefore, I examined the phosphorylation of ERK and Akt in MSP-treated hepatocytes. HGF addition promoted the phosphorylation of ERK and Akt (Figure 2B and C), but MSP did little extent (Figure 2B and C).

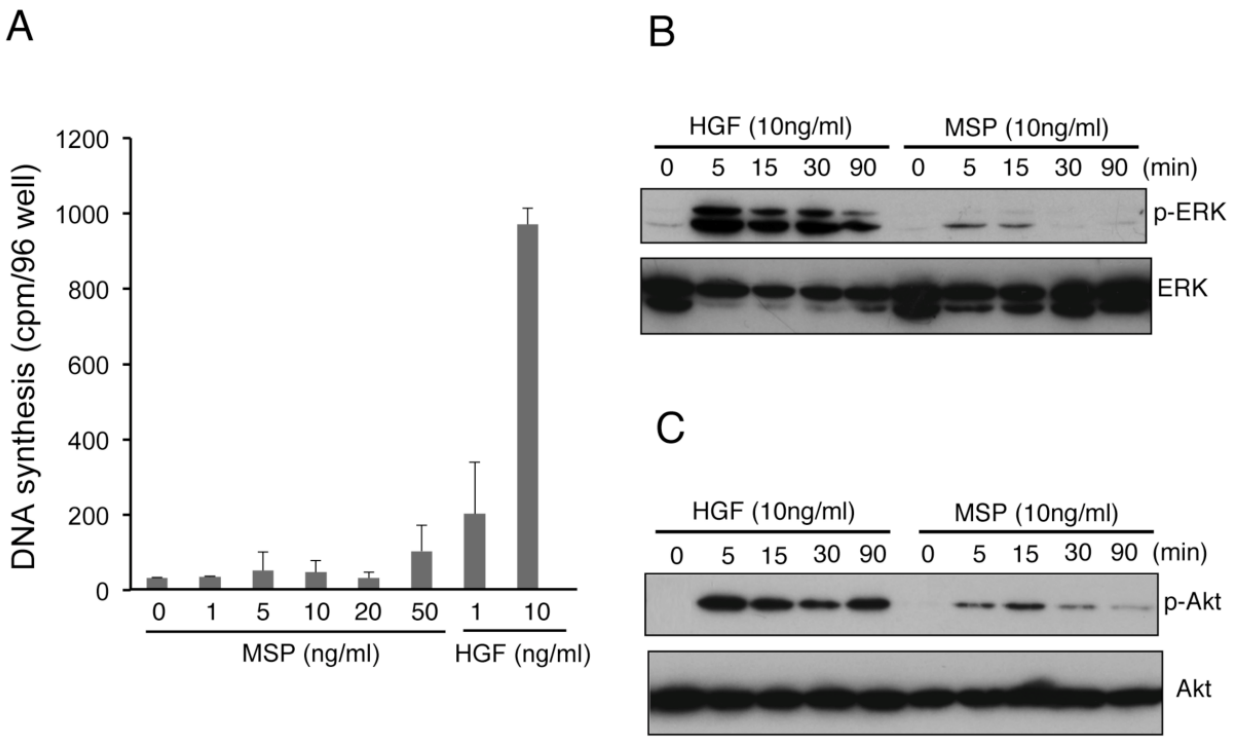

Figure 2

Little mitogenic activity of MSP-Ron in primary culture of hepatocytes. (A) Effects of MSP on DNA synthesis in the range of 2-50 ng/ml. 1 and 10 ng/ml of HGF were used as positive controls. Each value and vertical bar represents the mean ± S.D. of three wells. (B) Phosphorylation of ERK and Akt after MSP (10 ng/ml) and HGF (10 ng/ml) treatment at 5, 15, 30, and 90 min. Total ERK and Akt were used for loading control.

3.3. HGF induce Ron expression in the primary culture of hepatocyte

It was unknown whether HGF could regulate Ron expression. I observed that HGF addition induced Ron expression in the primary hepatocyte by immunoblot assay (Figure 3A). Moreover, HGF up-regulated *Ron* mRNA in hepatocytes (Figure 3B). In this study HGF-Met enhances Ron expression in the cultured hepatocytes (Figure 3C).

4. DISCUSSION

Through MSP binding, Ron activation leads to the trans-autophosphorylation of several intracellular C-terminal tyrosine residues. These phosphorylated tyrosine residues serve as high-affinity docking sites for effector proteins, including son of sevenless (SOS), Ras, PI3K, phospholipase C・ and growth factor receptor-bound 2 (Grb2). However, in the present study, I could not find the activation of MAP kinase (MAPK)-ERK and PI3K-Akt pathway. This fact is at least in part derived from the lower level of Ron expression in primary hepatocytes comparing with some kinds of cancers. Actually, some types of human tumor and cell lines show an increased expression of MSP and Ron (Leonis et al., 2007, Sugie et al., 2016, Wang et al., 2009, Logan-Collins et al., 2010). Therefore, MSP-Ron signaling was insufficient for activation MAPK-ERK and PI3K-Akt pathways, and therefore could not exhibit substantial mitogenetic activity in the normal hepatocytes.

Ron overexpression has been well-known in cancer cells (Leonis et al., 2007, Sugie et al., 2016). But it is still unclear what factor regulates Ron expression in normal cells. In the present study, I found that HGF up-regulated the Ron expression in primary culture of hepatocytes. HGF acts on broad roles in epithelial cells through Met receptor (Nakamura et al., 2010).However, it exists the question why HGF up-regulates the Ron expression. For example, HGF-Met and MSP-Ron signaling could play synergistically various

roles. Some reports suggest the correlation between Met and Ron. Cross-talk between Met and Ron often occurs in some cancer cells, and especially activation of HGF-Met signaling display activation of Ron (Benvenuti et al., 2011). In contrary, HGF-Met and MSP-Ron signalings may be modulated by relative levels of receptors and ligands (Zhao et al., 2013). HGF treatment of Ron knockdown cells caused an increase in intensity and duration of Met signaling, thus HGF-MET signaling may recover loss of MSP-RON signaling (Zhao et al., 2013). This finding could demonstrate that MSP-Ron signaling could be a negative regulator for HGF-Met signaling. Combined with the present finding, HGF might have the negative feedback mechanism via up regulating MSP-RON signaling for a negative feedback mechanismin normal hepatocytes.Further experiments are needed to clarify the importance of induced Ron on HGF-stimulated hepatocytes.

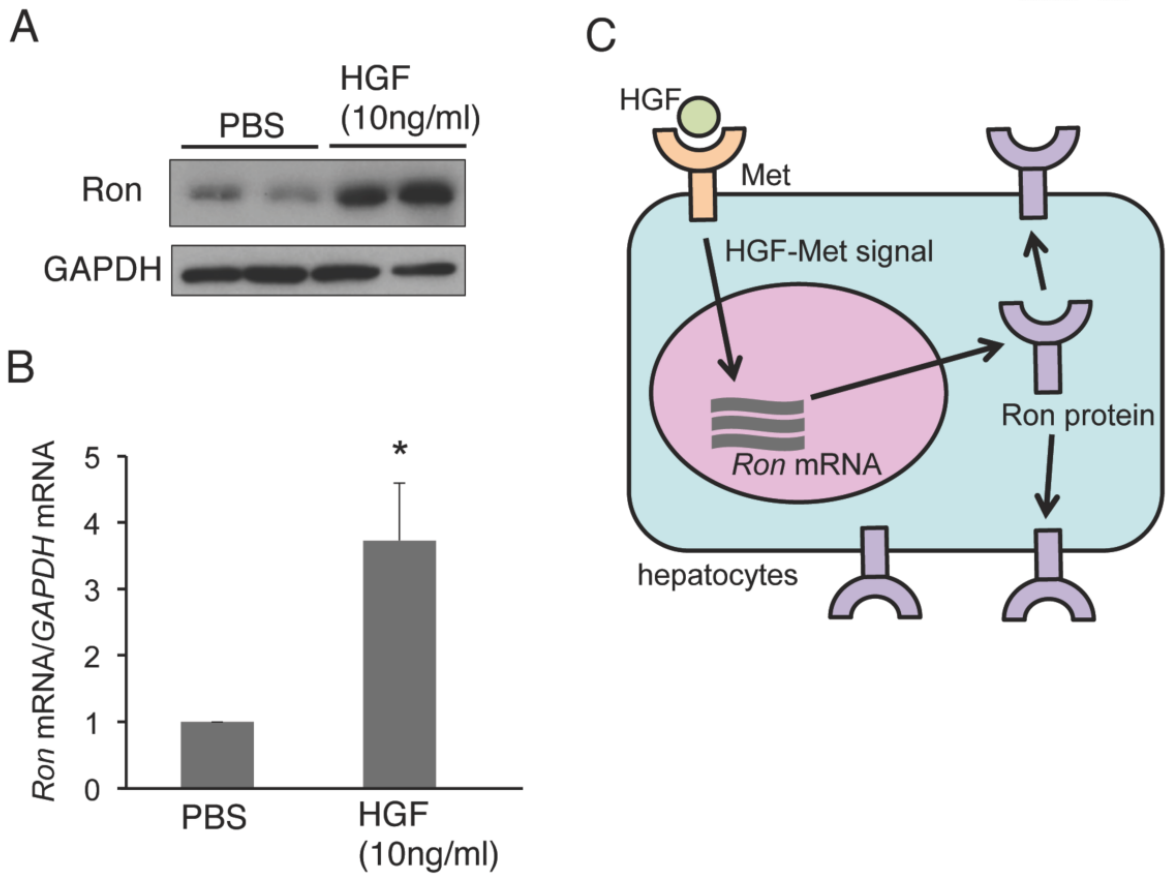

Figure 3

Ron expression induced by HGF treatment. (A) Immunoblotting for Ron expression in HGF-treated primary hepatocytes. (B) Real-time PCR for *Ron* mRNA in HGF-treated primary hepatocytes. Each value and vertical bar represents the mean ± S.D. of three samples. *P-values ≤ 0.05 are shown.

5. CONCLUSION

1.Primary cultured hepatocytes express Ron, MSP receptor.

2.MSP cannot sufficiently induce proliferation of primary cultured hepatocytes.

3.HGF might increase the Ron expression and thus enhance MSP-Ron signaling.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

FINANCIAL DISCLOSURES

None.

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