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Blockade of endogenous reactive oxygen species by *N*-acetyl-L-cysteine suppresses the invasive activity of rat hepatoma cells by modulating the expression of hepatocyte growth factor gene

Yutaka Miura*, Saho Tsukamoto and Kazumi Yagasaki
*Department of Applied Biological Science, Tokyo Noko University, Fuchu, Tokyo 183-8509, Japan(*Author for correspondence: E-mail: eiyouym@cc.tuat.ac.jp; fax: +81-42-367-5713)*

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Abstract

We have already reported that exogenously added reactive oxygen species (ROS) could potentiate the invasive activity of rat hepatoma cell line of AH109A by activating autocrine loop of hepatocyte growth factor (HGF)-c-Met pathway. In this report, we examined the involvement of endogenous ROS in the invasive activity of hepatoma cells by using a cell-permeable antioxidant, *N*-acetyl-L-cysteine (NAC). NAC could certainly scavenge intracellular ROS when directly added to the media at the concentration of 1 or 5 mM and could significantly suppress hepatoma cell invasion, although it showed a little effect on hepatoma cell proliferation at these concentrations. NAC also decreased the content of HGF mRNA and the secretion of HGF at these concentrations, leading to suppression of their invasion. In the present study, blockade of endogenous ROS by NAC proved to efficiently suppress the invasive activity of hepatoma cells by down-regulating HGF gene expression, suggesting the importance of endogenous ROS in cellular signaling of tumor cell invasion.

Introduction

Reactive oxygen species (ROS) are very small, rapidly diffusible and highly reactive radicals and have recently been recognized to act as signaling intermediates for various physiological phenomena, for example, inflammatory diseases (Lo and Cruz, 1995; Rahman et al. 1998). ROS encompass species such as superoxide anion, hydrogen peroxide, hydroxyl radicals and nitric oxide (Halliwell and Gutteridge, 1990). Oxidative stress, that is, an excess production of ROS causes many pathological conditions. ROS also have numerous effects on cell functions including induction of growth, regulation of kinase activity and inactivation of nitric oxide (Larsson and Cerutti, 1988; Staal et al. 1994; Sundaresan et al. 1995). There are many ROS generating systems in cells, such as NADH/NADPH oxidase or xanthine oxidase (Ushio-Fukai et al., 1996). Inflammatory cytokines increase intracellular ROS level by activating these oxidative enzymes (Tiku et al. 1990; Takano et al. 2002). ROS are also generated in the course of regular metabolism, especially in the respiratory chain of mitochondria. Tumor cells are known to produce larger amounts of ROS than do normal cells, because they proliferate more rapidly than do normal cells (Szatrowski and Nathan, 1991). Therefore, ROS are thought to be deeply involved in tumor cell biology (Guyton and Kensler, 1993).

We have already reported that exogenously added ROS could potentiate the invasive activity of rat hepatoma cell line of AH109A, using co-culture system between AH109A cells and mesentery-

derived mesothelial cells (M-cells), and that food factors with antioxidative activities could cancel the potentiation of invasion by scavenging ROS (Kozuki et al. 2000; Kozuki et al. 2001). Recently we have reported that ROS activate the gene expression of hepatocyte growth factor (HGF) in AH109A cells and that secreted HGF activate the motility of hepatoma cells by binding c-Met receptor on their cell surface (Miura et al. 2003). These results suggest that autocrine pathway of HGF-c-Met was involved in AH109A cell invasion. However, in these papers, we only examined the effect of exogenously added ROS on the invasion of AH109A cells. The significance of endogenous ROS produced by AH109A cells in their invasion is not fully understood. In this report, we have investigated the role of endogenous ROS in hepatoma cell invasion by using a cell-permeable antioxidant, *N*-acetyl-L-cysteine (NAC). By decreasing the intracellular oxidative level by NAC, the invasive activity of AH109A cells was suppressed. At the same time, NAC down-regulated the gene expression of HGF. These results suggest the importance of endogenous ROS in hepatoma cell invasion and further support the possibility that antioxidative substances can be utilized as anti-invasive reagents.

Materials and methods

AH109A cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University, Sendai, Japan and maintained in the peritoneal cavity of Donryu rats (NRC Haruna, Gunma, Japan) as described previously (Miura et al. 1997). Animals were treated in accordance with the guidelines established by the Animal Care and Use Committee of Tokyo Noko University. AH109A cells cultured in MEM (Nissui Pharmaceutical Co., Tokyo, Japan) containing 10% calf serum (JRH, Lenexa, KS, USA) (10% CS/MEM) for at least 2 weeks after preparation from accumulated ascites were used for the following experiments.

The effect of NAC on the proliferation of AH109A cells were investigated by counting cell number by hemocytometer, after culturing cells for 24 h at the presence of various concentrations of NAC in 10% CS/MEM. The proliferative activities were indicated as percent of control.

The invasive activity of AH109A was assessed by co-culturing primary cultured rat mesentery-derived M-cells and AH109A cells, as described previously (Miura et al. 1997). Briefly, M-cells were isolated from rat mesenteries by trypsin digestion, seeded in 6 cm ϕ culture dishes with 2-mm grids (Corning, NY, USA) and primarily cultured to the confluent state in 10% CS/MEM. AH109A cells were seeded onto M-cell monolayers and the number of invaded cells and colonies were counted under phase contrast microscope after 24 h co-culture.

Amounts of HGF mRNA in AH109A cells were measured by RT-PCR as described previously (Miura et al. 2003). Briefly, total RNA prepared form AH109A cells was reverse transcribed using a random primer (random 9-mers, Takara Shuzo, Shiga, Japan) by Superscript^{TM} II (Invtirogen, Carlsbad, CA, USA), and HGF or G3PDH (as an internal control) cDNAs were amplified by LA Taq^{TM} DNA polymerase (Takara Shuzo Co. Ltd., Shiga, Japan). Intensities of amplified cDNA fragments were measured using NIH Image 1.44 after agarose gel electrophoresis.

Concentrations of HGF in medium were measured by ELISA as described previously (Miura et al. 2003). Briefly, conditioned media and HGF standards were coated overnight to a 96-well plate (Maxisorp^{TM}, Nalge Nunc International, Tokyo, Japan) and wells were blocked with 1% bovine serum albumin (Sigma–Aldrich Japan, Tokyo, Japan) in Ca^{2+}, Mg^{2+}-free phosphate buffered saline (PBS), pH 7.4 for 2 h. After washing with 0.1% Tween-20/PBS, anti-rat HGF monoclonal antibody (1:1000 dilution, Toyobo, Osaka, Japan) was added to each well and incubated for 2 h. After washing each well, biotin-conjugated anti-mouse IgG goat antibody (1:1000 dilution, TAGO, Burlingame, CA, USA) was added and incubated for further 2 h. Bound antibodies were detected using streptavidin-POD conjugate (Boehringer Mannheim GmbH, Germany) and TMB peroxidase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) according to the manufacturers' instructions.

Intracellular peroxide levels were assessed by flow cytometric analyses using a fluorometric probe (2′, 7′-dichlorofluorescin diacetate; DCFH-DA, Molecular Probes, Eugene, OR, USA) according to the method described by Bass et al.

(1983) with EPICS ELITE EPS (Beckman–Coulter, Hialeah, FL, USA).

Data were indicated as means ± SEM and statistically analyzed by one-way analysis of variance followed by Dunnett multiple comparisons test. Values of $p < 0.05$ were considered statistically significant.

Results and discussion

Figure 1a shows the effect of NAC on the proliferative activities of AH109A cells. When AH109A cells were cultured in the presence of 10 mM NAC, the proliferation significantly decreased. However, 1 or 5 mM NAC showed no or little effect on their proliferation. Flow cytometric analyses showed that NAC certainly decreased the intracellular peroxide level at these concentrations (Figure 1b). These results indicated that 1 or 5 mM NAC could be incorporated into cells and effectively block endogenous ROS without any effect on the proliferation of AH109A cells. Under these conditions, the invasive activities of AH109A cells were assessed. The invasive activities of AH109A cells were significantly suppressed at the dose of 1 or 5 mM NAC (Figure 2). Although NAC could not completely suppress the intracellular peroxide level because of its cytotoxicity at higher concentrations (Figure 1a and unpublished results), the invasive activity of AH109A cells seemed to sensitively respond to a slight decrease in endogenous ROS level (Figures 1b and 2). The blockade of endogenous ROS by inducing ROS scavenging enzymes, such as catalase or superoxide dismutases (SODs), was reported to affect tumor cell invasion (Lam et al. 1999). Our results also indicate the importance of endogenous ROS in hepatoma invasion. However, we could not identify which ROS is most important in signaling of hepatoma cell invasion at present. The induction or repression of catalase and/or SOD by transfecting these genes or antisense oligonucleotides will give more helpful information about this problem and should be done in near future.

As already mentioned, we have found the involvement of HGF in exogenous ROS-induced invasion (Miura et al. 2003). Therefore, we have

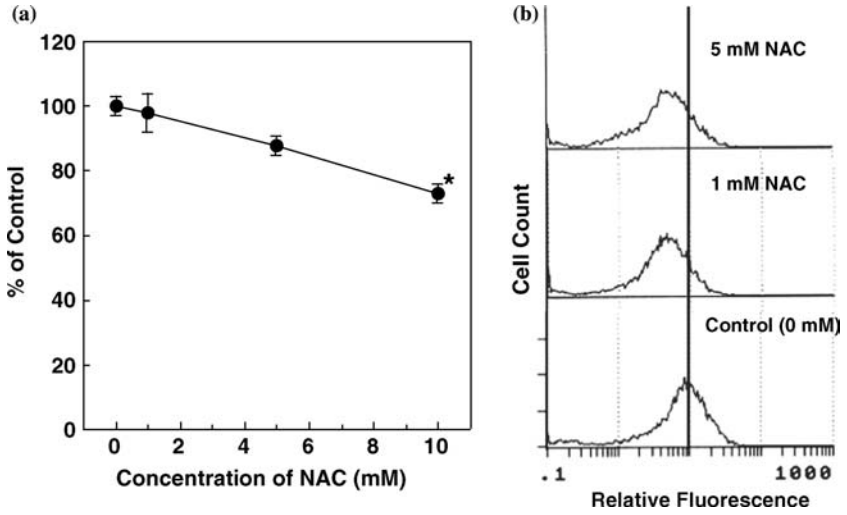

Figure 1. Effect of NAC on the proliferation and intracellular peroxide level in AH109A cells (a) AH109A cells (1×10^6 cells) were cultured in the presence of the various concentrations of NAC for 24 h. The proliferative activities of AH109A cells were assessed as described in Materials and methods. Data were indicated as percent of control (0 mM NAC). Data represent mean ± SEM for 4 wells. In the control group, the average cell number after 24 h incubation is $2.12 \pm 0.09 \times 10^6$ cells/well. An Asterisk indicates a statistical significance compared with control ($p < 0.05$). Three similar experiments were performed, and provided identical results. A representative result is shown. (b) AH109A (5×10^5 cells) were cultured in the presence of various concentration of NAC for 24 h and DCFH-DA (25 μM) was added followed by incubation for a further 20 min. Cells were collected and analyzed by flow cytometer. A vertical line in the figure is the peak position of control cells. More than three similar experiments were done, providing identical results. A representative result is shown.

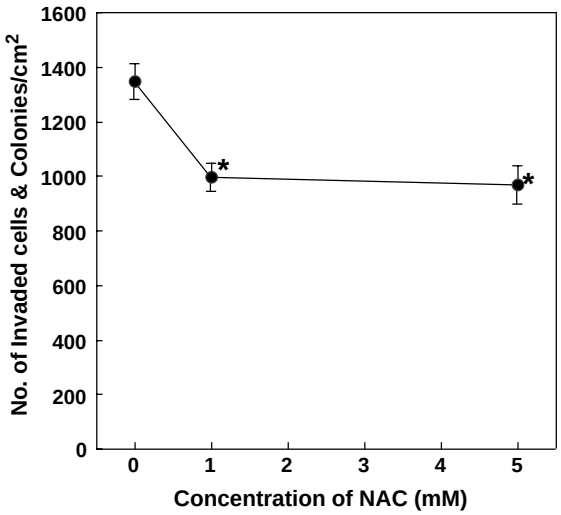

Figure 2. Effect of NAC on the invasion of AH109A cells AH109A cells (2.5×10^5 cells) were overlaid onto confluent M-cells monolayers cultured in a 6 cmϕ culture dish with 2-mm grids in the presence of the various concentration of NAC. After 24-h culture, invasive activity was assessed as described in Materials and methods. Data represent mean ± SEM of 10 areas. An asterisk indicates a statistical significance compared with control ($p < 0.05$). A representative result of two similar experiments is shown.

investigated the effect of NAC on the gene expression of HGF in AH109A cells. Figure 3a shows the result of semi-quantitative RT-PCR for HGF mRNA in AH109A cells cultured at the various concentrations of NAC. NAC dose-dependently decreased the relative content of HGF mRNA (Figure 3b). The secretion of HGF in the medium was also dose-dependently suppressed by NAC (Figure 3c). These results clearly indicate that NAC by its antioxidative activity down-regulated the gene expression of HGF in AH109A cells, leading to the suppression of HGF-c-Met autocrine pathway. They also indicate that endogenous ROS also stimulate the invasive activity of AH109A cells by activating this autocrine pathway. HGF reportedly induces invasion in various cancer cells, and some cancer cells secrete HGF (Rahimi et al. 1996; To and Tsao, 1998). 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

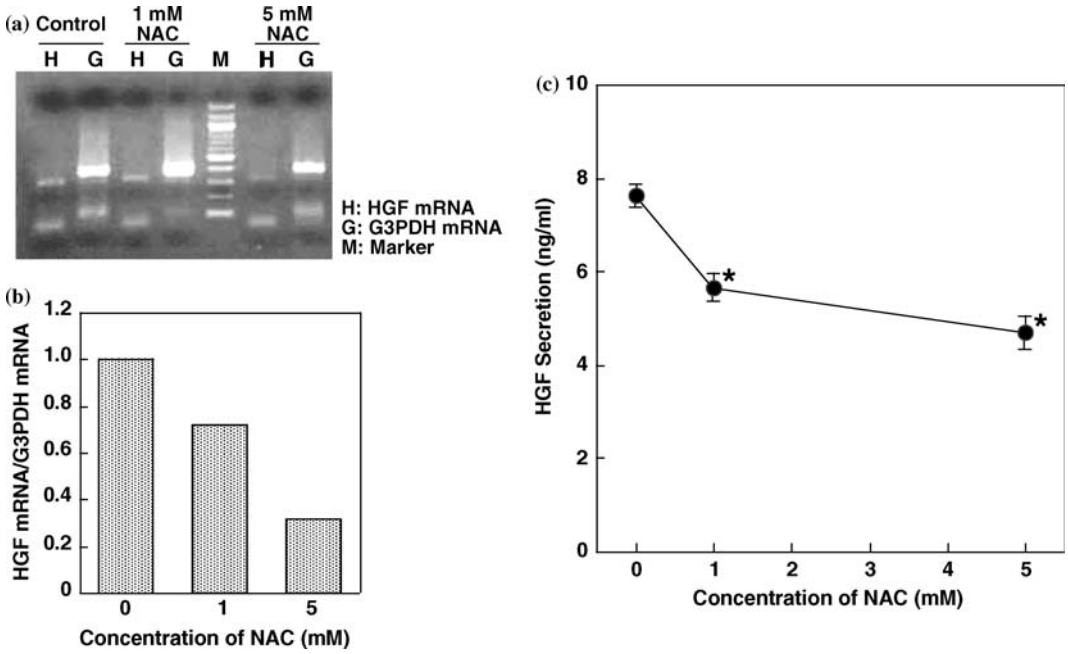

Figure 3. Effect of NAC on HGF mRNA content and secretion of HGF in AH109A cells (a) AH109A cells were cultured in the presence of various concentration of NAC for 4 h. Total RNA was prepared and RT-PCR was performed as described in Materials and methods. Amplified cDNA fragments from AH109A cells were analyzed by agarose gel electrophoresis. (b) Relative amount of HGF mRNA (HGF mRNA/G3PDH mRNA) was calculated from the result of (a). (c) AH109A cells were cultured in the presence of NAC for 4 h. Concentration of HGF in the conditioned medium was measured by ELISA. Data represent mean ± SEM of four samples. An asterisk shows statistical significance ($p < 0.05$) compared to control (0 mM NAC). Three similar experiments were performed, and provided identical results. A representative result is shown.

self-secreted HGF via the autocrine activity of HGF (Rahimi et al. 1996). AH109A cells also invade M-cell monolayers using HGF in an autocrine fashion and our data suggest that endogenous ROS play the important role in the regulation of HGF gene expression.

ROS are known to show their biological effects on gene expression by activating NF-κB or AP-1 (Abate et al. 1990; Schreck et al. 1991; Kim et al. 2002). Most of the genes induced by ROS contain binding sites for NF-κB, AP-1 or both in their promoter regions. The HGF promoter region (Liu et al. 1994; Plaschke-Schlutter et al. 1995) does not contain obvious NF-κB binding site, but does contain AP-1 binding sites. At present, whether endogenous ROS induce HGF mRNA by activating AP-1 or other transcription factors remains unknown. The precise molecular mechanisms for the induction of HGF mRNA by ROS should be determined in future.

In conclusion, we found that endogenous ROS were deeply involved in hepatoma cells invasion, by utilizing a cell-permeable antioxidant, NAC. These results and our previous results, that antioxidative food factors could effectively suppress tumor cell invasion, suggest the possibilities of antioxidative food components for the use of secondary prevention of tumor metastasis.

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References

Abate C., Patel L., Rauscher R.J. and Curran T. 1990. Redox regulation of fos and jun DNA-binding activity in vitro. Science 249: 1157–1161.

Bass D.A., Parce J.W., Dechatelet L.R., Szejda P., Seeds M.C. and Thomas M. 1983. Flow cytometric studies of oxidative product formation by neutrophils: a graded response to membrane stimulation. J. Immunol. 130: 1910–1917.

Guyton K.Z. and Kensler T.W. 1993. Oxidative mechanisms in carcinogenesis. Brit. Med. Bull. 49: 523–544.

Halliwell B. and Gutteridge J.M. 1990. Role of free radicals and catalytic metal ions in human disease: an overview. Methods Enzymol. 186: 1–85.

Kim H.J., Jung K.J., Yu B.P., Cho C.G., Choi J.S. and Chung H.Y. 2002. Modulation of redox-sensitive transcription factors by calorie restriction during aging. Mech. Ageing Dev. 123: 1589–1595.

Kozuki Y., Miura Y. and Yagasaki K. 2000. Inhibitory effects of carotenoids on the invasion of rat ascites hepatoma cells in culture. Cancer Lett. 151: 111–115.

Kozuki Y., Miura Y. and Yagasaki K. 2001. Resveratrol suppresses hepatoma cell invasion independently of its antiproliferative action. Cancer Lett. 167: 151–156.

Lam E.W.N., Zwecka R., Seftor E.A., Nieva D.R.C., Davidson B.L., Engelhardt J.F., Hendrix M.J.C. and Oberley L.W. 1999. Effects of antioxidant enzyme overexpression on the invasive phenotype of hamster cheek pouch carcinoma cells. Free Radical Biol. Med. 27: 572–579.

Larsson R. and Cerutti P. 1988. Oxidants induce phosphorylation of ribosomal protein S6. J. Biol. Chem. 263: 17452–17458.

Liu Y., Michalopoulos G.K. and Zarnegar R. 1994. Structural and functional characterization of the mouse hepatocyte growth factor gene promoter. J. Biol. Chem. 269: 4152–4160.

Lo Y.Y. and Cruz T.F. 1995. Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes. J. Biol. Chem. 270: 11727–11730.

Miura Y., Shiomi H., Sakai F. and Yagasaki K. 1997. Assay systems for screening food components that have anti-proliferative and anti-invasive activity to rat ascites hepatoma cells: in vitro and ex vivo effects of green tea extract. Cytotechnology 23: 127–132.

Miura Y., Kozuki Y. and Yagasaki K. 2003. Potentiation of invasive activity of hepatoma cells by reactive oxygen species is mediated by autocrine/paracrine loop of hepatocyte growth factor. Biochem. Biophys. Res. Commun. 305: 160–165.

Plaschke-Schlutter A., Behrens J., Gherardi E. and Birchmeier W. 1995. Characterization of the scatter factor/hepatocyte growth factor gene promoter; Positive and negative regulatory elements direct gene expression to mesenchymal cells. J. Biol. Chem. 270: 830–836.

Rahimi N., Tremblay E., McAdam L., Park M., Schwall R. and Elliott B. 1996. Identification of a hepatocyte growth factor autocrine loop in a murine mammary carcinoma. Cell Growth Differ. 7: 263–270.

Rahman A., Kefer J., Bando M., Niles W.D. and Malik A.B. 1998. E-selectin expression in human endothelial cells by TNF-α-induced oxidant generation and NF-κB activation. Am. J. Physiol. 275: L533–L544.

Schreck R., Rieber P. and Baeuerle P.A. 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. EMBO J. 10: 2247–2258.

Staal F.J., Anderson M.T., Staal G.E., Herzenberg L.A., Gitler C. and Herzenberg L.A. 1994. Redox regulation of signal transduction: tyrosine phosphorylation and calcium influx. Proc. Natl. Acad. Sci. USA 91: 3619–3622.

Sundaresan M., Yu Z.X., Ferrans V.J., Irani K. and Finkel T. 1995. Requirement for generation of H_2O_2 for platelet-derived growth factor signal transduction. Science 270: 296–299.

Szatrowski T.P. and Nathan C.F. 1991. Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res. 51: 794–798.

Takano M., Meneshian A., Sheikh E., Yamakawa Y., Wilkins K.B., Hopkins E.A. and Bulkley G.B. 2002. Rapid upregulation of endothelial P-selectin expression via reactive oxygen species generation. Am. J. Physiol. Heart. Circ. Physiol. 283: H2054–H2061.

Tiku M.L., Liesch J.B. and Robertson F.M. 1990. Production of hydrogen peroxide by rabbit articular chondrocytes. Enhancement by cytokines. J. Immunol. 145: 690–696.

To C.T. and Tsao M.S. 1998. The role of hepatocyte growth factor/scatter factor and met receptor in human cancers. Oncol. Rep. 5: 1013–1024.

Ushio-Fukai M., Zafari M., Fukui T., Ishizaka N. and Griendling K.K. 1996. p22phox is a critical component of the superoxide-generating NADH/NADPH oxidase system and regulates angiotensin II-induced hypertrophy in vascular smooth muscle cells. J. Biol. Chem. 271: 23317–23321.