Down-regulation of survivin suppresses uro-plasminogen activator through transcription factor JunB

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Abbreviations: HGF, hepatocyte growth factor; uPA, uro-plasmino-gen activator

Abstract

Survivin, a member of the inhibitors of apoptosis protein family, is expressed during development and in various human cancers. However, the clinical relevance of survivin in cancer is still a matter of debate. Genes induced by hepatocyte growth factor (HGF) were screened using cDNA microarray technology in the stomach cancer cell lines, NUGC3 and MKN28. The levels of JunB, survivin, and uro-plasminogen activator (uPA) were up-regulated in cells treated with HGF in a dose-dependent manner. HGF-induced up regulation of JunB, survivin, and uPA was inhibited by pre-treatment with a MEK inhibitor (PD 98059). HGF-induced up-regulation of uPA was repressed by survivin knockdown. HGF enhanced the binding activity of JunB to the survivin promoter in control cells, but not in the JunB-shRNA cells. Transfection with survivin-shRNA resulted in a decrement of cell proliferation, as determined with MTT assays. In an *in vitro* invasion assay, significantly fewer cells transfected with survivin shRNA than control cells were able to invade across a Matrigel membrane barrier. In conclusion, survivin appeared to play an important role in the up-regulation of uPA induced by HGF *via* JunB and might contribute to HGF-mediated tumor invasion and metastasis, which may serve as a promising target for gastric cancer therapy.

Keywords: BIRC5 protein, human; hepatocyte growth factor; neoplasm metastasis; transcription factor AP-1; urokinase-type plasminogen activator

Introduction

Gastric cancer is one of the leading causes of cancer death in Korea. In spite of some progress in treatment, the diagnosis of gastric cancer still carries a poor prognosis. An alteration in the balance between apoptosis and cell proliferation could result in a disturbance of tissue homeostasis, and dysregulation of apoptosis is associated with various cancers (Hets, 1998). Considerable interest has focused on the regulation of apoptosis, which may potentially contribute to the development of cancer.

Survivin is a novel member of the inhibitors of apoptosis family. Survivin is expressed during human embryonic development in most tumor tissues, but not expressed in terminally differ-entiated normal tissues (Altieri, 2003a; Chiou *et al.*, 2003). A series of studies have indicated that survivin has a dual role in blocking cell apoptosis and regulating cell proliferation; over-expression of survivin correlates with the occurrence and de-velopment of gastric cancer (Altieri, 2003b; Miyachi *et al.*, 2003). Moreover, the over-expression of survivin has been shown to be correlated with aggressive and histologically-unfavorable neu-roblastoma (Adida *et al.*, 1998).

Hepatocyte growth factor (HGF) is a pleiotropic cytokine/growth factor capable of inducing a variety of biological activities, including cellular prolifer-ation, migration, and invasiveness (Jeffers *et al.*, 1996; Comoglio *et al.*, 2001). Patients with in-operable hepatocellular carcinoma (HCC) have higher levels of serum HGF that healthy controls, and serum HGF is negatively corrected with survival times. A serum HGF level ≥ 1.0 ng/ml in HCC patients is suggestive of a grave prognosis, indicating that HGF plays important and active
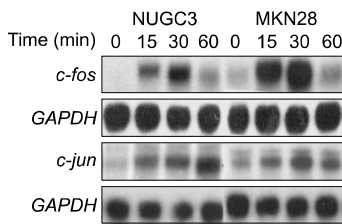
Figure 1. Induction of c-Jun and c-fos by HGF. Cells were serum-starved and treated with HGF (10 μg/ml) for the indicated times. RNA (10 μg) was separated on a 1% formaldehyde agarose gel and transferred to a Hybond N$^+$ membrane. The membrane was hybridized with a ^{32}P-labeled *c-jun* or *c-fos* probe and exposed to X-ray films. Equal loading of RNA was estimated with a *GAPDH* probe.

roles in disease progression (Vejchapipat *et al.*, 2004). The Jun family (C-Jun, JunB, and Jun D) are core members of activator protein-1 (AP-1). The role of the individual components of the Jun family in tumorogenesis is unclear. JunB may act to promote or inhibit neoplasia. Over-expression of JunB has been reported to be associated with neoplastic transformation of thyroid cells (Vallone *et al.*, 1997; Battista *et al.*, 1998).

The pivotal involvement of cell surface plasminogen activation associated with extracellular matrix (ECM) degradation has been extensively documented over the past decade (Brinckerhoff and Matrisian, 2002; Menshikov *et al.*, 2002). Tumor cell invasion and the metastatic process have been associated with elevated levels of cell surface uroplasminogen activator (uPA), and clinical studies suggest that inhibition of cell surface uPA is associated with reduced tumor cell invasion and metastasis, and improved clinical outcome (Foekens *et al.*, 2000; Giavazzi and Taraboletti, 2001). Using cDNA microarray analysis for HGF-inducible genes, we have provided evidence that survivin is a HGF-inducible gene in human gastric cancer cells. However, the role of HGF in the activation of survivin and its specific role in the regulation of expression of uPA have not been elucidated.

In this study, we found that the levels of survivin, JunB and uPA were increased in HGF-treated gastric cancer cells and investigated the role of HGF-induced survivin activation in the expression of uPA.

Results

Induction of c-fos and c-Jun by HGF

Since it is well-known that HGF induces up-regulation of *c-fos* and *c-jun* levels in a variety of cells, we determined whether or not NUGC3 and MKN28 cells also showed HGF-mediated *c-fos* and *c-jun* induction by real time RT-PCR. As expected, the levels of expression of *c-fos* and *c-jun* mRNA were increased with HGF in the early phase (to 30 min), then decreased in both cell lines (Figure 1). The results suggested that HGF exerts its effect in both cells.

Identification of HGF-responsive genes by cDNA microarray in NUGC3

In an attempt to explore differentially expressed genes in NUGC-3 cells treated with HGF, we used 17 k human cDNA microarrays. The initial analysis of the cDNA microarray expression data indicated that the presence of 26 genes changed by $\geq$ 2-fold after HGF treatment. A variety of genes were shown to be differentially expressed. The expression of several genes (Survivin [3.6-fold], Kiss-1 [9.3-fold], Bcl2 antagonist of cell death [BAD, 3.71-fold], histone deacetylate 5 [HDAC5, 3.26-fold], X-ray repair complementing defective repair 1 [XRCC1, 3.10-fold], and interleukin-1 [IL-1b, 3.25-fold]) increased 3-fold or more after HGF treatment. The genes were selected and the expression was confirmed by RT-PCR. RT-PCR showed that the level of expression of survivin was increased after HGF-treatment (Figure 2A). The survivin protein
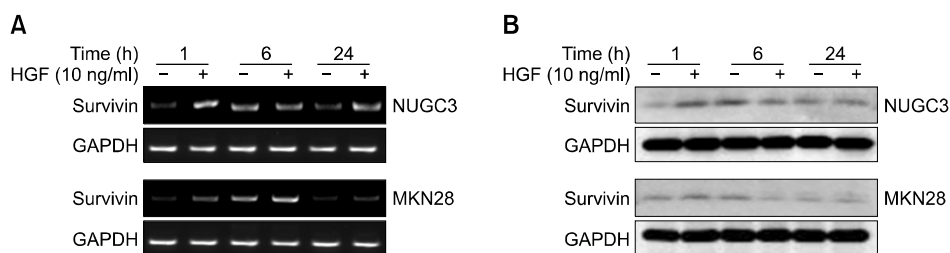

Figure 2. Effects of HGF on the level of expression of survivin in NUGC3 and MKN28 cells. Cells were serum-starved for 24 h, treated with or without HGF 10 ng/ml for the indicated times, and harvested. The levels of expression of survivin RNA and protein were confirmed by reverse transcription-polymerase chain reaction analysis (A) and Western blot (B). This illustrates representative data from three independent experiments.
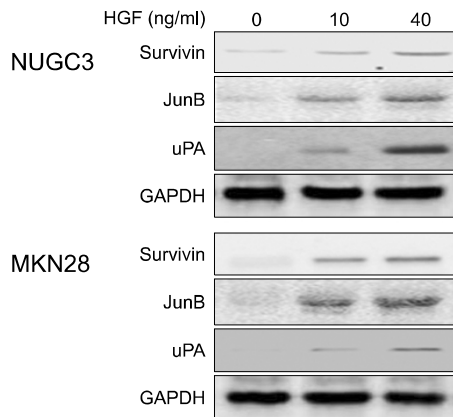
Figure 3. Expression of survivin, JunB and uPA on HGF dose-dependent treatment. Serum-starved cells were treated with HGF 0, 10, and 40 ng/ml for 1 h and harvested. The levels of expression of survivin, JunB and uPA was confirmed by Western blotting. This illustrates representative data from three independent experiments.

level was also enhanced by HGF treatment and confirmed by Western blot analysis (Figure 2B).

Up-regulation of survivin, JunB and uPA after treatment with HGF and signal pathway

Whether or not HGF plays an important role in the regulation of survivin, JunB and uPA expression was determined by measuring the levels of protein after treatment with HGF. As expected, HGF enhanced these protein expression in a dose-dependent manner in both cell lines (Figure 3). We have previously reported that the phosphorylation of ERK is induced by HGF in a gastric cancer cell line (Lee *et al.*, 2006). To further elucidate that the signal transduction pathways regulating survivin,

JunB, and uPA induction by HGF in gastric cancer cells, we measured the effect of a MEK1 and MEK2 inhibitor on HGF-induced survivin up-regulation. Pre-treatment with PD98059 repressed survivin up-regulation induced by HGF treatment. However, pre-treatment with LY294002, PI3 kinase inhibitor, did not repress survivin. These results suggested that HGF-induced survivin up-regulation is mediated through a common ERK activation in gastric cancer cells (Figures 4A and 4B).

Binding of JunB to a survivin promoter

We analyzed the promoter sequence of survivin genes to identify the putative JunB binding sequence using the TESS program (Schug and Overton, 1997). Two putative JunB binding sites were identified within the survivin promoter. The JunB binding site for the survivin promoter was located within the proximal promoter region upstream of the transcriptional start site (Figure 5A). To verify whether or not a comparable JunB binding site functions in the survivin promoter, JunB-shRNA cells and control cells were treated with 10 ng/ml of HGF and binding of JunB to putative JunB binding sites was measured by the ChIP assay. HGF enhanced the binding activity of JunB to the survivin promoter with relatively strong constitutive activity in control cells, but not in the JunB shRNA cells. These results suggested that the differentially-regulated JunB binding activities contribute to JunB-mediated survivin levels induced by HGF in NUGC3 and MKN28 cells (Figure 5B).
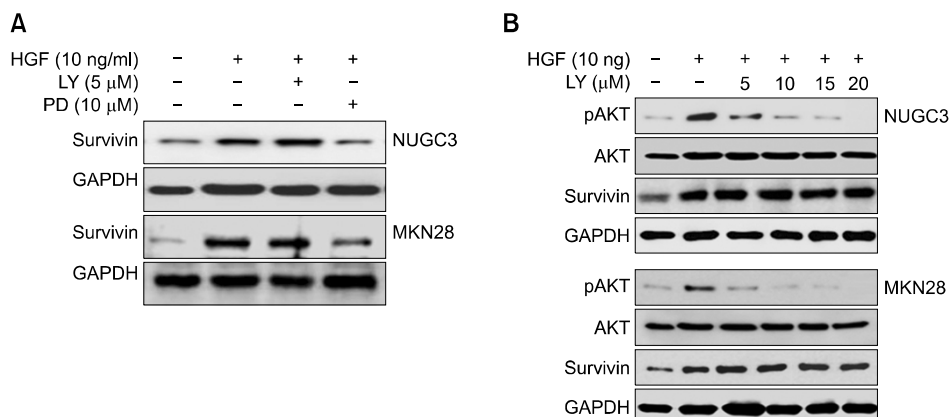

Figure 4. Effects of survivin on the inhibitor of AKT and ERK. Cells were starved for 24 h. Starved cells treated with or without LY (5 μM) and PD (10 μM) for 30 min, then treated with HGF (10 ng/ml). The expression of survivin was analyzed with Western blotting (A). To find out the effects of survivin on the inhibitor of AKT according to LY dosage, cells were starved for 24 h. Starved cells treated with or without LY (5, 10, 15, 20 μM) for 30 min, then treated with HGF (10 ng/ml). The expression of surviving and pAKT were analyzed with Western blotting (B). This illustrates representative data from three independent experiments.
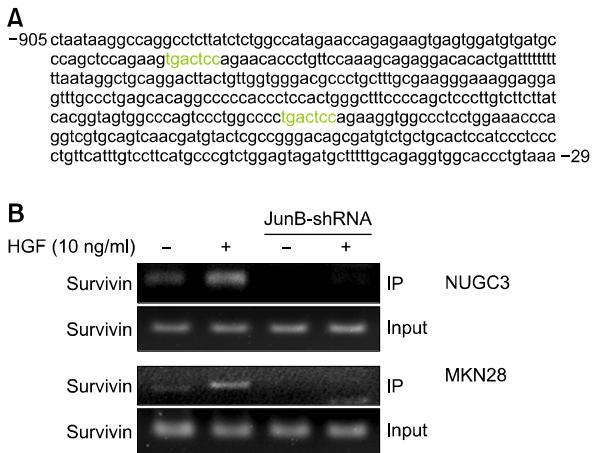
A

−905 ctaataaggccaggcctcttatctctggccatagaaccagagaagtgagtggatgtgatgc
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ctgttcatttgtccttcatgcccgtctggagtagatgcttttttgcagaggtggcaccctgtaaa −29

B

Figure 5. JunB binds to the survivin promoter and is responsible for survivin up-regulation by HGF. HGF enhances JunB binding at the promoter sequences of survivin. (A) sequence of the proximal survivin promotors. Dark line marks the location of JunB binding site. (B) CHIP assay results show the amplification of a fragment of the proximal survivin promotor containing the JunB binding site. Immunoprecipitation was carried out using an anti-JunB antibody. This illustrates representative data from three independent experiments.

Effects of JunB knockdown on HGF-induced up-regulation of survivin and the luciferase reporter gene assay

In order to further determine the role of JunB in up-regulation of survivin induced by HGF in gastric cancer cells, JunB level was knocked-down with a specific JunB- shRNA in both cells. Knockdown of JunB RNA and protein levels in stable JunB-shRNA cells was confirmed by RT-PCR and Western blot analysis (data not shown). The up-regulation of survivin induced by HGF was repressed by JunB knockdown. These results suggest that JunB might play an important role in

HGF-induced up-regulation of survivin (Figure 6A). To further confirm the functional role of HGF in activation of the promoter of genes identified by ChIP analysis, both cells were co-transfected with survivin promoter constructs with a vector containing the JunB-shRNA gene, and cultured with or without additional HGF. Figure 6B showed that the knockout of the JunB gene decreased the basal and HGF-induced survivin promoter activity in both cells. These findings provide direct evidence that the portion of the survivin promoter containing JunB sites is optimal and activated by HGF-induced JunB.

Effects of survivin knock down on the HGF induced up-regulation of uPA

To further determine the role of survivin in up-regulation of uPA induced by HGF in stomach cancer cells, the survivin level was knocked-down with a specific survivin-shRNA in both cells. Knock-down of survivin RNA and protein levels in stable survivin-shRNA cells were confirmed by RT-PCR and Western blot analysis (data not shown). Survivin-shRNA cells were treated with HGF and the level of expression of uPA proteins was measured by Western blotting. The up-regulation of uPA induced by HGF was repressed by survivin knock-down. These results suggested that survivin plays an important role in HGF-induced up-regulation of uPA (Figure 7).

Effects of survivin on cell proliferation and apoptosis

To determine whether or not survivin plays a role in cell proliferation, the MTT assay was performed after treatment of the cells with HGF in survivin-
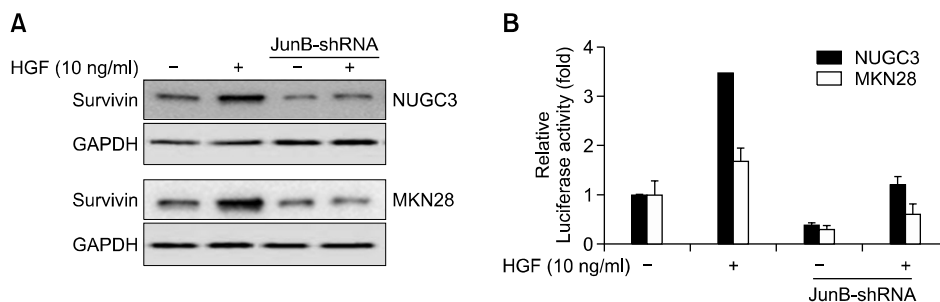
A

B

Figure 6. Effects of survivin on stable JunB-shRNA cells. Control cells and stable JunB-shRNA cells (1 × 10^6/well) were plated overnight in complete medium, starved for 24 h, treated with or without 10 ng/ml HGF for 1 h and harvested. The levels of expression of survivin were analyzed by Western blotting (A). To demonstrate HGF and JunB induced survivin promoter activity, stable JunB-shRNA cells and control cells were co-transfected with the plasmid containing survivin promoter sequence and stimulated with or without 10 ng/ml of HGF for 1 h. The promoter activity was analyzed in each well of the cultured medium using a Dual Glo^{TM} luciferase assay system with a Turner Designs instrument (B). The measured luminescence of firefly luciferase was divided by renilla luciferase and the resulting quotient corresponded to the relative amounts of luciferase.
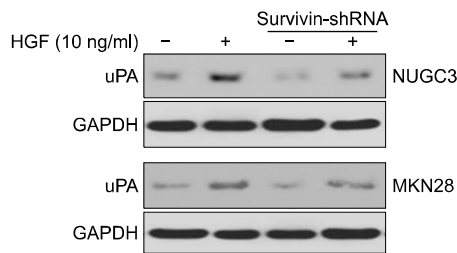
Figure 7. Effects of survivin on uPA expression. Control cells and stable survivin-shRNA cells (1 × 10⁶/well) were plated overnight in complete medium, starved for 24 h, treated with or without 10 ng/ml of HGF for 1h and harvested. The levels of expression of uPA were analyzed by Western blotting. This illustrates representative data from three independent experiments.
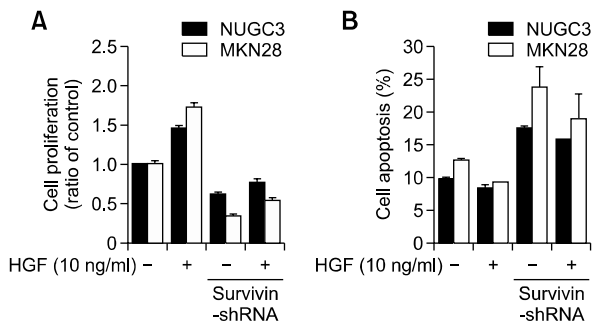

Figure 8. Effects of survivin on cell proliferation and apoptosis. Cells (1 × 10³/well) and stable survivin-shRNA cells were seeded in 96-well plates with DMEM media supplemented with 5% FBS and incubated for 24 h. After serum-starvation for 24 h, cell were treated with 10 ng/ml of HGF for 72 h. Cell proliferation was measured by MTT assays and expressed as a percentage of HGF-untreated control cells (A). Values are the means ± SD of three independent experiments. To see effects of survivin on HGF-mediated apoptosis, stable survivin-shRNA cells and control cells were treated with or without 10 ng/ml of HGF for 1 h and propidium iodide (PI). Fluorescence intensity of propidium iodide was analyzed by flow cytometry (B). Values are the means ± SD of three independent experiments.

shRNA stable cells. Following a 72-h incubation, HGF increased proliferation in control cells, but survivin-shRNA cells exhibited inhibition of proliferation (Figure 8A). And also we found that survivin plays a role in anti-apoptosis. Survivin knockdown induced an increase in apoptosis, confirmed by propidium iodide staining (Figure 8B).

Effects of survivin on cell invasion

To assess the role of JunB in HGF-mediated cell invasion, *in vitro* invasion using a matrigel migration chamber assay was measured in both transfected cells. JunB-shRNA cells showed a decrease in HGF-mediated cell invasion compared to the control cells (Figure 9A). Similarly, HGF-mediated cell invasion was also decreased in survivin-shRNA cells (Figure 9B), suggesting that survivin
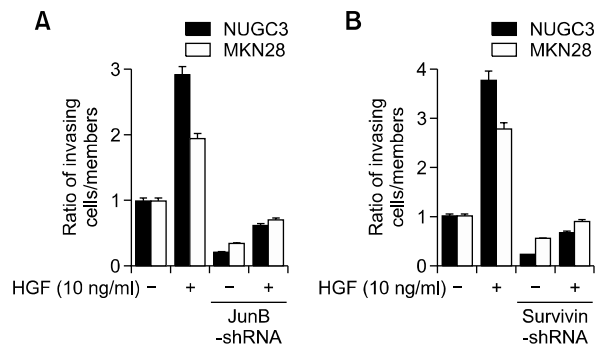

Figure 9. Effects of JunB and survivin on HGF-mediated cell invasion. Stable JunB-shRNA cells and control cells were treated with or without 10 ng/ml of HGF for 48 h. Cell invasion capacity was measured using the standard two-chamber invasion assay with Matrigel migration chambers (A). To see effects of survivin on HGF-mediated cell invasion, stable survivin-shRNA cells and control cells were treated with or without 10 ng/ml of HGF and measured (B). Values are the means ± SD of three independent experiments.

may play an important role in HGF-mediated cell invasion in gastric cancer cells through JunB.

Discussion

Gastric cancer is a worldwide problem. When gastric cancer is diagnosed, it is generally in its advanced stage with lymph node metastasis. Looking for molecular markers to predict the depth of invasion and lymph node metastasis of gastric cancer has important significance. One of the important activities of HGF in promoting tumorigenesis and metastasis is to stimulate ECM proteolysis and angiogenesis by a direct effect on uPA and endothelial growth (Bussolino *et al.*, 1992; Grant *et al.*, 1993; Lee and Kim, 2009). In the present study, we investigated the potential role of HGF-induced survivin in the expression of uPA and transcription factor. We demonstrated that HGF induced up-regulation of the ECM degradation protein, uPA, *via* the JunB/survivin pathway in gastric cancer cells, suggesting an important novel mechanism by which HGF promotes tumor invasion and metastasis.

Repression of apoptosis has been observed in gastric cancer (Lauwers *et al.*, 1995). Survivin, a new member of the inhibitors of apoptosis protein family, is a powerful apoptosis repression factor. Sustained over-expression of survivin is a characteristic feature of gastric cancer, and plays in important role in tumor progression by inhibiting apoptosis and facilitating mitosis, thus giving cancer cells a survival and growth advantage (Wakana *et al.*, 2002; Miyachi *et al.*, 2003). The inhibition of survivin has been demonstrated to be

an effective molecular target therapy in the treatment of a variety of cancers, such as lung (Falleni *et al.*, 2003), breast (Tananka *et al.*, 2006), liver (Kountouras *et al.*, 2005), and colon cancers (Lin *et al.*, 2003).

However, since survivin was identified several years ago, there have been contradictory reports about the clinical relevance of survivin expression in cancer. Gianani *et al.* (2001) reported that expression of survivin was not a specific marker of the colon, but showed characteristic patterns of expression in normal colonic mucosa. In gastric carcinoma, Lu *et al.* (1998) showed that survivin could promote tumor cell viability, whereas Okada *et al.* (2001) indicated that survivin expression in the nuclei of tumor cells was predictive of a favorable prognosis. In recent years, molecular target therapy using survivin inhibition has been applied in the treatment of gastric cancer. However, few reports have shown inhibitory effects of shRNA against survivin in gastric cancer *in vitro* and *in vivo*. Lee *et al.* (2006) reported that survivin may play an important role in carcinogenesis by stimulating tumor-angiogenesis in human gastric cancer.

In a study involving a mouse xenograft model *in vivo*, Uchida *et al.* (2004) reported that intratumoral injection with recombinant adenoviral vectors encoding shRNA against survivin exerted a powerful anti-tumor effect. This novel model may be a promising tool for the study of cancer gene therapy *in vivo*. In the current study, we constructed survivin-shRNA plasmids and successfully transfected these plasmids into gastric cancer cell lines (NUGC3 and MKN28). The results showed that stable survivin-shRNA cells decreased the expression of uPA and cell invasion in a Matrigel two-chamber assay.

Wang *et al.* (2004) reported the expression of survivin in primary and metastatic gastric cancer cells obtained by laser-capture micro-dissection. There was a statistically significant difference in survivin expression between primary gastric cancer cell (80%) and adjacent morphologically-normal gastric epithelial cells (23%). Of the metastatic gastric cancer cell groups from lymph nodes, 95% had clear expression of the survivin gene. The high rate of expression in metastatic lesions suggests a possible role of survivin in cancer invasiveness and metastasis, and may contribute to the detection of gastric cancer micro-metastases as a potential molecular marker.

Among the different cellular mechanisms controlling AP-1 activity, post-translational modification and regulation of protein turnover are crucial.

Post-translational modification of the Jun proteins are most extensively documented in the case of mitogen and cellular stress-induced hyperphosphorylation, with a particular emphasis on the activation of *c-Jun* by JNK (Karin and Gallagher, 2005). In contrast, JunB phosphorylation has rarely been studied. The fact that the specific JNK phophorylation sites found in *c-Jun* are not conserved in JunB suggests that the latter protein is not a substrate for JNK (Dérijard *et al.*, 1994). In our previous study (in press), ERK and p38 kinase phosphorylated JunB to stimulate tumor invasion-related protein, MMP-9, and uPA expression, and showed a decrement in uPA expression in stable JunB-shRNA gastric cancer cells.

In recent years, antisense oligonucleotide has been used to explore gene function, and exhibits a great potential in prevention and treatment of neoplasms. Our study has established a basis for further exploring the roles of survivin in biological behaviors of gastric cancer and the regulatory mechanisms. Our findings may contribute to the detection of micrometastases in gastric cancer as a potential molecular marker. Further, our findings also provide a new era of vision and an important method for the targeted therapy of gastric cancer.

Methods

Cell culture

We used two human gastric cancer cell lines, a poorly differentiated adenocarcinoma (NUGC3), and a moderately differentiated tubular adenocarcinoma (MKN28), which were obtained from the Korea Cell Line Bank (Seoul, Korea). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 2 mM L-glutamine, $2 \times$ vitamin solution, and 50 U/ml penicilline/streptomycin (Life Technologies, Inc., Gaithersburg, MD). Unless otherwise noted, cells underwent passage and were removed from flasks when 70-80% confluent.

Reagents and antibodies

Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were purchased from Bio-Rad Laboratories (Philadelphia, PA). Recombinant human HGF (R&D Systems, Inc, Minneapolis, MN) and rabbit polyclonal antibody against human survivin were purchased Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Human recombinant HGF was purchased from Becton Dickinson Lab (Beverly, MA). uPA was obtained from American Diagnostica (Greenwich, CT). PD 98059 was purchased Biomol Reasearch Laboratories, Inc. (Butler Pike, PA). LY294002 was purchased from Calbiochem Inc. (San Diego, CA).

Semi-quantitative reverse transcription-polymerase chain reaction

Complementary DNA (cDNA) was synthesized from total RNA using MMLV reverse transcriptase (Promega Corp., Madison, WI) by the oligo (dT) priming method in a 10 μl reaction mixture. The polymerase chain reaction (PCR) was performed in a 10 μl reaction volume containing 10 mM Tris-HCl (pH 8.5), 50 mM KCl, 1 μl cDNA, 200 μM dNTPs, 1 mM MgSO$_4$, 1 U platinum pfx Taq polymerase, and 2 μM primers. The PCR reactions as follows: an initial denaturation at 95°C for 4 min; 27 cycles at 94°C for 15 s, 60°C for 15 s, and 72°C for 30 s; and a final extension at 72°C for 10 min. The PCR products were separated on a 1.5% agarose gel containing ethidium bromide and visualized on a UV transilluminator.

Western blot analysis

Cells were harvested and incubated with a lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1 mM EDTA, 1% Trion X-100, 10% glycerol, 1 mM PMSF, 1 mM sodium vanadate, and 5 mM NaF) with protease inhibitors and centrifuged at 15,000 rpm for 10 min at 4°C. Proteins (50 μg) were separated on 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were soaked with 5% non-fat dried milk in TTBS (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.05% Tween-20) for 30 min, then incubated overnight with a primary antibody at 4°C. After washing 6 times with TTBS for 5 min, membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 90 min at 4°C. The membranes were rinsed 3 times with TTBS for 30 min and antigen-antibody complexes were detected using an enhanced chemiluminescence detection system.

3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay

The cells (1,500/well) were seeded in 96-well plates in DMEM supplemented with 5% FBS and incubated for 24 h. The cells were then serum-starved for 24 h and treated for 72 h with or without HGF (10 ng/ml). At the end of the incubation period, 50 μl of 2 mg/ml 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) solution was added and the cells were allowed to incubate for 3 h at 37°C. The supernatant was carefully removed by aspiration, and the converted dye was dissolved with 100 μl of DMSO. The plates were placed in a microplate shaker for 5 min, and the absorbance was measured at 570 nm using a Biorad multiscan plate reader.

Survivin knock-down with short hairpin RNA

The human survivin-specific short hairpin (sh) RNA expression vector containing the survivin-targeted shRNA sequence (AAACCCAGGGCTGCCTTGGAAAAG) was purchased from Open Biosystems (survivin-shRNA, RHS3979-97062015, Huntsville, AL). Cells were transfected with survivin-shRNA using Lipofectamine (Life Technologies Inc.). Clonal selection was conducted by culturing with puromycin (10 μg/ml), followed by serial dilution of the cells. Stable transfectant clones with a low expression of the target genes were identified by RT-PCR. The primers for survivin in real-time (RT)-PCR as follows: sense, 5'-gacttggcaggtgcctgt-3'; and antisense, 5'-agctgctgcctcca-aaga-3'.

Standard two-chamber invasion assay

Control cells and transfected cells (1 $\times$ 10^4) were placed in the upper chamber of a Matrigel migration chamber with 0.8 μm pores (Fisher Scientific, Houston, TX) in media containing 5% FBS with/without HGF (10 ng/ml). After incubation for 48 h, cells were fixed and stained using a HEMA 3 stain set (Curtis Matheson Scientific, Houston, TX), according to the manufacturer's instructions. The stained filter membrane was cut and placed on a glass slide. The migrated cells were counted under light microscopy (10 fields at 200 $\times$ power).

Chromatin immunoprecipitation (ChIP) assay

The chromatin immunoprecipitation (ChIP) assay was done using a chips assay kit (Upstate Biotechnology, Waltham, MA) following the manufacturer's directions. Briefly, cells were fixed with 1% formaldehyde at 37°C for 10 min. Cells were washed twice with ice-cold PBS with protease inhibitors (1 mM phenylmethylsulphonyl fluoride, 1 mg/ml aprotinin, and 1 mg/ml pepstatin A), scraped, and pelleted by centrifugation at 48°C. Cells were resuspended in a lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris-HCl [pH 8.1]), incubated for 10 min on ice, and sonicated to shear DNA. After sonication, the lysate was centrifuged at 13,000 rpm for 10 min at 48°C. The supernatant was diluted in CHIP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 16.7 mM Tris-HCl [pH 8.1], 167 mM NaCl, and protease inhibitors). Primary antibodies were added and incubated overnight at 48°C with rotation. The immunocomplex was collected by protein A/G agarose beads and washed with low-salt washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl [pH 8.1], and 150 mM NaCl), high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 200 mM Tris-HCl [pH 8.1], and 500 mM NaCl), LiCl washing buffer (0.25 M LiCl, 1% NP40, 1% deoxycolate, 1 mM EDTA, and 10 mM Tris-HCl [pH 8.1]), and finally 1 $\times$ TE buffer (10 mM Tris-HCl and 1 mM EDTA [pH 8.0]). The immunocomplex was then eluted with elution buffer (1% SDS, 0.1 M NaHCO$_3$, and 200 mM NaCl), and the cross-links were reversed by heating at 65°C for 4 h. After the reaction, the samples were adjusted to 10 mM EDTA, 20 mM Tris-HCl (pH 6.5), and 40 mg/ml proteinase K, and incubated at 45°C for 1 h. DNA was recovered and subjected to PCR amplification of the survivin promoter region (-29 to -905) with the following primers: 5'-ctggacggctaataaggcca-3' (forward); and 5'-cgtct-ccaccgtgggacatt-3' (reverse).

Survivin promoter analysis

The transcriptional regulation of survivin by HGF and JunB was examined using transient transfection with an survivin promoter luciferase reporter construct (survivin-pMetluc reporter). Cell transfection was performed using Lipofec-

tamine[TM]2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. For the luciferase reporter gene assay, control cells and sh-JunB expression cells were co-transfectioned with 1 μg of survivin-pMetluc-reporter plamids and 0.05 μg of pHYK plasmids, which was used as an internal transfection-efficiency control. Transfected cells were stimulated with or without 10 ng/ml HGF for 1 h. The promoter activity was analyzed in each well of the cultured medium using a Dual Glo[TM] luciferase assay system with a Turner Designs instrument lumino-meter (Turner Designs, Sunnyvale, CA). The measured luminescence of firefly luciferase was divided by renilla luciferase and the resulting quotient corresponded to the relative amount of luciferase.

Analaysis of apoptosis by flow cytometry

Cell were harvested, washed twice with PBS, and fixed with 70% ethanol at -20°C for 1 h. Following the washing of cells with PBS conaining 2% FBS and 0.01% $CaCl_2$, RNase (1% w/v) was added and incubated at 37°C for 30 min. Peopidium iodide (50 μg/ml) was added, and cells were incubated for 30 min. The intracellular propidium iodide fluorescence intensity of each population 10,000 cells was measured in each sample using a Becton-Dickinson FACS Caliber flow cytometer, and the cell cycle was analyzed by Cell Quest software (Becton-Dickinson, San Jose, CA).

Acknowledgements

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