Transactivation of Epidermal Growth Factor Receptor Is Involved in Leptin-Induced Activation of Janus-Activated Kinase 2 and Extracellular Signal–Regulated Kinase 1/2 in Human Gastric Cancer Cells

Dai Shida, Joji Kitayama, Ken Mori, Toshiaki Watanabe, and Hirokazu Nagawa

Department of Surgical Oncology, University of Tokyo Graduate School of Medicine, Tokyo, Japan

Abstract

Leptin is known to act as a growth factor through the Janus-activated kinase (JAK)/signal transducer and activator of transcription signaling pathway as well as the mitogen-activated protein kinase pathway. In this study, we showed a novel signal transduction pathway using two human gastric cancer cell lines, MKN28 and MKN74. Both gastric cancer cells expressed leptin and its receptors (Ob-R) at the protein level. We found that leptin, even at as low as 0.1 ng/mL, induced significant tyrosine phosphorylation of epidermal growth factor receptor (EGFR). Time-course experiments revealed that phosphorylation was maximal after 5 minutes of stimulation and declined thereafter. We also revealed that tyrosine phosphorylation of EGFR induced by leptin was significantly attenuated by two inhibitors, an EGFR tyrosine kinase inhibitor, AG1478, and a broad-spectrum matrix metalloproteinase inhibitor, GM6001. This indicates that the pathway of EGFR transactivation induced by leptin is dependent on proteolytically released EGFR ligands. Leptin induced JAK2 activation and extracellular signal–regulated kinase (ERK) 1/2 activation in these gastric cancer cells, both of which occurred after the peak of EGFR transactivation. Pretreatment of gastric cancer cells with AG1478 significantly reduced the degree of phosphorylation of both JAK2 and ERK1/2. These findings indicate the involvement of EGFR transactivation in the activation of JAK2 and ERK1/2. Our results reveal that EGFR transactivation is involved in the leptin signaling pathway in gastric cancer cells, which extends the physiologic action of leptin beyond its central effects in the hypothalamus to regulate body weight. (Cancer Res 2005; 65(20): 9159-63)

Introduction

Epidemiologic studies have indicated that adiposity contributes to increased incidence and/or death from various cancers, such as breast, colon, prostate, kidney, and esophagus, and it has been estimated that 15% to 20% of all cancer deaths in the United States can be attributed to overweight and obesity (1). These facts strongly suggest that adipocyte-derived factors may play positive roles in tumor progression. Leptin, the 16 kDa protein product of the obese gene, is a hormone with multiple biological actions. The main role of leptin has been thought to be the homeostasis of body weight by regulating food intake and energy expenditure via hypothalamic-mediated effects. However, recent studies have revealed that leptin stimulates the proliferation of various cell types, leading to consideration of this protein as a novel growth factor (2). Leptin exerts its action through the leptin receptor, termed Ob-R, which bears strong sequence homology to the interleukin 6 (IL-6)-type class 1 cytokine receptor superfamily. It consists of a single membrane-spanning protein that, through alternative mRNA splicing, exists in several isoforms. Ob-R is highly expressed in the hypothalamus but is also found in many peripheral tissues. For example, Ob-R is detected in normal gastric mucosa, especially in chief cells and parietal cells, and in some gastric cancer cells (3, 4). Furthermore, in addition to adipocytes and the placenta, the gastric mucosa has been shown to produce a significant amount of leptin (5). These reports indicate that leptin has some pathophysiologic functions in normal and malignant gastric tissues. Leptin receptors lack intrinsic kinase activity. Hence, their association with intracellular Janus-activated kinase 2 (JAK2) is crucial for activation of downstream signaling molecules (6). A second intracellular pathway recruited by Ob-R is the mitogen-activated protein kinase (MAPK) cascade (6). However, other leptin-induced signal transduction pathways and the interaction of these pathways remain largely unknown. Cross talk between different members of receptor families has become a well-established concept in signal transduction. Tyrosine phosphorylation of epidermal growth factor receptor (EGF) receptor (EGFR) in response to activation of many G protein-coupled receptors (GPCR), which was designated “trans-activation,” has been shown to have important physiologic consequences and has received considerable attention in recent years. EGFR has been shown to be phosphorylated by various ligands for GPCR such as lysophosphatidic acid and thrombin (7). In addition to various ligands of GPCR, growth hormone and prolactin, of which receptors are members of the IL-6 receptor family of class 1 cytokine receptors, have been reported to induce EGFR tyrosine phosphorylation (8). This led us to design experiments to investigate whether the leptin signal can interact with the EGFR signal in human gastric cancer cells. Then, we investigated the molecular mechanism of EGFR transactivation induced by leptin and the contribution of EGFR tyrosine kinase activity to the JAK2 signaling pathway as well as the extracellular signal–regulated kinase (ERK) signaling pathway in leptin stimulation.

Materials and Methods

Materials. Human recombinant leptin was purchased from Sigma Chemical Co. (St. Louis, MO). An EGFR tyrosine kinase inhibitor, AG1478,
was purchased from Biomol, Inc. (Plymouth Meeting, PA). Recombinant human EGF was purchased from PeproTech (London, United Kingdom). Mouse anti-human phosphotyrosine monoclonal antibody (mAb; PY20), mouse anti-erb2 mAb, and rabbit anti-human phospho-JAK2 polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The broad-spectrum matrix metalloproteinase (MMP) inhibitor GM6001 was purchased from Calbiochem (La Jolla, CA). Rabbit anti-phospho-ERK1/ERK2 polyclonal antibody was purchased from R&D Systems, Inc. (Minneapolis, MN).

**Cell culture.** The human gastric cancer cell lines MKN28 and MKN74 were obtained from the Riken Cell Bank (Tsukuba, Japan). MKN28 and MKN74 are cell lines established from moderately differentiated adenocarcinomas. These cells were maintained in DMEM supplemented with 10% FCS (Sigma), 100 units/mL penicillin, and 100 μg/mL streptomycin (Life Technologies, Inc., Grand Island, NY).

**Proliferation assay.** Proliferation assays were done as previously described (9). Briefly, MKN28 cells (5 x 10^4 cells in 100 μL per well) were seeded in a 96-well plate in DMEM containing 0.1% fatty acid–free bovine serum albumin and cells were subjected to 30 minutes of preincubation with either DMSO (control) or 250 nmol/L AG1478 before stimulation as previously described (10). Then, cells were stimulated with various concentrations of leptin, which was added every 24 hours. After incubation at 37°C and 5% CO2 for 120 hours, the number of living cells was measured using an MTS assay (Promega Co., Madison, WI) according to the instructions of the manufacturer. Briefly, MTS solution was added to each well and the cells were further incubated for 3 hours. The number of living cells was determined by measuring absorbance at 490 nm.

**Immunoprecipitation and Western blot analysis.** Immunoprecipitation and Western blot analysis were done as previously described (11, 12). In brief, MKN28 and MKN74 cells were grown to 80% to 90% confluence in 10-cm dishes. These cells were serum starved for 24 hours and then leptin or EGF was added to the culture thereafter. After stimulation of starved cells with leptin for various times, cellular protein lysates (1 mL/dish/tube) were obtained and then all proteins were incubated with antibodies against EGFR (20 μL/tube). Immunoprecipitates were collected with protein A-agarose. Immunoprecipitated proteins were electrophoresed in SDS-7.5% polyacrylamide gel for 35 minutes at 200 V. Then, the protein was transferred onto an Immobilon transfer membrane (Millipore Co., Bedford, MA) for sequential incubation with 5% reconstituted nonfat milk powder to block nonspecific sites, dilutions of mouse anti-phosphotyrosine mAb, and then horseradish peroxidase–labeled sheep anti-mouse immunoglobulin G (Amersham, Inc., Buckinghamshire, United Kingdom). Some cells were pretreated with 250 mmol/L AG1478 or 25 μmol/L GM6001 for 30 minutes before stimulation. All membranes were stripped and immunoblotted with antibodies against EGFR as a control.

**Janus-activated kinase 2 and extracellular signal–regulated kinase phosphorylation.** Cell lysates were electrophoresed in SDS-7.5% polyacrylamide gel for 35 minutes (for detection of Ob-R and JAK2) or SDS-15% polyacrylamide gel for 45 minutes at 200 V (for detection of leptin and ERK). Then, Western blot analysis was done as described above.

**Results**

**Leptin induced tyrosine phosphorylation of epidermal growth factor receptor in human gastric cancer cells.** First, we examined whether human gastric cancer MKN28 and MKN74 cells express Ob-R by Western blot analysis. As shown in Fig. 1A, both cells highly expressed the long and short forms of Ob-R. They also expressed a significant level of leptin receptor (Fig. 1B).

Then, we investigated whether exogenously added leptin trans-activates EGFR in human gastric cancer cells. MKN28 and MKN74 cells were incubated with 100 ng/mL leptin for 2 to 60 minutes, and tyrosine phosphorylation of EGFR was examined. As shown in Fig. 2A, leptin induced significant tyrosine phosphorylation of EGFR in both cell types. The degree of phosphorylation induced by leptin was as great as that induced by 1 ng/mL EGF although it was less than that induced by 10 ng/mL EGF (Fig. 2C). Time-course experiments revealed that phosphorylation was maximal after 5 minutes of stimulation and declined thereafter, suggesting that leptin induced rapid and transient tyrosine phosphorylation of EGFR. This time course of EGFR phosphorylation is similar to that induced by GPCR ligands previously reported. The amount of EGFR immunoprecipitation was the same with or without leptin (Fig. 2D). In reverse experiments, phosphotyrosine immunoprecipitation and EGFR immunoblotting revealed the same results (Fig. 2E).

We next examined the degree of EGFR phosphorylation using various concentrations of leptin. As shown in Fig. 2C, leptin significantly induced tyrosine phosphorylation of EGFR even at as
low as 0.1 ng/mL and the phosphorylation of EGFR appeared to plateau with a leptin concentration of 100 to 1,000 ng/mL in MKN28 cells, in clear contrast to EGFR activation by EGF, which showed a dose-dependent increase (data not shown).

Leptin induced proliferation of human gastric cancer cells at higher concentration. Leptin is known to be a growth factor for gastrointestinal cells as well as these cancer cells (2, 3). We next examined the effects of leptin on proliferation of MKN28 cells measured by MTS assay using 5 to 5,000 ng/mL leptin. Leptin at 5 to 500 ng/mL showed no significant proliferative effect on MKN28 cells (Fig. 1C). In contrast, a much higher concentration of leptin (5,000 ng/mL) significantly induced proliferation of MKN28 cells (120 ± 3%, P < 0.001; Fig. 1C).

Figure 3. Tyrosine phosphorylation of EGFR in response to leptin in human gastric cancer cells. A, human gastric cancer MKN28 and MKN74 cells were serum starved for 24 hours, pretreated with 250 nmol/L AG1478, 25 μmol/L GM6001, or an equal volume of vehicle (DMSO) for 30 minutes, and stimulated for 5 minutes with 100 ng/mL leptin or 10 ng/mL EGF. After cell lysis, EGFR was immunoprecipitated using anti-EGFR mAb and immunoprecipitates were immunoblotted with anti-phosphotyrosine mAb. Then the membrane was stripped and immunoblotted with anti-EGFR to detect EGFR at M, 170 kDa as a control. B, human gastric cancer MKN28 cells were serum starved for 24 hours, pretreated with 10 to 100 μmol/L GM6001 or an equal volume of vehicle (DMSO) for 30 minutes, and stimulated for 5 minutes with 100 ng/mL leptin. After cell lysis, EGFR was immunoprecipitated using anti-EGFR mAb and immunoprecipitates were immunoblotted with anti-phosphotyrosine mAb. Then the membrane was stripped and immunoblotted with anti-EGFR to detect EGFR at M, 170 kDa as a control.

Transactivation of epidermal growth factor receptor was dependent on epidermal growth factor receptor tyrosine kinase activity and metalloproteinase function in gastric cancer cells. To elucidate the mechanisms of transactivation, we did several experiments using specific inhibitors. The EGFR inhibitor AG1478 (250 nmol/L) markedly inhibited leptin-induced tyrosine phosphorylation of EGFR in both MKN28 and MKN74 cells (Fig. 3A). EGFR transactivation induced by GPCR ligands is also known to require MMP activation, which results in cleavage of the membrane-anchored growth factor precursor pro-HB-EGF in some cells (10, 13). We next examined the effect of the MMP inhibitor GM6001. As shown in Fig. 3A, leptin-induced tyrosine phosphorylation of EGFR at the 5-minute time point was almost totally abolished by 30-minute preincubation with GM6001 (25 μmol/L) in both cell types. The inhibition by GM6001 showed clear dose dependency (Fig. 3B).

Epidermal growth factor receptor was implicated in leptin-induced Janus-activated kinase 2 signaling pathway and extracellular signal–regulated kinase pathway in gastric cancer cells. The JAK/signal transducer and activator of transcription signaling pathway plays a critical role in mediating the effects of many cytokines and growth factors, and activation of the ERK/MAPK pathway is a key step in the regulation of important cellular responses such as cell proliferation. Leptin is known to induce JAK2 and ERK1/2 activation. We therefore
investigated the effect of leptin on both JAK2 and ERK activity in MKN28 cells. We revealed that leptin (100 ng/mL) induced JAK2 and ERK activation, both of which were significantly detected as early as 7.5 minutes after stimulation and peaked within 15 minutes (Fig. 4A). Thus, activation of both molecules occurred after the peak of EGFR transactivation, suggesting the possibility that intrinsic EGFR tyrosine kinase activity plays a role in JAK2 and ERK activation induced by leptin. Moreover, AG1478 (250 nmol/L) partially, but significantly, inhibited activation of both in MKN28 cells (Fig. 4B). Taken together, these results imply that EGFR transactivation acts upstream of JAK2 and ERK in leptin-induced signal pathways.

**Discussion**

Various studies have shown a critical role of interreceptor cross talk between EGFR and GPCRs (7, 14). In this study, we showed that leptin can induce transactivation of EGFR in human gastric cancer cells. The phosphorylation of EGFR induced by leptin reached a maximum in a few minutes and declined thereafter. Moreover, the phosphorylation was inhibited by the MMP inhibitor GM6001, suggesting that leptin rapidly induces proteolytic cleavage of transmembrane precursors, such as pro-HB-EGF, via MMP activation. Both the time course and MMP dependency were mostly consistent with the observation of EGFR phosphorylation induced by GPCR ligands.

The time course of EGFR phosphorylation induced by leptin was mostly consistent with the observation of EGFR phosphorylation induced by various ligands of GPCRs such as lysophosphatidic acid and endothelin-1 (7, 10–13). It is certainly an interesting question why this EGFR phosphorylation peaks within 5 minutes and then disappears, although a precise explanation has not been proposed. As for EGF itself, it is well known that addition of EGF induced rapid and also transient activation of EGFR (15, 16). In that case, Sturani et al. (15) showed that only about 10% of cellular EGFRs are phosphorylated on tyrosine on binding to the specific ligand and that phosphorylated EGFR is rapidly dephosphorylated by phosphatases, a process in which protein kinase C plays a significant role. They also revealed that removal of bound EGF leads to rapid receptor dephosphorylation, indicating, on one hand, that the autokinase activity in vivo is exclusively associated with the EGF-receptor complex and, on the other hand, that the phosphorylated form is largely associated with the cell surface even after long exposure to EGF (15). From these standpoints, the well-established concept of EGFR transactivation induced by GPCR ligands is that MMP-dependent cleavage of EGFR ligands induced on activation of GPCRs is not only rapid but also transient (13). This means that the proteolytically released EGFR ligands only occur transiently. From our results, the same mechanism can be considered to work in leptin-induced EGFR transactivation. As a result, leptin phosphorylates EGFR within 5 minutes and then disappears. This mechanism remains an interesting research target but is beyond the scope of this study.

As shown in Fig. 3A, an MMP inhibitor inhibited EGFR activation induced by leptin, which is the same as the phenomenon observed in EGFR transactivation induced by various ligands of GPCRs, indicating that EGFR transactivation induced by leptin is also dependent on the proteolytically released EGFR ligands. Thus, it is suggested that these leptin-induced responses occur in a paracrine manner (i.e., in the cellular microenvironment). On the other hand, we could not detect leptin-induced release of EGF into the cell culture medium after 5 to 60 minutes of stimulation with leptin (0.1-5,000 ng/mL), measured by a human EGF ELISA kit (Quantikine, R&D Systems; data not shown). The discrepancy between our findings of MMP dependency and the result that EGF could not be detected in the cell culture medium on leptin stimulation may be because on proteolytic processing, EGFR ligands may remain associated with the heparan sulfate proteoglycan matrix before interaction with their high-affinity receptors (13) and because it may occur only in the cellular microenvironment.

In addition to GPCR ligands, growth hormone and prolactin, of which receptors are members of the IL-6 receptor family of class I cytokine receptors, have been reported to induce EGFR phosphorylation (8). In that study, EGF was shown to be directly phosphorylated by JAK2, which couples cytokine stimulation to MAPK activation and c-fos gene transcription via the transactivated EGFR. In our time-course experiments, however, leptin first induced transactivation of EGFR and then subsequently induced JAK2 and ERK activation. Moreover, blockade of EGFR activation by AG1478 significantly reduced the phosphorylation of JAK2 and ERK. We also confirmed that pretreatment of cells with a
JAK2 inhibitor, AG-490, did not induce any significant effect on leptin-induced EGFR transactivation (data not shown). From these findings, we conclude that the mechanism of leptin-induced EGFR transactivation is not dependent on JAK2.

This indicates some discrepancy between the mechanism of EGFR transactivation induced by leptin and that induced by both growth hormone and prolactin, although all belong to the class I cytokine receptors. In the same way as GPCR ligands induced EGFR transactivation, which is proposed to be mediated by both extracellular and intracellular processes, these members of the cytokine family may induce EGFR transactivation via several pathways such as MMP activation and JAK2 activation, although the contribution of each pathway may differ with the ligand.

EGFR has been implicated in ERK activation induced by various GPCR ligands such as lysophosphatidic acid (7, 17). In addition, EGFR signals can also induce JAK2 activation (18). Our results were compatible with these previous reports and implied that in various signaling pathways induced by leptin, EGFR transactivation acts upstream of both the JAK2 and ERK signaling pathways.

There may be a question why exogenous treatment with leptin is needed for EGFR transactivation whereas gastric cancer cells constitutively express leptin, as shown in Fig. 1. A novel signal transduction pathway is induced by leptin. Leptin, at physiologic concentrations, transactivates the EGF receptor via MMP activation, which is involved in activation of the JAK2 and ERK pathways in gastric cancer cells (20). This indicates that leptin may be a potent stimulator of gastric cancer, presumably in an autocrine or paracrine manner, using EGFR as a downstream signaling partner. Leptin might critically affect the behavior of gastric cancer through cross-interaction with the EGFR signal.

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**References**

1. Calle EE, Kaaks R. Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nat Rev Cancer 2004;4:579–91.
2. Hardwick JC, Van Den Brink GR, Offerhaus GJ, Van Deventer SJ, Peppelenbosch MP. Leptin is a growth factor for colonic epithelial cells. Gastroenterology 2001; 121:79–90.
3. Schneider R, Bornstein SR, Chrousos GP, Boxberger S, Ehniger G, Breidert M. Leptin mediates a proliferative response in human gastric mucosa cells with functional receptor. Horm Metab Res 2001;33:1–6.
4. Mix H, Widaja A, Jandl O, et al. Expression of leptin and leptin receptor isoforms in the human stomach. Gut 2000;47:481–6.
5. Bado A, Levasseur S, Attou S, et al. The stomach is a source of leptin. Nature 1998;394:790–3.
6. Sweeney G. Leptin signalling. Cell Signal 2002;14: 655–63.
7. Daub H, Weiss FU, Wallach C, Ulrich A. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature 1996;379:557–60.
8. Yamauchi T, Ueki K, Tobe K, et al. Tyrosine phosphorylation of the EGF receptor by the kinase Jak2 is induced by growth hormone. Nature 1997;390: 91–6.
9. Shida D, Kitayama Y, Yamaguchi H, et al. Lysophosphatidic acid (LPA) enhances the metastatic potential of human colon carcinoma DLD1 cells through LPA1. Cancer Res 2003;63:1706–11.
10. Gschwind A, Prenzel N, Ulrich A. Lysophosphatidic acid-induced squamous cell carcinoma cell proliferation and motility involves epidermal growth factor receptor signal transactivation. Cancer Res 2002;62: 6329–36.
11. Shida D, Watanabe T, Aoki J, et al. Altered MAP kinase (ERK1,2) concentration and advanced gastrointestinal cancers: a case controlled study. BMC Cancer 2004;4:29.
12. Andl CD, Mizushima T, Oyama K, Bowser M, Nakagawa H, Rustgi AK. EGFR-induced cell migration is mediated predominantly by the JAK-STAT pathway in primary esophageal keratinocytes. Am J Physiol Gastroint Liver Physiol 2004;287:G1227–37.
13. Shida D, Kitayama Y, Yamaguchi H, et al. Dual mode regulation of migration by lysophosphatidic acid in human gastric cancer cells. Exp Cell Res 2004;301: 168–78.
14. Prenzel N, Zwick E, Daub H, et al. EGFR receptor transactivation by G-protein-coupled receptors requires metaproteinase cleavage of proerbB-EGF. Nature 1999; 402:884–8.
15. Shida D, Kitayama Y, Yamaguchi H, et al. Altered MAP kinase activity of epidermal growth factor receptor in intact A431 cells. Mol Cell Biol 1988;8: 1345–51.
16. Xing C, Imagawa W. Altered MAP kinase (ERK1,2) regulation in primary cultures of mammary tumor cells: elevated basal activity and sustained response to EGF. Carcinogenesis 1999;20:1201–8.
17. Cunnick JM, Dorsey JF, Standley T, et al. Role of tyro- sine kinase activity of epidermal growth factor receptor in the lysophosphatidic acid-stimulated mitogen-activated protein kinase pathway. J Biol Chem 1998;273: 14468–75.
18. Andl CD, Mizushima T, Oyama K, Bowser M, Nakagawa H, Rustgi AK. EGFR-induced cell migration is mediated predominantly by the JAK-STAT pathway in primary esophageal keratinocytes. Am J Physiol Gastroint Liver Physiol 2004;287:G1227–37.
19. Shida D, Watanabe T, Aoki J, et al. Altered MAP kinase (ERK1,2) concentration and advanced gastrointestinal cancers: a case controlled study. BMC Cancer 2004;4:29.
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