Suppression of Adriamycin-induced Apoptosis by Sustained Activation of the Phosphatidylinositol-3′-OH kinase-Akt Pathway*

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The mechanisms by which growth factors trigger signal transduction pathways leading to protection against apoptosis are of great interest. In this study, we investigated the effect of hepatocyte growth factor (HGF/SF) and epidermal growth factor (EGF) on Adriamycin (ADR)-induced apoptosis. Treatment of human epithelial MKN74 cells with ADR, a DNA topoisomerase IIα inhibitor, caused apoptosis. However, cells pretreated with HGF/SF, but not those pretreated with EGF, were resistant to this apoptosis. The protective effect of HGF/SF against the ADR-induced apoptosis was abolished in the presence of either LY294002, an inhibitor of phosphatidylinositol-3′-OH kinase (PI3-K) or 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecycarbonate, an inhibitor of Akt, thus implicating the role of PI3-K-Akt signaling in the protective action of HGF/SF. Immunoblotting analysis revealed that HGF/SF stimulated the sustained phosphorylation of Akt for several hours but that EGF stimulated the phosphorylation only transiently. Furthermore, ADR-induced activation of caspase-9, a downstream molecule of Akt, was inhibited for at least 24 h after HGF/SF stimulation, but it was not affected by EGF stimulation. Cell-surface biotin-labeling analysis showed that the HGF/SF receptor remained on the cell surface until at least 30 min after HGF/SF addition but that the EGF receptor level on the cell surface was attenuated at an earlier time after EGF addition. These results indicate that HGF/SF, but not EGF, transmitted protective signals against ADR-induced apoptosis by causing sustained activation of the PI3-K-Akt signaling pathway. Furthermore, the difference in ant apoptotic capacity between HGF/SF and EGF is explained, at least in part, by the delayed down-regulation of the HGF/SF receptor.

Chemotherapy for the treatment of cancer was introduced into the clinic more than 50 years ago. Recently, apoptosis has been shown to represent the cytotoxic endpoint for many chemotherapeutic drugs. The intracellular machinery responsible for apoptosis depends on a family of cysteine asparagines (caspases), and the two main apoptotic pathways, the death receptor and mitochondrial pathways, are activated by caspase-8 and caspase-9, respectively. Apoptotic triggers such as chemotherapeutic drugs activate the latter pathway, which requires disruption of the mitochondrial membrane and the release of cytochrome c from the mitochondria. Cytochrome c functions with Apf-1 to induce activation of caspase-9, thereby activating a set of caspases (1, 2). Furthermore, mitochondrial membrane permeabilization is controlled by the opposing actions of pro- and antiapoptotic Bcl-2 family members (3–6).

The effectiveness of cancer chemotherapy has suffered from a range of confounding factors, including toxicity against normal cells and the development of a chemotherapy-resistant phenotype. Drug resistance is thought to arise from a number of molecular changes in cellular transport and drug metabolism, mutations of tumor suppressor genes, and overexpression of oncogenes (7, 8). In addition, several studies indicate that growth factors such as insulin-like growth factor I and epidermal growth factor (EGF)1 promote the survival of a variety of cell types and block apoptosis induced by diverse apoptotic stimuli (9–14). Moreover, recent studies showed hepatocyte growth factor (HGF/SF) to protect various epithelial and carcinoma cell types against apoptosis and cytotoxicity induced by DNA-damaging agents (15–20).

HGF/SF is a mesenchyma-derived cytokine that acts as mitogen, motogen, and morphogene toward various target cells (21–25). Two tyrosine residues of the HGF/SF receptor’s β-chain are phosphorylated upon HGF/SF binding to its receptor, which allows the receptor to transmit signals via association with p60
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1 The abbreviations used are: EGF, epidermal growth factor; HGF/SF, hepatocyte growth factor/scatter factor; ADR, doxorubicin (Adriamycin); PI3-K, phosphatidylinositol-3′-OH kinase; FCS, fetal calf serum; PARP, poly(ADP-ribose) polymerase; HRP, horseradish peroxidase; GSK, glycogen synthase kinase; LY, LY294002.

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pathway has emerged as the major mechanism by which growth factors promote cell survival (reviewed in Ref. 50).

In this study, we found that doxorubicin (Adriamycin; ADR)-induced apoptosis of MKN74 cells was significantly inhibited by HGF/SF but was not changed by EGF. HGF/SF induced sustained activation of survival signals of PI-3-K and Akt in MKN74 cells. It also inhibited ADR-induced caspase-9 activation in an Akt-dependent manner. Because caspase-9 is intimately associated with the initiation of apoptosis, HGF/SF seemed to exert protective action against ADR-induced apoptosis by stimulating caspase-9 activity via the PI-3-K-Akt survival signaling pathway. On the other hand, EGF also activated survival signals of PI-3-K and Akt, but its effects on PI-3-K and Akt were transient. Furthermore, EGF had no effect on ADR-induced caspase-9 activation. These results suggest that the long-lasting activation of PI-3-K and Akt is critical for the protective action of growth factors against ADR. Because the resistance to apoptosis is a major cause of failure of treatment for malignancies, the PI-3-K/Akt survival signaling pathway is a promising target for drug design in the treatment of several types of human cancer.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant human HGF/SF was provided by the Research Center of Mitsubishi Pharma Corporation (51, 52). EGF (ultrapure) from mouse submaxillary glands was purchased from Toyo Co., Ltd. (Osaka, Japan). Fetal calf serum (FCS), phenylmethylsulfonyl fluoride, pepstatin A, leupeptin, and aprotinin came from Sigma. RPMI 1640 medium was from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Antibodies used and their sources were as follows: anti-phosphotyrosine antibody (PY20) was from BD Transduction Laboratories; anti-caspase-9 p10 antibody (H9262) and anti-p-caspase-9 (Ser 196) antibody, and anti-CbI (C-15) antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-poly(ADP-ribose) polymerase (PARP) antibody was from Oncogene Research Products (Cambridge, MA); anti-ACTIVE MAP kinase antibody and anti-pS657/658 Akt antibody were from Promega; anti-Akt antibody was from Cell Signaling Technology, Inc. (Beverly, MA); anti-phospho-Akt1/2/3 (Thr-308) antibody and anti-Gab1, C terminus antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); anti-phospho-Akt1 (Thr-308) antibody and anti-HEF1 antibody were from Upstate Biotechnology (Lake Placid, NY); anti-PI-3-K (AB61) antibody was from Cell Signaling Technology, Inc. (Beverly, MA); swine horseradish peroxidase (HRP)-linked anti-rabbit Ig antibody was from DAKO (Glostrup, Denmark); and sheep HRP-linked anti-mouse Ig antibody was from Amersham Biosciences. Phosphatidylinositol 3,4,5-trisphosphate was synthesized from Matreya Inc. (Pleasant Gap, PA).

Cell Cultures—Human gastric adenocarcinoma MKN74 cells were cultured to subconfluence in RPMI 1640 medium supplemented with 10% FCS and used for all of the experiments.

**Treatment of Cells with ADR**—For most experiments, subconfluent cultures in 60- or 100-mm dishes were preincubated with or without 100 ng/ml of HGF/SF or EGF for 48 h and then treated with 20 μM ADR for 2 h. After exposure to the ADR, the cultures were washed twice to remove the drug and then incubated at 37 °C for 24 h in RPMI 1640 medium supplemented with 5% FCS. Cells were then harvested for assays of DNA fragmentation or for immunoblotting.

**DNA Fragmentation Assays**—The DNA fragmentation assay was performed as described previously (53). Briefly, after the indicated times of treatment with ADR, adherent cells and floating cells were harvested by centrifugation and washed twice in phosphate-buffered saline. DNA was extracted and purified from the pellet by use of IsoQuick (ORCA Research Inc., Bothell WA), and it was dissolved in gel loading buffer and then analyzed by 2% agarose gel electrophoresis for visualization of “DNA ladder,” the electrophoresed gel was soaked in Tris-borate/EDTA solution containing 0.1 mg/ml ethidium bromide.

**Preparation of Cellular Lysates and Immunoblotting**—Cells were seeded at a density of 1.6 × 10⁵ cells/100-mm dish and cultured for 2 days. The cells were washed with buffer A (25 mM HEPES/NaOH, pH 7.4, containing 135 mM NaCl) supplemented with a mixture of protease inhibitors (1 μg/ml aprotinin, 1 μg/ml leupeptin, 2 μg/ml leupeptin, 1 μM sodium orthovanadate, 50 mM sodium fluoride, and 30 μM Na₃VO₄) for 10 min at 4 °C. The lysates were then incubated on ice for 30 min and clarified by centrifugation at 12,000 × g for 10 min at 4 °C. The supernatant fractions were incubated overnight at 4 °C with anti-phosphotyrosine antibody (PY20), anti-Gab1 antibody, or anti-c-Cbl antibody. Immune complexes were collected on Protein-G-Sepharose (Amersham Biosciences). Bound proteins were washed five times with phosphate-buffered saline with buffer B and eluted in Laemmli SDS sample buffer containing 2-mercaptoethanol. Eluted proteins were subjected to SDS-PAGE and immunoblotted as described above.

**PI-3-K Activity Assay**—PI-3-K activity was examined according to the method of Tanimura et al. (39). Briefly, MKN74 cells were seeded at a density of 9.6 × 10⁶ cells/100-mm dish and incubated in RPMI 1640 medium supplemented with 5% FCS for 3 days. The cultures were then pretreated or not with 10 μM LY294002 (LY) for 90 min and stimulated with HGF/SF for various times at 37 °C. Immunoprecipitates prepared from cell lysates with anti-phosphotyrosine antibody (PY20) were incubated in an assay mixture containing 40 mM Tris/HCl, pH 7.4, 0.5 mM EGTA, 5 mM MgCl₂, 0.2 mM phosphatidylinositol 4,5-diphosphate (Sigma), 0.2 mM phosphatidylerine (Avanti Polar-Lipids, Inc.), 50 μM ATP, 25 μCi of [γ-³²P]ATP (3000 Ci/mmol) (PerkinElmer Life and Analytical Sciences) and resolved by thin layer chromatography in CHCl₃/methanol/acetic acid/H₂O (7:2:2:2).

**Akt Activity Assay**—Akt activity was examined according to the instruction manual of the Akt kinase assay kit used (New England Biolabs, Inc.). Briefly, MKN74 cells were pretreated or not with Akt inhibitor for 3 h, stimulated with HGF/SF for 5, 15, and 30 min, and washed with buffer A containing a mixture of protease inhibitors. Subsequently, the cells were lysed in buffer C (20 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Na₃VO₄, 1 μM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The lysates were incubated on ice for 30 min and clarified by centrifugation at 12,000 × g for 10 min at 4 °C. The supernatant fractions were incubated for 3 h at 4 °C with a slurry of immobilized anti-Akt antibody. The immune complexes were washed twice with buffer C and equilibrated with kinase buffer containing 5 μM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM sodium orthovanadate, and 10 mM MgCl₂; then, a kinase reaction mixture containing 200 μM ATP and 1 μg of GSK-3β fusion protein was added to the equilibrated immune complexes. The reaction was carried out at 30 °C for 30 min and stopped by the addition of Laemmli SDS sample buffer. Samples were subjected to SDS-PAGE and immunoblotted with anti-phospho-GSK-3β antibody.

**Surface Labeling**—Cells were seeded at a density of 9.6 × 10⁶ cells/100-mm dish and cultured for 2 days. They were then washed with buffer A containing a mixture of protease inhibitors and next washed with buffer D (100 mM HEPES/NaOH, pH 8.0, containing 150 mM NaCl). The cells were then incubated in buffer D containing 0.2 mg/ml of BSA and buffer B, the cell lysates were immunoprecipitated with anti-HGF/SF receptor antibody or anti-EGF receptor antibody as described above. Immunoprecipitates were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore). The membranes were sequentially incubated with HRP-conjugated streptavidin (Amersham Biosciences) for 1 h, and the samples were then developed with ECL Western blotting detection reagents (Amersham Biosciences) and exposed to autoradiography film (X-Omat Blue XB-1; PerkinElmer Life and Analytical Sciences).

**RESULTS**

Initially, we evaluated the abilities of HGF/SF and EGF to rescue MKN74 cells from apoptosis induced by the DNA-dam
aging agent ADR. Pretreatment of the cells with 100 ng/ml HGF/SF for 48 h markedly suppressed the cell death induced by ADR, whereas 100 ng/ml EGF pretreatment had no effect on the cell death (Fig. 1A). Next, to evaluate whether this ADR-induced cell death resulted from apoptosis, we looked for DNA fragmentation after exposing the cells to 20 μM ADR for different times. As illustrated in Fig. 1B, ADR induced DNA fragmentation, and this fragmentation became apparent at an early time (3 h) after the addition of the drug. Pretreatment with HGF/SF, but not EGF, markedly protected the cells against DNA fragmentation induced by ADR treatment for 3, 6, 12, or 24 h. This protective action of HGF/SF against ADR was dose- and time-dependent, and maximal protection required pretreatment with 100 ng/ml of HGF/SF for 48 h (data not shown). Therefore, cells were pretreated in this fashion in subsequent experiments.

The requirement of prolonged pretreatment with HGF/SF for protection against apoptosis suggests that maximal protection may require new protein synthesis. Several recent studies indicate that certain growth factors can suppress apoptosis through modulating the process of apoptosis (55, 56). Thus, we determined the effect of HGF/SF and EGF on the levels of key anti-apoptotic proteins (i.e., Bcl-2 and Bcl-XL). As shown in Fig. 2, ADR increased the expression of the Bcl-2 protein, and pretreatment with HGF/SF or EGF had no effect on the ADR-induced Bcl-2 expression. As for the Bcl-XL protein, another antiapoptotic member of the Bcl-2 family, ADR did not change its level irrespective of the presence or absence of HGF/SF or EGF.

HGF/SF binding to its cognate receptor results in receptor activation and autophosphorylation on tyrosine residues, which then leads to activation of the PI3-K-dependent signaling pathway and the Ras-MAP kinase cascade. To determine a causal link between the activation of PI3-K and the antia apoptotic action of HGF/SF, we tested the effect of LY294002 (LY), a selective PI3-K enzyme inhibitor, on the protective action of HGF/SF. Cells were preincubated with 10 μM LY for 90 min before HGF/SF treatment, which was followed by exposure to ADR and postincubation as usual. LY had no significant effect on cell viability in control or ADR-treated cells, but it reduced the degree of HGF/SF-mediated protection against ADR (Fig. 3A). In contrast to LY, the MAP kinase kinase inhibitor PD98059 did not significantly alter the ability of HGF/SF to protect against the ADR effect. This conclusion was confirmed by the findings that the protective action of HGF/SF against ADR-induced DNA fragmentation was inhibited by LY but not by PD98059 (Fig. 3B). We next determined whether LY actually blocked PI3-K activity in a specific manner. As shown in Fig. 3C, when the cells were stimulated with HGF/SF for 3 or 10 min, increased incorporation of 32P into the phosphatidylinositol 3,4,5-triphosphate was observed. However, the PI3-K activation was not detectable in LY-pretreated cells. On the other hand, LY did not significantly alter HGF/SF-stimulated phosphorylation of MAP kinase (Fig. 3D). These findings indicate that HGF/SF-induced protection is caused, at least in part, by activation of PI3-K.

To address the mechanism by which HGF/SF, but not EGF, increased cell survival against ADR-induced apoptosis of

Fig. 1. HGF/SF protects MKN74 cells against apoptosis induced by ADR. A, MKN74 cells were pretreated or not with 100 ng/ml HGF/SF or 100 ng/ml EGF for 48 h. The cells were then treated with 20 μM ADR for 2 h and incubated in ADR-free medium. The phase-contrast micrographs shown were taken 24 h after incubation in ADR-free medium. Scale bar, 100 μm. B, cells were harvested at the indicated times after incubation in ADR-free medium and used for the DNA fragmentation assay described under “Experimental Procedures.” C, no pretreatment with HGF/SF or EGF; H, HGF/SF treatment; E, EGF treatment.

Fig. 2. Effect of HGF/SF and EGF on ADR-induced Bel-2 and Bcl-XL protein expression. Cells were treated with HGF/SF or EGF for 48 h and then with ADR for 2 h, as described in Fig. 1. They were then incubated for an additional 0, 3, 6, or 12 h in ADR-free medium. Next, total cell protein was extracted from the cells, and aliquots of protein (20 μg per lane) were electrophoresed on 13.5% SDS-PAGE gels, transferred to Immobilon-P membranes, and immunoblotted with anti-Bcl-2 antibody (top) and anti-Bcl-XL antibody (bottom), as described under “Experimental Procedures.” Experiments were repeated three times.
MKN74 cells, we analyzed PI3-K activity and signaling pathways downstream of PI3-K in HGF/SF- and EGF-treated cells. Cells were treated with either HGF/SF or EGF, and cell lysates were prepared and immunoprecipitated with anti-phosphotyrosine antibody and then used for immunoblot analysis of the regulatory subunit of PI3-K. As shown in Fig. 4, HGF/SF stimulated intensively tyrosine phosphorylation of the subunit, which level was sustained for at least 60 min. In contrast, EGF induced transient tyrosine phosphorylation of PI3-K, and its level was lower. We next examined the activation of Akt by these growth factors. Akt is a protein kinase downstream of PI3-K that has been implicated in growth factor signaling related to cell survival (57–59). Cells were incubated in the presence of HGF/SF or EGF for several periods of time, and cell lysates were prepared from these cells to determine the phosphorylation of Akt by immunoblotting (Fig. 5A). Increased phosphorylation of Akt on serine 473 and threonine 308 became evident 15 min after HGF/SF addition, and sustained
phosphorylation of both residues was observed up to about 6 h. In EGF-treated cells, the phosphorylation level on both residues in Akt reached maximum earlier (2 min) and then rapidly declined to the unstimulated level. Fig. 5B shows that the HGF/SF-induced phosphorylation of Akt was inhibited by LY294002, suggesting that the Akt phosphorylation occurs downstream of PI3-K activation.

To explore whether HGF/SF exerted its antiapoptotic action against ADR through activation of Akt, we tested the effect of 1L-6-hydroxymethyl-chiro-inositol 2-(R)-2-O-methyl-3-O-octadecycarbonate, a selective Akt inhibitor, on the antiapoptotic action of HGF/SF. Cells were treated with the inhibitor and HGF/SF, exposed to ADR, and examined for the ADR-induced cytotoxicity (Fig. 6, A and B). In the absence of ADR, the Akt inhibitor had no effect on cell viability or DNA fragmentation whether HGF/SF was present or not. However, the inhibitor decreased the degree of HGF/SF-mediated protection against 20 μM ADR. To confirm the specific action of the inhibitor on Akt activity, we prepared cell lysates from HGF/SF-treated MKN74 cells and performed an in vitro Akt kinase assay. As shown in Fig. 6C, when the cells were stimulated with HGF/SF for 5, 15, or 30 min, increased phosphorylation of the GSK-3 fusion protein was observed, indicating an increase in Akt activity in response to HGF/SF. However, pretreatment with the Akt inhibitor blocked the HGF/SF-induced Akt activation. In contrast to the effect on Akt activity, the inhibitor had no effect on HGF/SF-induced PI3-K phosphorylation or MAP kinase phosphorylation (Fig. 6, D and E).

The apoptotic pathway is activated by a family of cysteine-dependent aspartate-specific proteases known as the caspases. Cardone et al. (60) reported that Akt induced the phosphorylation of pro-caspase-9, thereby inhibiting its protease activity. Therefore, we examined the phosphorylation of pro-caspase-9 by HGF/SF or EGF. Cells were incubated in the presence of each growth factor for several times, and cell lysates were then prepared from these cells to determine the phosphorylation of pro-caspase-9 by immunoblotting (Fig. 7A). In the same way that the phosphorylation of Akt was affected by these growth factors, HGF/SF produced sustained phosphorylation of pro-caspase-9 at least up to 6 h, but EGF induced only transient phosphorylation of it. We next determined the time course for the conversion of pro-caspase-9 into caspase-9 after exposure of MKN74 cells to ADR. Immunoblot analysis, using an antibody that detected both pro-caspase-9 and one of the cleaved caspase-9 fragments, revealed that ADR treatment decreased the pro-caspase-9 level and concomitantly caused the appearance of a cleaved caspase fragment (Fig. 7B, top). A similar ADR-induced decrease in the pro-caspase-9 level was seen in EGF-pretreated cells. However, HGF/SF pretreatment suppressed the ADR-induced cleavage of pro-caspase-9. One of the major downstream substrates for activated caspase-9 is PARP. Along with the activation of caspase-9, full-length PARP (112 kDa) was decreased by ADR in a time-dependent manner (Fig. 7B, bottom). This ADR-induced cleavage of PARP was blocked by pretreatment with HGF/SF but not by that with EGF. The HGF/SF-induced suppressive effects on the cleavage of pro-caspase-9 and PARP were completely inhibited by pretreatment with LY294002, suggesting that this suppression is dependent on PI3-K/Akt activity (Fig. 7C). Fig. 7D shows that pro-caspase-9 phosphorylation induced by HGF/SF was also inhibited by the Akt inhibitor, suggesting that the Akt-dependent pathway is involved in the protection of MKN74 cells against ADR through the phosphorylation of pro-caspase-9.

EGF transiently activated the PI3-K/Akt signaling pathway. In contrast, HGF/SF activated the signaling pathway for a longer period. Both growth factors elicit their biological activities through the activation of their intrinsic receptors induced by autophosphorylation in response to these factors. We reported previously that HGF/SF and EGF induced autophosphorylation of their specific receptors in MKN74 cells (61). Therefore, the differential action of both growth factors on the PI3-K/Akt signaling pathway must be ascribed to processes after the receptor autophosphorylation. To examine the association of the autophosphorylated receptors with PI3-K, we treated cells with HGF/SF or EGF and then immunoprecipitated the cell lysates with anti-HGF/SF receptor antibody or anti-EGF receptor antibody. Immunoblot analysis revealed that the regulatory subunit of PI3-K was detected in neither of the immunoprecipitates (data not shown). Many reports have shown that adapter proteins such as Gab1 (36) or c-Cbl (62) are necessary for the association between tyrosine kinase receptors and PI3-K. We next incubated MKN74 cells with 10 ng/ml HGF/SF or 10 ng/ml EGF for various times and evaluated the association between PI3-K and Gab1 or c-Cbl. As shown in Fig. 8A, PI3-K was co-precipitated with Gab1 in HGF/SF-pretreated cells but not detected in EGF-pretreated cells. Furthermore, tyrosine phosphorylation of Gab1 occurred concomitantly with associa-
tion between PI3-K and Gab1 in HGF/SF-treated cells. Fig. 8B shows that maximal tyrosine-phosphorylation of c-Cbl occurred 15 min after EGF stimulation but became maximal at 120 min or later after HGF/SF stimulation. However, no association between c-Cbl and PI3-K was detected in cells treated with either HGF/SF or EGF (data not shown). It thus seems that PI3-K is activated via its association with the Gab1 adaptor protein.

c-Cbl plays an essential role in the ligand-induced ubiquitination of receptor tyrosine kinases (63). This mechanism participates in the down-regulation of tyrosine kinase receptors and thus in the loss of their function. Earlier activation of c-Cbl in EGF-treated cells (see Fig. 8B) may indicate that the EGF receptor is down-regulated earlier than the HGF/SF receptor after stimulation with growth factors. To explore this possibility, we treated cells with EGF or HGF/SF for different times, labeled the cells with sulfo-NHS-biotin, and immunoprecipitated the receptors in the cell lysates with antibody against HGF/SF receptor or EGF receptor. Immunoblot analysis showed, in fact, that the level of biotin-labeled EGF receptor had been attenuated at 15 min but that the HGF/SF receptor remained on the cell surface for at least 30 min (Fig. 9). This prolonged presence of HGF/SF receptors on the cell surface could endow MKN74 cells with resistance to ADR-induced apoptosis through long-lasting activation of the PI3-K/Akt signaling pathway.

**DISCUSSION**

Diverse chemotherapeutic drugs can kill tumor cells by activating apoptotic pathways. The resistance to apoptosis can be acquired by cancer cells through a variety of strategies and is a major cause of treatment failure in malignancies. The most commonly occurring loss of a proapoptotic regulator through mutation involves the p53 tumor suppressor gene. The functional inactivation of its product, the p53 protein, results in the removal of a key component of the DNA damage sensor that can induce the apoptotic effector cascade (64, 65). In addition, recent studies suggest that certain growth factors and cytokines can suppress apoptosis of cancer cells (55, 56). In this study, we showed that HGF/SF possessed the ability to protect MKN74 cells from ADR-induced apoptosis. On the other hand, EGF did not affect the ADR-induced apoptosis in the same cells. Because HGF/SF and HGF/SF receptor are overexpressed during the progression from noninvasive tumors to invasive carcinomas and their overexpression may contribute to resistance against apoptosis, it is important to understand how
induced apoptosis was inhibited by the caspase-9 inhibitor. HGF/SF contributes to resistance to chemotherapeutic drugs.

The intracellular machinery responsible for apoptosis depends on a family of caspases. Caspase-9 is a key caspase involved in ADR-induced apoptosis (66). In fact, ADR induced caspase-9 activation in ADR-sensitive MKN74 cells, and ADR-induced apoptosis was inhibited by the caspase-9 inhibitor, which stimulates the release of cytochrome c through a heterodimer formation of Bad/Bcl-XL. Because phosphorylation of Bad sequesters Bad from Bcl-XL, Akt may function to promote survival through the phosphorylation and inactivation of Bad (47, 68, 69). However, this possibility is not likely, because ADR induced an increase in the amount of cytosolic cytochrome c in MKN74 cells, but this increase was not changed by the pretreatment with HGF/SF (data not shown).

One of these targets is Bad, a pro-apoptotic Bcl-2 family member, which stimulates the release of cytochrome c through a heterodimer formation of Bad/Bcl-XL. Because phosphorylation of Bad sequesters Bad from Bcl-XL, Akt may function to promote survival through the phosphorylation and inactivation of Bad (47, 68, 69). However, this possibility is not likely, because ADR induced an increase in the amount of cytosolic cytochrome c in MKN74 cells, but this increase was not changed by the pretreatment with HGF/SF (data not shown).

Another mechanism whereby Akt functions to promote survival is through the phosphorylation and inactivation of procaspase-9, because Akt has been found to phosphorylate procaspase-9 and thereby inhibit its protease activity (60). In the present study, HGF/SF, but not EGF, induced prolonged phosphorylation of procaspase-9, and this phosphorylation was suppressed to the control level in the presence of an Akt inhibitor. Thus, it seems that the primary mechanism used by Akt to prevent apoptosis is Akt-induced caspase-9 phosphorylation. Furthermore, prolonged, but not transient, activation of P13K/Akt pathway may be essential for HGF/SF to transmit its protective signal against ADR-induced apoptosis.

Our immunoblot analysis using antibody against biotin-labeled HGF/SF receptor or EGF receptor revealed that the HGF/SF receptor was present on the cell surface for a longer period of time than was the EGF receptor. c-Cbl plays an essential role in the ligand-induced ubiquitination of tyrosine kinase receptors and participates in the down-regulation of these receptors (63). c-Cbl has been shown to be a major substrate of protein tyrosine kinases after activation of a broad
range of cell-surface receptors (reviewed in Ref. 70). In this study, tyrosine phosphorylation of c-Blk continued for longer period in response to HGF/SF than in response to EGF. We do not know now whether tyrosine phosphorylation of c-Blk proteins affects their ubiquitination activity for tyrosine kinase receptors. However, it is possible that the HGF/SF-induced sustained phosphorylation of c-Blk enables HGF/SF receptors to escape receptor degradation by the 20S proteasome.

Gab1 has been identified as a multisubstrate adaptor protein of the insulin-responsive substrate 1 family that associates with phosphotyrosine residues in receptors such as the HGF/SF receptor and the EGFR receptor (30, 36). Gab1 contains multiple Tyr phosphorylation sites that function as binding sites for Src homology 2 domains of a variety of signaling proteins, including PI3-K. Therefore, Tyr phosphorylation sites located in the Gab1 protein as well as in tyrosine kinase receptors are able to interact with PI3-K (26, 36). When cell lysates prepared from MKN74 cells were immuno precipitated with antibody against Gab1 or phosphotyrosine and immunoblotted with antibody against Gab1 or phosphotyrosine and immunoblotted with anti-P13-K antibody, a similar amount of P13-K was recovered in both immunoprecipitates, indicating that tyrosine phosphorylation of P13-K occurred mainly through its association with Gab1 in MKN74 cells.

HGF/SF has been reported to protect a variety of tumor cells against apoptosis induced by DNA-damaging agents, including ADR (15–19). In these reports, Bcl-XL, an antiapoptotic protein against apoptosis induced by DNA-damaging agents, including anticancer drugs.

The authors concluded that sustained phosphorylation of c-Blk enables HGF/SF receptors to escape receptor degradation by the 20S proteasome. However, it is possible that the HGF/SF-induced sustained phosphorylation of c-Blk affects their ubiquitination activity for tyrosine kinase receptors, but not known now whether tyrosine phosphorylation of c-Blk proteins affects their ubiquitination activity for tyrosine kinase receptors. Therefore, Tyr phosphorylation sites located in the Gab1 protein as well as in tyrosine kinase receptors are able to interact with PI3-K (26, 36). When cell lysates prepared from MKN74 cells were immuno precipitated with antibody against Gab1 or phosphotyrosine and immunoblotted with anti-P13-K antibody, a similar amount of P13-K was recovered in both immunoprecipitates, indicating that tyrosine phosphorylation of P13-K occurred mainly through its association with Gab1 in MKN74 cells.

HGF/SF has been reported to protect a variety of tumor cells against apoptosis induced by DNA-damaging agents, including ADR (15–19). In these reports, Bcl-XL, an antiapoptotic protein related to Bcl-2, is suggested as a component of the protective mechanism. However, our study indicated that HGF/SF treatment changed the level of neither Bcl-2 nor Bcl-XL. Recently, it was shown that insulin-like growth factor-1, but not interleukin-6, stimulated sustained activation of Akt and nuclear factor-κB (33). The authors concluded that sustained activation of these molecules caused up-regulation of anti-apoptotic protein such as XIAP and cIAP-2. However, we investigated the effect of HGF/SF and EGF on expression of XIAP and cIAP-2 in MKN74 cells, we observed no effect on their expression levels (data not shown). A variety of growth factors are intimately associated with resistance of tumor cells to apoptosis, but there seem to be a variety of mechanisms by which growth factors control tumor cells with resistance to anticancer drugs.

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