Involvement of the Akt signaling pathway in ER-α36/GRP94-mediated signaling in gastric cancer

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Abstract. Glucose-regulated protein 94 (GRP94) has been implicated in the promotion of tumor proliferation and metastasis. Previous studies have found that GRP94 is involved in the malignant growth of gastric carcinoma cells through estrogen receptor-α36 (ER-α36)-mediated estrogen signaling, but the underlying mechanism remains unclear. In the present study, we examined the expression levels of GRP94 and ER-α36 in tumor specimens from gastric cancer patients by immunohistochemistry, and found that both GRP94 and ER-α36 were highly expressed in the cytoplasms of gastric carcinoma cells. Furthermore, treatment with 17β-estradiol at a concentration of 10-12 M for 24 h increased the expression levels of GRP94 and ER-α36, and the phosphorylation levels of Akt at the Ser473 site (Ser473-Akt). In established SGC7901 gastric cancer cells with knockdown of ER-α36 expression, the levels of GRP94 and Ser473-Akt expression were significantly reduced. Thus, the Akt signaling pathway is a potentially important signaling pathway in ER-α36-GRP94-mediated gastric carcinogenesis.

Introduction

Glucose-regulated protein 94 (GRP94) is a molecular chaperone in the endoplasmic reticulum that binds to misfolded proteins and unassembled complexes, with an important role in the maintenance of cellular homeostasis and the suppression of cell death in stress conditions. GRP94 is highly expressed in cancer tissues, and previous studies have shown that GRP94 is involved in tumor proliferation, metastasis, drug resistance and immunotherapy (1,2). In gastric carcinoma, GRP94 overexpression has been associated with increased tumor size, increased depth of invasion, lymphatic and venous invasion, and advanced stage (3). GRP94 cleavage, induced by honokiol through calpains, has been shown to induce apoptosis in human gastric cancer cells and reduce gastric tumor growth (4). However, the involvement of GRP94 in carcinogenesis has not been well established.

Gastric cancer is the fourth most common type of cancer and the second leading cause of cancer-related mortality worldwide (5,6). Epidemiological studies have demonstrated a global gastric cancer predominance in males. Tamoxifen, an antiestrogen agent, has been shown to accelerate tumor progression and increase the overall risk of gastric adenocarcinoma (7,8). These findings suggest an association between estrogen signaling and the pathogenesis of gastric cancer. Previous studies have found that estrogen receptor-α36 (ER-α36), a novel variant of ER-α, is highly expressed in human gastric cancer, and that ER-α36 expression levels were positively correlated with lymph node metastasis and GRP94 expression levels (3,9,10). However, the molecular mechanism by which ER-α36 functions through GRP94 in the pathogenesis of gastric cancer remains unclear.

In the present study, GRP94 and ER-α36 expression levels in gastric cancer samples were examined. To clarify the mechanism of GRP94 involvement in gastric carcinogenesis through ER-α36 signaling, SGC7901 human gastric adenocarcinoma cells were treated with 17β-estradiol (E2) and the expression levels of GRP94 and ER-α36, and the phosphorylation levels of Akt at the Ser473 site (Ser473-Akt) were measured. GRP94 and Ser473-Akt levels were also determined in established gastric cancer cells with knockdown of ER-α36 expression.

Materials and methods

Antibodies and chemicals. 17β-E2 was purchased from Sigma-Aldrich (St. Louis, MO, USA). Polyclonal rabbit anti-mouse, anti-rat, anti-cow, anti-dog and anti-human GRP94 antibody was obtained from Abcam (Cambridge, UK). The monoclonal rabbit anti-human phospho-Akt at Ser473 (Ser473-Akt) antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The rabbit anti-ER-α36 antibody was generated and characterized as previously described (11). The mouse anti-β-actin antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Bicinchoninic acid protein detection kits, goat anti-mouse peroxidase-conjugated secondary antibody, chemiluminescence substrate kits and polyvinylidene...
Cell culture. The SGC7901 human gastric adenocarcinoma cell line was obtained from the Cell Center of Basic Medicine, Chinese Academy of Medical Sciences (Beijing, China). SGC7901-low36, a gastric cancer cell line with knockdown of ER-α36 expression was established using the lentiviral small hairpin RNA method (11). The SGC7901 and SGC7901-low36 cells were cultured in RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS; HyClone Laboratories, Inc., Logan, UT, USA) at 37˚C in a 5% CO2 atmosphere.

Western blot analysis. Western blot analysis was performed according to methods previously established (12). Briefly, the cells were lysed and homogenized with RIPA buffer. The protein concentration was then estimated with the biocinchoninic acid kit according to the manufacturer’s instructions. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to the PVDF membranes. The membranes were blocked with 5% non-fat milk dissolved in TBS-Tween-20 (containing 50 mm Tris HCl, pH 7.6, 150 mM NaCl and 0.2% Tween-20) for 1 h and probed with the primary antibodies (1:1,000) at 4˚C overnight. The blots were then incubated with monoclonal goat anti-mouse or polyclonal goat antibodies (1:1,000) at 4˚C overnight. The blots were then incubated with the secondary antibody (horseradish peroxidase-conjugated polyclonal goat anti-rabbit Ig; 1:100; Invitrogen Life Technologies) for 30 min. Diaminobenzidine served as a chromagen and the slides were counterstained with hematoxylin. Images were observed and captured using an Olympus BX53 microscope and an Olympus DP72 digital camera, respectively (Olympus Corporation, Tokyo, Japan).

Statistical analysis. SPSS 12.0 software (SPSS, Inc., Chicago, IL, USA) was used to conduct the analysis. All analyses were performed using Student’s t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

GRP94 and ER-α36 are highly expressed in gastric tumors. The GRP94 and ER-α36 expression levels were examined in the specimens from gastric carcinoma patients using IHC assay. GRP94 and ER-α36 were highly expressed in the cytoplasms of gastric carcinoma cells (Fig. 1).

E2 increases the protein expression levels of GRP94, ER-α36 and Ser473-Akt. To investigate the involvement of GRP94 in gastric cancer estrogen signaling, the SGC7901 human gastric adenocarcinoma cells were treated with 10^{-12} M E2 for 24 h (14). Western blotting and quantitative analysis revealed a significant increase in Ser473-Akt, GRP94 and ER-α36 expression levels (P<0.01 for each; Fig. 2).

ER-α36-mediated signaling regulates GRP94 expression through the Akt signaling pathway. To further analyze the function of the Akt signaling pathway in ER-α36-GRP94 signaling, GRP94, ER-α36 and Ser473-Akt expression levels were examined in SGC7901-Low36 cells with knockdown of ER-α36 expression. Significant reductions in GRP94 and Ser473-Akt expression levels were observed in these cells compared with SGC7901 control cells transfected with an empty expression vector (P<0.01 for each; Fig. 3). These results suggest that the Akt signaling pathway is involved in ER-α36-mediated estrogen signaling through GRP94 in gastric carcinogenesis.

Discussion

GRP94 is a chaperone in the endoplasmic reticulum that, under basal expression, controls normal physiological functions; however, GRP94 is also induced in pathological conditions, for example, hypoxia and nutrient deprivation (15). Under malignant conditions, GRP94 expression is upregulated and has
been shown to be involved in the pathogenesis, growth, invasion and metastasis of gastric carcinoma (16). In our previous study, GRP94 was found to be highly expressed in human gastric adenocarcinoma tissues (3). The levels of GRP94 expression were significantly correlated with gender, tumor stage, lymph node metastasis and the expression levels of ER-α, which is highly expressed in human gastric cancer and is involved in the malignant growth of gastric carcinoma cells (9,10). As estrogen is known to induce the expression of GRPs (3), this suggested that GRP94 may be involved in gastric carcinogenesis through ER-α-mediated estrogen signaling.

Akt (also known as protein kinase B) is a serine/threonine protein kinase known to regulate the balance between cell survival and apoptosis (17). Activated Akt expression induces cell survival, whereas inhibition of Akt activity stimulates apoptosis (18,19). Studies have shown that the overexpression and/or activation of Akt occurs in gastric cancer (20,21), and that the phosphoinositide 3-kinase/Akt
signaling pathway is important in the chemoresistance of gastric cancer cells against the cell death induced by etoposide and doxorubicin (22). These results suggest that the Akt signaling pathway is involved in tumor proliferation and drug resistance. In addition, E2 promptly activates the PI3K/Akt signaling pathway in Ishikawa cells in ER-dependent and ER-independent manner in HEC-1A cells (23). E2 treatment has been demonstrated to increase the phosphorylation of Akt on Ser473 (Akt1), but does not activate Akt2 (on Ser474); furthermore, ER-α is required for Akt1 activation (24). Akt1 may increase ER-α protein levels, while simultaneously reducing transcriptional activity (25). In the present study, to clarify the mechanisms involving GRP94 in the pathogenesis of gastric cancer induced by the ER-α36 signaling pathway, SGC7901 cells were treated with E2; increased GRP94 and ER-α36 expression levels, as well as increased phosphorylation levels of Akt at Ser473, were observed. By contrast, in established gastric cancer cells with knockdown of ER-α36 expression, GRP94 and Ser473-Akt expression levels were significantly reduced. These results suggest that Akt may be a key downstream effector of ER-α36-GRP94-mediated signaling in gastric carcinogenesis.

In conclusion, in the present study, high expression levels of GRP94 and ER-α36 were identified in gastric cancer tissues. Furthermore, E2 treatment increased the expression levels of GRP94, ER-α36 and Ser473-Akt. In established gastric cancer cells with knockdown of ER-α36 expression, GRP94 and Ser473-Akt expression levels were significantly reduced. Thus, the Akt signaling pathway is a potentially important signaling pathway involved in ER-α36/GRP94-mediated signaling in gastric carcinogenesis.

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