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Potentiation of invasive activity of hepatoma cells by reactive oxygen species is mediated by autocrine/paracrine loop of hepatocyte growth factor

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Abstract

We have already reported that reactive oxygen species (ROS) promote rat ascites hepatoma cell invasion beneath mesentery-derived mesothelial cell monolayer. To investigate the mechanism for this, we examined the involvement of motility factors, particularly hepatocyte growth factor (HGF). Rat ascites hepatoma cell line of AH109A expressed HGF and c-Met mRNAs. Treatment with ROS augmented amounts of HGF mRNA in AH109A and HGF concentration in the medium. ROS also induced HGF gene expression in mesothelial cells. Exogenously added HGF enhanced invasive activity of AH109A cells, but exerted no effect on proliferation. AH109A cells pretreated with ROS showed an increased invasive activity, which was cancelled by simultaneous pretreatment with anti-HGF antibody. These results suggest that the invasive activity of AH109A is mediated by the autocrine and paracrine pathways of HGF, and ROS potentiate invasive activity by inducing gene expression of HGF in AH109A and mesothelial cells.

Keywords: Invasion; Metastasis; Hepatocyte growth factor; Hepatoma; Reactive oxygen species; Autocrine action; Paracrine action; c-Met

Metastasis is a complex and sequential cascade of events leading to the dissemination of tumor cells to distant tissue sites. In many cases, metastasis may prove lethal in the course of clinical treatment of cancer. Invasion is the most important and characteristic event in tumor metastasis. Inhibition of invasion is thought to lead to the inhibition of metastasis. The precise mechanisms involved in tumor cell invasion are therefore the subject of intense scrutiny. Liotta et al. [1] reported that tumor cell invasion comprises three steps: adhesion of tumor cells to normal cell layer; motility of tumor cells; and degradation of extracellular matrices. Inhibition of any of these steps may effectively suppress invasion and inhibit metastasis.

We have been investigating the effects of food factors on tumor cell invasion by co-culturing rat ascites hepatoma cell line of AH109A with rat mesentery-derived mesothelial cells [2–4] and found that the anti-oxidative properties of food factors are crucial to anti-invasive activity. Food factors displaying anti-oxidative activity, suppressing the invasion of AH109A cells across mesothelial monolayers. In addition, the invasive activity of AH109A cells is potentiated by reactive oxygen species (ROS), with this potentiation cancelled by anti-oxidative food factors [3,4]. Tumor cells are known to produce larger amount of ROS than normal cells [5]. Although numerous reports have indicated the importance of ROS in tumor cell invasion, the precise mechanisms remain controversial. Niitsu and Plate [6] reported that ROS directly stimulate the intracellular signaling pathway in the invasion of 3LL or Meth-A cells. Yang et al. [7] reported a relationship between ROS and matrix metalloproteinases. On the other hand, Lam et al. [8] reported that overexpression of manganese superoxide dismutase (MnSOD) gene in hamster cheek pouch carcinoma cells resulted in increased invasiveness, while co-overexpression of MnSOD and catalase genes partially cancelled this increase. However, few studies have reported the effect of ROS on tumor cell motility factors.

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During the above-mentioned investigation, we noticed that ROS induce the expression of hepatocyte growth factor (HGF) gene and stimulate the autocrine action of HGF in invading AH109A cells. HGF, which is identical to scatter factor, is known to represent a multipotent growth factor predominantly secreted by mesenchymal cells [9,10]. HGF acts via its receptor, c-Met. Numerous tumor cells express HGF, c-Met or both, and the HGF-c-Met axis is thought to be deeply involved in tumor cell invasion and metastasis [11]. In fact, the effect of NK4, the antagonistic fragment of HGF, on tumor cells is under active investigation and is considered to be a promising anti-metastatic agent [12,13]. However, the relationship between ROS and HGF has yet to be elucidated. In this study, we report that ascites hepatoma cells express both HGF and c-Met genes and that ROS increase HGF secretion. We also report that ROS induce HGF gene expression in mesothelial cell monolayer, in which HGF mRNA is scarcely detectable under normal culture conditions. HGF secreted from both hepatoma and mesothelial cells may act synergistically on hepatoma cells, resulting in augmented invasiveness. This is the first report to describe the involvement of HGF in the potentiation of tumor cell invasion by ROS.

Materials and methods

Male Donryu rats were purchased from NRC Haruna (Gunma, Japan). Animals were treated in accordance with the guidelines established by the Animal Care and Use Committee of Tokyo Noko University. AH109A cells were obtained from the Institute of Development, Aging and Cancer, Tohoku University Sendai, Japan and maintained in the peritoneal cavity of Donryu rats, as described previously [2]. AH109A cells cultured in MEM (Nissui Pharmaceutical, Tokyo, Japan) containing 10% calf serum (JRH, Lenexa, KS) (10% CS/MEM) for at least 2 weeks after preparation from accumulated ascites were used for the following experiments.

The invasive activity of AH109A was assessed by co-culturing primary cultured rat mesentery-derived mesothelial cells (mesothelial cells) and AH109A cells, as described previously [2]. Briefly, mesothelial cells were isolated from rat mesenteries by trypsin digestion, seeded in 6-cm ∅ culture dishes with 2-mm grids (Corning, NY), and primarily cultured to the confluent state in 10% CS/MEM. AH109A cells were seeded onto mesothelial cell monolayers and the number of invaded cells and colonies were counted under phase contrast microscope after 24-h co-culture.

ROS were generated by adding both hypoxanthine (HX, 4 μg/ml, Sigma–Aldrich, Japan, Tokyo) and hypoxanthine oxidase (XO, 7×10^{-4} U/ml, Sigma–Aldrich, Japan, Tokyo). Pretreatment of AH109A cells by ROS was performed, as described previously [3]. Recombinant rat HGF and anti-rat HGF mouse monoclonal antibody were purchased from Toyobo (Osaka, Japan).

Amounts of HGF mRNA in AH109A, mesothelial cells, and L929 mouse fibroblasts (obtained from Riken Cell Bank, Tsukuba, Japan) were measured by RT-PCR according to the methods described by Neaud et al. [14]. The amount of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was also measured as an internal control. Briefly, total RNA prepared from AH109A cells, mesothelial cells, or L929 mouse fibroblasts using the AGPC method [15] was reverse transcribed using random primers (random 9-mers, Takara Shuzo, Shiga, Japan) by Superscript II (Invitrogen, Carlsbad, CA), and HGF or G3PDH cDNAs were amplified by LA Taq DNA polymerase (Takara Shuzo, Shiga, Japan) by HGFS primer (5′-TGATCCCCCA TGAACACAGC-3′)/HGFAS primer (5′-TGATGTCATGCTTGTG GGGG-3′) and G3PDHS primer (5′-GTTCAACGGCACAGTCAA GG-3′)/G3PDHAS primer (5′-GAGTGGCAGTGATGGCATGG-3′), respectively. The PCR (35 cycles) was as follows; 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min. Intensities of amplified cDNA fragments were measured using NIH Image 1.44 after agarose gel electrophoresis. Presence of c-Met mRNA in AH109A cells or mesothelial cells was also confirmed by RT-PCR using c-Met S primer (5′-TGTGCATTCCCCATCAAATA-3′) and c-Met AS primer (5′-AT GCTCAACAATAACTTCTG-3′). All PCR primers used in this study were designed based on reported sequences for cDNAs in GenBank.

Concentrations of HGF in medium were measured by ELISA according to standard procedures. Briefly, conditioned media were coated overnight to a 96-well plate (Maxisorp, Nalge Nunc International, Tokyo, Japan) and wells were blocked with 1% bovine serum albumin (Sigma–Aldrich, Tokyo, Japan) in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline (PBS, pH 7.6) for 2 h. After washing with 0.1% Tween 20/PBS, anti-rat HGF monoclonal antibody (1:1000 dilution) was added to each well and incubated for 2 h. After washing each well, the second antibody (biotin-conjugated anti-mouse IgG goat antibody, 1:1000 dilution, TAGO, Burlingame, CA) was added and incubated for further 2 h. Bound antibodies in each well were detected using streptavidin-POD conjugate (Boehringer–Mannheim GmbH, Germany) and TMB peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD) according to the manufacturer's instructions.

The tyrosine phosphorylation state of the c-Met receptor was investigated according to the methods described by Delédinghen et al. [16]. Briefly, cultured AH109A cells were collected and washed with ice-cold PBS. Cells were then lysed with RIPA buffer (1% Igepal CA-630, 0.5% sodium deoxycholate, and 0.1% SDS/PBS) containing protease and phosphatase inhibitors. The lysate (500 μg of cellular protein) was precleared with normal mouse IgG (sc-2025: Santa Cruz Biotechnology, Santa Cruz, CA) and protein A–Sepharose (Amersham Biosciences, Tokyo, Japan) for 1 h, then incubated with anti-c-Met monoclonal antibody (sc8057: Santa Cruz Biotechnology,) under rotation for 2 h at 4 °C. Immunoprecipitates were collected with protein A–Sepharose, washed four times with RIPA buffer, and eluted by boiling in 1× Laemelli's buffer. Immunoprecipitates were then run on 8% polyacrylamide gels and analyzed by immunoblotting with anti-phosphotyrosine monoclonal antibody (P-Tyr-100: Cell Signalling Technology, Beverly, MA). Blots were then reprobed with anti-c-Met antibody.

Intracellular peroxide levels in AH109A cells were assessed by flow cytometric analyses using a fluorometric probe (2′,7′-dichlorofluorescein diacetate; DCFH-DA, Molecular Probes, Eugene, OR) according to the methods described by Bass et al. [17] with EPICS ELITE EPS (Beckman–Coulter, Hialeah, FL). The proliferative activity of AH109A cells was determined using a Cell Counting Kit (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions.

Data were statistically analyzed by one-way analysis of variance followed by Tukey's Q test or Student's t test. Values of $P < 0.05$ were considered statistically significant.

Results and discussion

To clarify the involvement of motility factors in the potentiation of invasive activity by ROS, we first examined as to which motility factors are expressed in AH109A cells and mesothelial cells using RT-PCR techniques. Fig. 1 shows that AH109A cells express HGF mRNA and c-Met mRNA, and that mesothelial

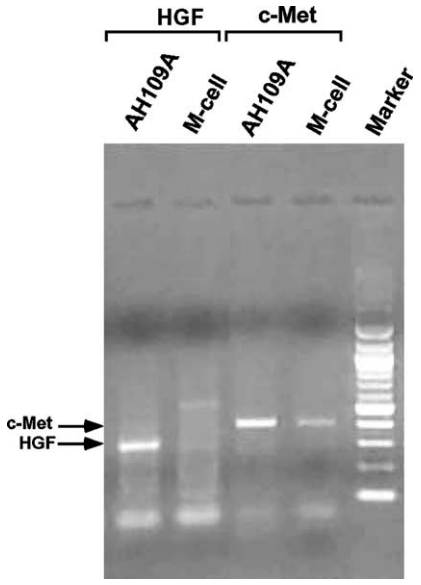

Fig. 1. Expression patterns of HGF and c-Met mRNA in AH109A and mesothelial cells. Total RNAs were prepared from AH109A cells and mesothelial cells. One microgram of total RNA was reverse transcribed, as described in Materials and methods and HGF or c-Met mRNAs were amplified using the respective primers. Amplified cDNAs were visualized by ethidium bromide staining after agarose gel electrophoresis. Arrows indicate amplified cDNA. A representative result is shown.

cells express c-Met mRNA. Mesothelial cells contained barely any HGF mRNA under normal culture conditions. AH109A and mesothelial cells did not express mRNA for autocrine motility factor or its receptor (data not shown). Warn et al. [18] recently reported that the HGF-c-Met system works in mesothelium in addition to the mesenchymal–epithelial axis and that the migration of mesothelial cells is mediated by HGF. Our results show that HGF mRNA is present in AH109A cells and that c-Met mRNA is present in both AH109A and mesothelial cells. This result is consistent with their results as mesothelial cells are mesothelial in origin.

As shown in Fig. 2, AH109A cells treated for 1 h by ROS generated by hypoxanthine (HX) and xanthine oxidase (XO) contain about 10-fold more HGF mRNA than controls (Figs. 2A and B) and secretion of HGF into medium also increased by 2-fold after 1–2 h treatment with ROS (Fig. 2C). ROS also induced gene expression of HGF in mesothelial cells, but showed no effect on the amount of HGF mRNA in L929 mouse fibroblasts (Fig. 2D). These results suggest that induction of HGF gene expression by ROS occurs cell-specifically. AH109A cells treated by ROS for 1 h contained more intracellular peroxides than control cells when analyzed by flow cytometer, using DCFH-DA as an indicator (Fig. 3). Although RT-PCR methods used in this study were not quantitative, exogenously added ROS must have entered into cells and induced expression of HGF, as the amount of secreted HGF increased in ROS-treated AH109A cells. ROS reportedly induce the expression of various genes. These genes include cell adhesion molecules [19] and matrix metalloproteinases [7,8,20], which are deeply involved in tumor cell motility and invasion. However, no reports have described ROS affecting the expression of HGF gene in both normal and tumor cells. This study is the first to indicate the involvement of the HGF-c-Met system in ROS-induced tumor cell motility and invasion. ROS are known to show biological effects on gene expression by activating NF-κB or AP-1 [21–23]. Most of the above-mentioned genes induced by ROS contain binding sites for NF-κB, AP-1, or both in their promoter regions. The HGF promoter region [24,25] does not contain obvious NF-κB binding site, but does

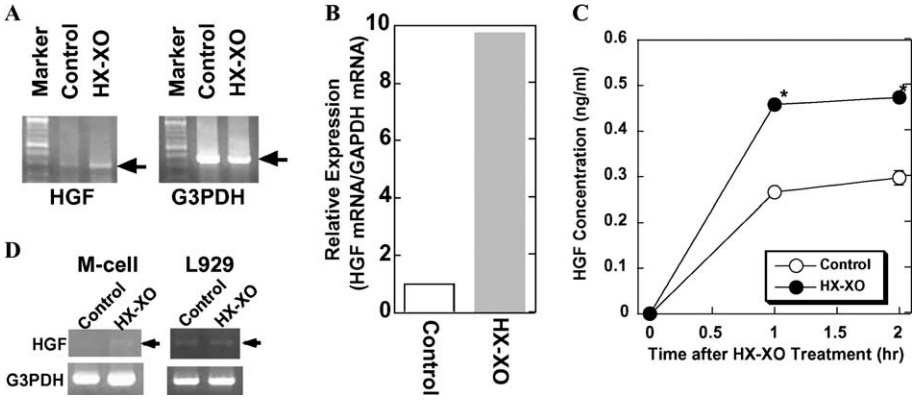

Fig. 2. Effects of ROS on amounts of HGF mRNA in AH109A cells, mesothelial cells (M-cells), and L929 fibroblasts and on secretion of HGF by AH109A cells. AH109A cells, M-cells, or L929 fibroblasts were cultured in the presence or absence of HX and XO for 1 h as described in Materials and methods. Total RNA was prepared and RT-PCR was performed. (A) Amplified cDNA fragments from AH109A cells were analyzed by agarose gel electrophoresis. (B) Relative amount of HGF mRNA (HGFmRNA/G3PDH mRNA) was calculated from the result of (A). (C) AH109A cells were cultured in the presence or absence of ROS for 1 or 2 h. Concentration of HGF in the conditioned medium was measured by ELISA. Data represent means ± SEM of four samples. An asterisk shows statistical significance ($P < 0.05$) compared to the corresponding control using Student's t test. (D) Amplified cDNA fragments from M-cells and L929 fibroblasts were analyzed by agarose gel electrophoresis. Three similar experiments were performed and provided identical results. A representative result is shown.

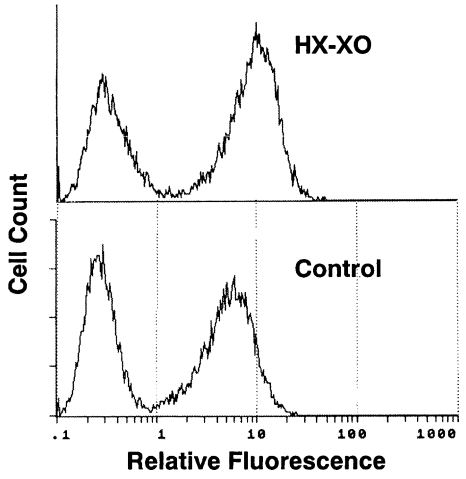

Fig. 3. Flow cytometric analyses of intracellular peroxide levels in AH109A cells. AH109A (1×10^6 cells) were cultured in the presence or absence of hypoxanthine and xanthine oxidase for 1 h and DCFH-DA (25 μM) was added followed by incubation for a further 20 min. Cells were collected and analyzed by flow cytometer. The first peaks in the histograms are cell debris and cells that cannot incorporate DCFH-DA. Although there is no difference in the first peaks between control cells and HX–XO-treated cells, cells incorporating DCFH-DA (second peaks) contained larger amounts of intracellular peroxide in HX–XO-treated cells than control cells. More than three similar experiments were done, providing identical results. A representative result is shown.

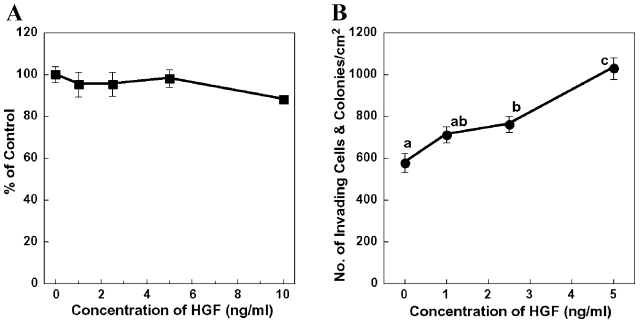

Fig. 4. Effect of exogenous HGF on proliferation and invasion of AH109A cells. (A) AH109A cells (5×10^4 cells) were cultured in the presence of the various concentrations of HGF indicated in the figure. After 20 h culture, WST-1 reagent was added and cells were further incubated for 4 h. Proliferation was assessed under spectrophotometry. Data indicated as percentage of control represent means ± SEM of 8 wells. (B) AH109A cells (2.5×10^5 cells) were overlaid onto confluent mesothelial cells monolayers cultured in a 6-cm ∅ culture dish with 2-mm grids in the presence of the various concentration of HGF indicated in the figure. After 24-h culture, invasive activity was assessed as previously described [2]. Data represent means ± SEM of 10 areas. [a,b,c] Values not sharing a common letter are significantly different ($P < 0.05$) by Tukey's Q test. A representative result of three similar experiments is shown.

contain AP-1 binding sites. At present, whether ROS induce HGF mRNA by activating AP-1 or other transcription factors remains unknown. In addition, ROS induced gene expression of HGF in a cell-specific manner. At present, no information is available regarding the mechanisms underlining cell specificity. The precise molecular mechanisms for the induction of HGF mRNA by ROS should be determined in future.

Exogenous HGF promotes the invasive activity of AH109A cells against mesothelial cell monolayer, but exerts no effect on proliferation of AH109A cells (Fig. 4). HGF reportedly induces invasion in various cancer cells and some cancer cells secrete HGF [26,27]. HGF was formerly thought to act in a paracrine manner. That is, HGF produced by mesenchymal cells induces motility in epithelial cells. However, recent reports suggest that some tumor cells can produce HGF and invade using self-secreted HGF—invading via the autocrine activity of HGF [26,27]. Our data suggest that AH109A cells also seem to invade mesothelial cell monolayers using HGF in an autocrine fashion. Moreover, ROS induced gene expression of HGF in mesothelial cells. HGF secreted by mesothelial cells may act on AH109A cells in a paracrine fashion and in synergy with HGF secreted by AH109A cells. However, the amount of HGF mRNA induced in mesothelial cells by ROS was very low compared to that in AH109A cells (Fig. 2). HGF secreted by mesothelial cells may therefore not contribute greatly to invasion by AH109A cells. In addition to this autocrine/paracrine activity in AH109A cells, HGF may affect mesothelial cell monolayers via c-Met receptors on their cell surface (Fig. 1). HGF potentiates the motility of AH109A cells and simultaneously induces the retraction of mesothelial cell monolayers, which may further accelerate the invasion of AH109A cells.

The potentiated invasive activity of AH109A cells pretreated with ROS for 4 h was completely cancelled by simultaneous treatment by anti-HGF antibody (Fig. 5). Pretreatment by both ROS and HGF (4 ng/ml) for 4 h showed no additive effect on invasion of AH109A and pretreatment by HGF and anti-HGF antibody did not affect invasion by AH109A cells (Fig. 5). These results clearly indicate that ROS induce expression of HGF, in turn inducing invasion by AH109A cells by binding to c-Met on their cell surface, and that anti-HGF antibody blocked the action of secreted HGF. In fact, c-Met receptors on AH109A cells pretreated with HX–XO were highly tyrosine phosphorylated compared with control and simultaneous treatment with HX–XO and anti-HGF antibody blocked this, while c-Met receptors in control cells were tyrosine phosphorylated because AH109A cells constitutively secrete HGF into medium (Fig. 5B). Numerous studies have reported that ROS potentiate the invasion of tumor cells [6,7,28]. In particular, Niitsu and Plate [6] reported that ROS affect the intracellular signaling pathway and induce activation of Rho and PKC, which induce morphological changes and motility in tumor cells. They did not mention the involvement of soluble factors in the induction of invasion by ROS. However, in our system, ROS stimulate the autocrine loop of HGF, resulting in the induction of

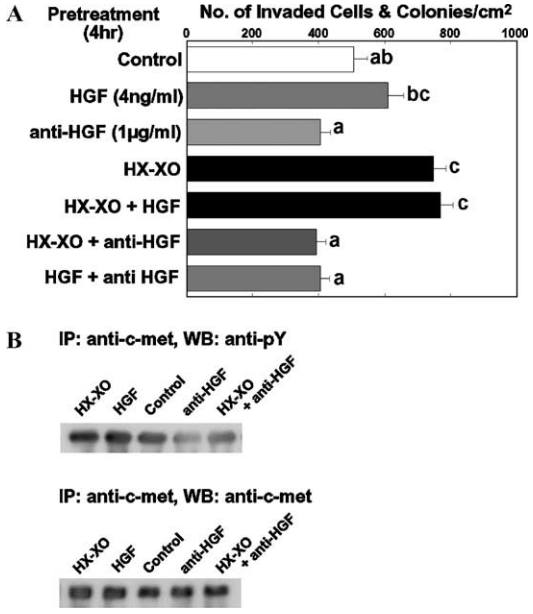

Fig. 5. Effect of anti-HGF antibody on ROS-potentiated invasive activity of AH109A cells. (A) AH109A cells (5×10^5 cells) were pretreated for 4 h as indicated in the figure. Cells were collected, washed, and overlaid onto confluently cultured mesothelial cells monolayers in 10% CS/MEM. After 24-h culture, invasive activity was assessed as previously described [2]. Each column and bar indicates mean ± SEM of 10 areas. [a,b,c] Values not sharing a common letter are significantly different ($P < 0.05$) by Tukey's Q test. A representative result of two similar experiments is shown. (B) AH109A cells (3×10^7 cells) were treated for 2 h as indicated in figure. Cells were collected, washed, and lysed. The phosphorylation state of c-Met receptor was analyzed, as described in Materials and methods. A representative result of two similar experiments is shown.

invasion. We cannot clearly explain the discrepancy between these results. However, we have already observed that B16F10 melanoma cells which secrete autocrine motility factor (AMF) contain greater amounts of AMF mRNA than controls, when treated by ROS (unpublished results). Thus, the effects of ROS on tumor cell motility factors may be a more general phenomenon, as tumor cells are known to produce large amounts of ROS compared to normal cells [5]. Recently, some drugs that augment intracellular ROS levels by inhibiting superoxide dismutases, were reported to have potential anti-tumor effects by inducing apoptosis of tumor cells with minimal side effects on normal cells [29]. Our results suggest that slight increases in intracellular levels of ROS may aggravate tumor by increasing invasive activity. Precise and intense evaluation of the effects of ROS on tumor cells should be carefully conducted in the near future.

Acknowledgments

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