THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 28, Issue of July 12, pp. 25203–25208, 2002

Printed in U.S.A.

Hepatocyte Growth Factor Inhibits Anoikis in Head and Neck Squamous Cell Carcinoma Cells by Activation of ERK and Akt Signaling Independent of NFκB*

Received for publication, February 15, 2002, and in revised form, April 16, 2002
Published, JBC Papers in Press, May 6, 2002, DOI 10.1074/jbc.M201598200

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Hepatocyte growth factor (HGF), also known as a scatter factor, regulates a variety of biological activities including cell proliferation, survival, migration, and angiogenesis. Importantly, HGF and its receptor c-Met have been found to be associated with metastasis of human head and neck squamous cell carcinoma (HNSCC). Because anoikis resistance plays an important role in tumor progression and metastasis, here we examined whether HGF suppressed suspension-induced apoptosis (anoikis) in HNSCC cells, and if so, we assessed downstream signaling pathways mediated by HGF. We found that HNSCC cells underwent anoikis upon loss of matrix contact, whereas HGF provided protection against it. HGF-induced anoikis resistance was found to be dependent on both ERK and Akt signaling pathways. The inhibition of either ERK or Akt activation abolished HGF-mediated survival. Furthermore, we found that HGF did not activate NFκB transcription in HNSCC cells and that HGF-mediated anoikis resistance was independent of NFκB. Taken together, our results suggest that anoikis resistance induced by HGF may also play an important role in the progression and metastasis of HNSCC.

HGF[1] is a pleiotropic growth factor that regulates cell proliferation, migration, survival, angiogenesis, and invasion (1–6). These diverse biological effects of HGF are mediated through its interaction with its high affinity tyrosine kinase receptor, c-*met* proto-oncogene (7). It is well known that HGF activates two important kinase cascades, the extracellular signal-related kinase (ERK) and the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways (4, 8–10). The ERK pathway plays an important role in stimulating cell proliferation, differentiation, survival, and growth. The activation of ERK is mediated by a cascade of phosphorylation events. Activated ERK can phosphorylate a variety of substrates including transcription factors, kinases, and phosphatases (11–15). The activation of the PI3K/Akt pathway has been shown to play an important role in cell survival induced by growth factors and oncogenes. Multiple pro-apoptotic and anti-apoptotic proteins have been shown to be modified by the activation of Akt (10, 11, 15). Additionally, HGF activates the stress-responsive transcription factor, nuclear factor κB (NFκB), in several human cancer cell lines (16). NFκB transcriptionally regulates a broad spectrum of genes that play an important role in cell cycle progression, survival, and inflammation (17, 18).

Importantly, growing evidence has demonstrated that the expression of HGF and/or c-Met plays an important role in tumor progression and metastasis (4). c-Met/HGF is found to be overexpressed in various human cancers including head and neck squamous cell carcinoma (HNSCC) and nasopharyngeal carcinoma (4, 19–23). Clinical studies have found that the c-Met protein expression level was inversely correlated with survival in patients with large-stage nasopharyngeal carcinoma (22). Serum HGF in head and neck cancer patients was significantly increased compared with healthy control subjects (19–20). HGF strongly induces the expression of angiogenic factors interleukin-8 and vascular endothelial growth factor in HNSCC cells, suggesting that HGF promotes tumor development by induction of angiogenesis (20). Matsumoto *et al.* (24) found that HGF induced tyrosine phosphorylation of focal adhesion kinase and promoted the migration and invasion by oral SCC cells (24). Moreover, an increase of c-Met has been found in lymph node metastases of HNSCC compared with primary tumors, which suggested that the Met receptor HGF ligand signaling may play a critical role in promoting the metastasis of HNSCC (25). In support of this notion, several rodent and human model systems have demonstrated that Met-HGF signaling induces metastatic behavior *in vivo* by stimulating cell invasion and angiogenesis (4, 26, 27).

In addition to gaining functions of invasion and angiogenesis, cell resistance to anoikis also appears to play an important role in tumor progression and metastasis as tumor cells lose matrix attachment during metastasis (28). Anoikis, also known as suspension-induced apoptosis, is a term used to describe programmed cell death (apoptosis) of epithelial cells induced by loss of matrix attachment. This process is important for maintaining normal cell and tissue homeostasis (28). Anoikis frequently occurs in adult organisms during regeneration of skin

* This work was supported by National Institutes of Health Research Grants R01-DE13848 and R01-DE13788 (to C.-Y. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HGF, hepatocyte growth factor; PI3K, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; HNSCC, head and neck squamous cell carcinoma; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; EMSA, electrophoretic mobility shift assay; IκBα, inhibitor of κBα; NFκB; nuclear factor κB; SR-IκBα, super-repressor form of IκBα; SCC, squamous cell carcinoma; TNF, tumor necrosis factor.

or colonic epithelia or during involution of the mammary gland following weaning (28–33). The precise signaling cascade that links caspase activation and cell adhesion has not been elucidated. Recently, the death receptor signaling pathway has been found to be required for the induction of anoikis. The expression of a dominant-negative mutant of FAS-associated death domain protein, FADD, blocks caspase activation in anoikis (29, 30). Gaining anoikis resistance or anchorage-independent survival is a hallmark of oncogenic transformation. For example, oncogenic Ras has been shown to confer resistance to anoikis in epithelial cells and to promote anchorage-independent growth. The ERK signaling pathway or the PI3K/Akt signaling pathway has been found to inhibit a loss of contact-induced apoptosis in rat intestinal cells or Madin-Darby canine kidney epithelial cells (28, 33). Although significant progress has been made in understanding apoptotic signaling mediated by chemotherapeutic drugs and irradiation, little is known regarding regulation of anoikis by growth factors. Moreover, many studies on anoikis are performed in primary or immobilized rodent cells, which may be irrelevant to human cancers (28–30, 33). Given the fact that the inhibition of anoikis is critical for tumor metastasis because tumor cells lack appropriate cell matrix contacts while traversing the blood and lymph system during metastasis, new findings involved in the regulation of anoikis of human tumor cells may provide new insight into mechanisms of tumor metastasis.

Because c-Met-HGF signaling is clinically associated with metastasis of HNSCC cells (24, 25), we performed experiments to determine whether HGF provided protection against apoptosis induced by a loss of matrix attachment. We found that HNSCC cells underwent apoptosis when placed in suspension growth conditions, whereas HGF provided potent protection against it. To explore the molecular mechanisms by which HGF inhibited anoikis, the activation of ERK, Akt, and NFκB by HGF was evaluated in HNSCC cells. We found that HGF-induced anoikis resistance was dependent on both ERK and Akt signaling pathways. The inhibition of either ERK or Akt with a specific chemical inhibitor was sufficient to abolish HGF-mediated anoikis resistance. Moreover, we found that in contrast to other cell types HGF could not stimulate NFκB transcription in HNSCC cells and that HGF-mediated survival was independent of NFκB. Taken together, our results suggest that the inhibition of HNSCC cell anoikis appears to be one of the mechanisms by which HGF promotes HNSCC metastasis.

EXPERIMENTAL PROCEDURES

Cell Culture and Retrovirus Transduction—Human HNSCC cell lines HNSCC1, HNSCC23, and HNSCC14A were derived from SCC of the head, neck, and oral cavity at The University of Michigan (Ann Arbor, MI). Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 unit/ml), and streptomycin (100 μg/ml). Chemical inhibitors used in this study for MEK1/2 (U0126) were purchased from Promega, and PI3K inhibitor LY294002 was purchased from Calbiochem. Retroviruses were generated by transfecting the retroviral construct encoding super-repressor IκBα (SR-IκBα) into 293T cells by the calcium phosphate method. The retrovirus-containing supernatant was harvested 48 h later and stored at −70 °C. To stably express SR-IκBα, cells were infected with retroviruses in the presence of 6 μg/ml polybrene. Forty-eight hours after infection, cells were selected with puromycin (1.5 μg/ml) for 1 week. The resistant clones were pooled and confirmed by Western blot analysis.

Anoikis Induction, Cell Death Enzyme-linked Immunosorbent Assay, Trypan Blue Exclusion Assay, and DNA Laddering—For induction of anoikis, cells were plated on 0.6% soft agar with or without HGF (40 ng/ml) (R&D Systems) in the presence of growth medium as described previously (34). 48–72 h after cell suspension, the cells were collected, washed in phosphate-buffered saline, and any cell aggregates were dispersed by trypsinization. Cell viability was determined by the trypan blue exclusion analysis. For cell death enzyme-linked immunosorbent assay, cell supernatants were collected and incubated with monoclonal antibodies against DNA and histone according to the manufacturer instructions. The reaction was measured with a plate reader at the wavelength of 405 nm. To examine DNA laddering, cells were lysed, and the soluble fraction was extracted with phenol-chloroform (Fisher Scientific). Fragmented DNA was separated on a 1.2% agarose gel as described previously (35).

Western Blot Analysis—Cells were harvested, washed with ice-cold phosphate-buffered saline, and pelleted. Whole cell lysates were prepared with radioimmune precipitation buffer containing 1% Nonidet P-40, 5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 100 mM sodium orthovanadate, 1:100 protease inhibitors mixture (Sigma). The protein concentrations were determined using the Bradford protein assay (Bio-Rad). The protein extracts were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane by electroblotting (Bio-Rad). The membranes were blocked with 5% nonfat dry milk, 1× TBST (25 mM Tris-HCl, 125 mM NaCl, 0.1% Tween 20) overnight at 4 °C and probed with primary antibodies for 1 h and then with horseradish peroxidase-conjugated secondary antibodies for 1 h. The immune complexes were visualized using the ECL kit (Amersham Biosciences) according to protocol of the manufacturer. For internal control, the blots were stripped with Tris buffer (62.5 mM, pH 8.0) containing 100 mM 2-mercaptoethanol, 2% SDS at 60 °C for 1 h and reprobed with an anti-α-tubulin monoclonal antibody. Primary antibodies were purchased from the following commercial sources. Polyclonal phospho-specific antibodies against ERK1/2 (1:1000), Akt (1:1000), and IκBα were from Cell Signaling. Monoclonal antibodies against α-tubulin (1:7500) were from Sigma, and secondary antibodies against rabbit or mouse IgG (1:7500) were from Promega.

Transfection, κB-dependent Luciferase and Electrophoretic Mobility Shift Assays (EMSA)—0.5×10^5 cells were plated in 6-well plates in triplicates. Cells were transfected using LipofectAMINE according to the recommendations by the manufacturer (Invitrogen). Plasmids were mixed with LipofectAMINE (1:4 ratio) in Opti-MEM I medium (Invitrogen), and complexes were incubated for 30 min at room temperature. For internal control, pRL-TK *Renila* luciferase reporter was co-transfected to normalize for transfection efficiency. The DNA-LipofectAMINE mixtures were added to the cells and incubated for 6 h at 37 °C. After incubation, cells were replenished with fresh medium. Twenty-four hours after transfection, cells were treated with HGF for an additional 24 h. Cells were then washed with 1× ice-cold phosphate-buffered saline and lysed in lysis buffer (Promega). Luciferase activities were measured using a dual luciferase system (Promega). For EMSA, cells were treated with TNF (20 ng/ml), HGF (40 ng/ml), or TNF plus HGF for the indicated times. Nuclear protein extraction and EMSA were performed as described previously (40).

RESULTS

HGF Suppressed Anoikis in HNSCC Cells—To induce anoikis, HNSCC1 cells were cultured on a cushion of 0.6% agar to prevent cell attachment as described previously (34). As shown in Fig. 1*A*, a loss of cell adhesion strongly induced DNA fragmentation and histone release in HNSCC1 cells as determined by cell death enzyme-linked immunosorbent assay. In contrast, significantly less DNA fragmentation and histone

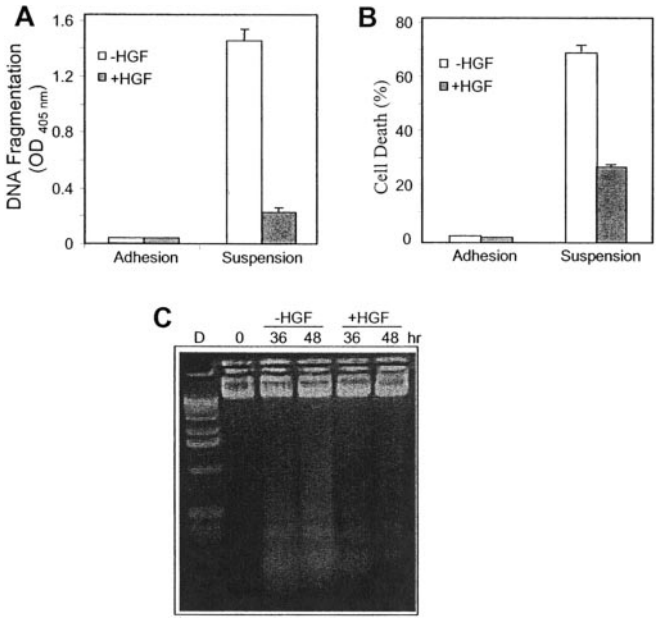

FIG. 1. **HGF suppresses apoptosis induced by loss of matrix contact in HNSCC1 cells.** *A*, HGF inhibited DNA fragmentation and histone release from HNSCC1 cells in suspension. HNSCC1 cells were plated on a cushion of 0.6% agar to prevent cell adhesion and were treated with or without human HGF (40 ng/ml) for 72 h. Control cells were seeded on tissue culture plates (adhesion condition) and treated with or without HGF (40 ng/ml). After treatment, cell supernatants were collected and stored at −70 °C. 20 μl of each sample was assessed by cell death enzyme-linked immunosorbent assay according to the instructions by the manufacturer. The reactions were quantitated with a plate reader by measuring the optical density at 405 nm. The assays were performed in duplicate, and the result represents one of four independent experiments. The *error bars* represent the mean ± S.D. *B*, HGF inhibited anoikis in HNSCC1 cells. The induction of anoikis and HGF stimulation were performed as described in *A*. The cell viability was determined with the trypan blue exclusion assay. The assays were performed in duplicate, and the result represents one of four independent experiments. The *error bars* represent the mean ± S.D. *C*, detection of apoptosis by DNA laddering. HNSCC1 cells were incubated with or without HGF (40 ng/ml) in suspension for the indicated time periods. Cells were then lysed, and soluble fractions were extracted with phenol-chloroform. Fragmented genomic DNA was separated on a 1.2% agarose gel as described previously (35).

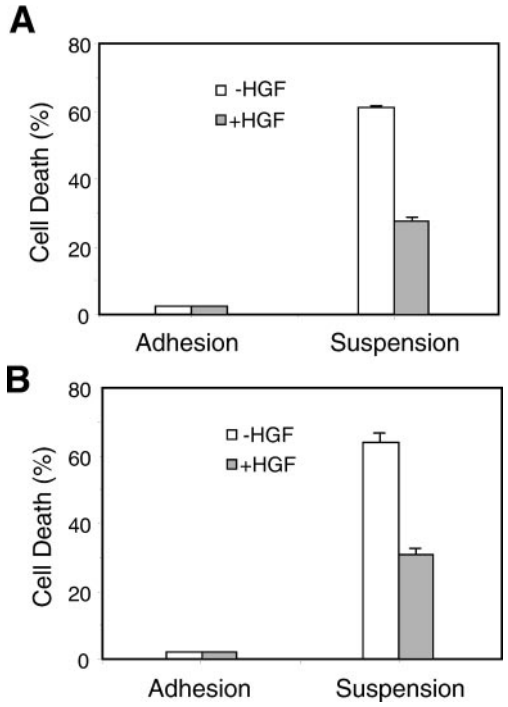

FIG. 2. **HGF inhibits anoikis in HNSCC14A and HNSCC23 cells.** *A*, HGF rescued HNSCC14A cells from anoikis. The induction of anoikis and HGF treatment were performed as described in Fig. 1*A*. Cell viability was determined with the trypan blue exclusion assay. *B*, HGF rescued HNSCC23 cells from anoikis.

release (over 3-fold) was detected when HNSCC1 cells were treated with HGF. Through trypan blue exclusion assay, we found that over 70% of cells were dead 72 h after a loss-of-matrix contact, but only 30% of cells treated with HGF were dead. Because some dead cells were rapidly lysed, the percentage of cell death in HNSCC1 without HGF treatment might be underscored. To further confirm that induction of anoikis in HNSCC1 cells was through the activation of an apoptotic program, we also isolated genomic DNA to examine the DNA laddering, a hallmark of apoptosis (36, 37), by agarose gel analysis. As shown in Fig. 1*C*, HGF stimulation also significantly inhibited the formation of DNA laddering. Additionally, we also determined whether HGF suppressed anoikis in other HNSCC cell lines. As shown in Fig. 2, *A* and *B*, HGF stimulation rescued both HNSCC14A and HNSCC23 cells from anoikis, respectively. Taken together, our results demonstrated that HGF provided protection against cell detachment-induced apoptosis.

Inhibition of Anoikis by HGF Was Independent of NFκB Transcription—HGF is known to activate several key signaling pathways including the NFκB pathway (16). NFκB is an important transcription factor that regulates cell proliferation and survival (17, 18). Studies have suggested that HGF activates the NFκB signaling pathway to stimulate cell growth and angiogenesis by induction of interleukin 8 (20). We and others have previously found (38–47) that NFκB plays an important role in the inhibition of TNF-mediated apoptosis. Because the death receptor signaling pathway has been demonstrated to be involved in anoikis (39, 30), we questioned whether HGF-mediated protection was dependent on NFκB activation. First, we determined whether HGF stimulated NFκB activation in HNSCC cells. Under most unstimulated conditions, NFκB is retained in the cytoplasm by IκB inhibitory family proteins. Upon stimulation, the IκB kinase complex is activated to phosphorylate IκBα, resulting in ubiquitination and degradation of IκBα. The liberated NFκB is then translocated to the nucleus where it activates gene transcription (17–18). Because IκB kinase activation plays an essential role in NFκB activation, we performed Western blot analysis to examine IκB kinase-mediated IκBα phosphorylation following HGF stimulation. As shown in Fig. 3*A*, *top panel*, no significant phosphorylation of IκBα was detected following HGF treatment. Also, HGF treatment did not induce IκBα degradation (Fig. 3*A*, *middle panel*). As a positive control, TNF treatment rapidly induced the phosphorylation and degradation of IκBα (Fig. 3*A*). Furthermore, HGF treatment did not stimulate NFκB reporter activity (Fig. 3*B*), indicating that HGF did not modify NFκB transcription.

The results above suggest that HGF-mediated protection is independent of NFκB. As a further confirmation, we established an HNSCC1 cell line (HNSCC1I) stably expressing NFκB inhibitor, SR-IκBα, by retroviral transduction (Fig. 3*C*). A control cell line (HNSCC1V) expressing empty vector was also obtained. Because SR-IκBα was FLAG-tagged, the molecular weight of SR-IκBα was slightly larger than that of endogenous IκBα. SR-IκBα, which contains two mutations at serine 32 and serine 36, cannot be phosphorylated by the IκB kinase complex and subsequently degraded by proteasomes, thereby preventing the nuclear translocation of NFκB. The inhibition of NFκB by SR-IκBα renders various cell types sensitive to TNF-mediated apoptosis (39–42). Because the promoter of IκBα is

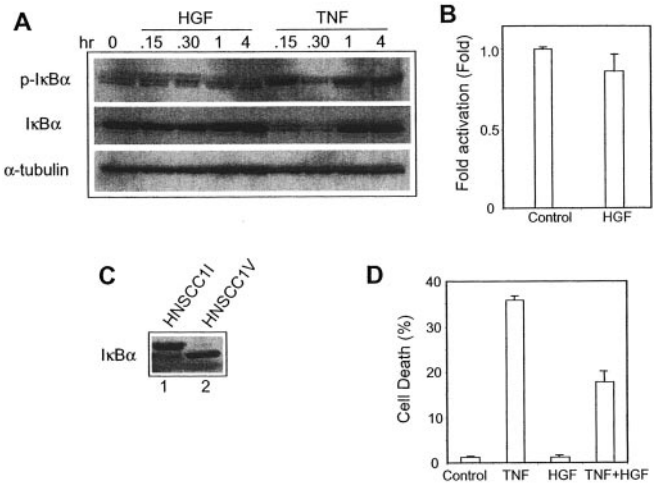

FIG. 3. **HGF does not activate the NFκB survival pathway in HNSCC cells.** *A*, HGF did not induce phosphorylation and degradation of IκBα in HNSCC1 cells. HNSCC1 cells were treated with HGF (40 ng/ml) or TNF (20 ng/ml) for the indicated times. The whole cell extracts were prepared with radioimmune precipitation buffer, and 50-μg aliquots of protein extracts were separated on a 12% SDS-PAGE gel. The blots were probed with polyclonal antibodies against phospho-specific IκBα (*top panel*) or non-phosphorylated IκBα (*middle panel*). To assess the equivalency of loading, the blots were stripped and reprobed with an anti-α-tubulin (1:5000) monoclonal antibody. *B*, HGF did not stimulate NFκB transcription. HNSCC1 cells were transfected with a κB-dependent luciferase reporter with LipofectAMINE. 24 h after transfection, cells were stimulated with HGF (40 ng/ml). Luciferase activities were determined as described under "Experimental Procedures." *C*, the establishment of HNSCC1 cells stably expressing SR-IκBα. HNSCC1 cells were transduced with retroviruses expressing SR-IκBα or control empty vector. Forty-eight hours after infection, cells were selected with puromycin (1.5 μg/ml) for 2 weeks, and the resistant clones were pooled. HNSCC1 cells stably expressing SR-IκBα (HNSCC1I) or control empty vector (HNSCC1V) were assessed for IκBα expression by Western blot analysis. *D*, HGF inhibited TNF killing independent of NF-κB. HNSCC1I cells were pretreated with or without HGF (40 ng/ml) for 2 h and then treated with TNF (20 ng/ml) for 24 h. Cell viability was determined with the trypan blue exclusion assay.

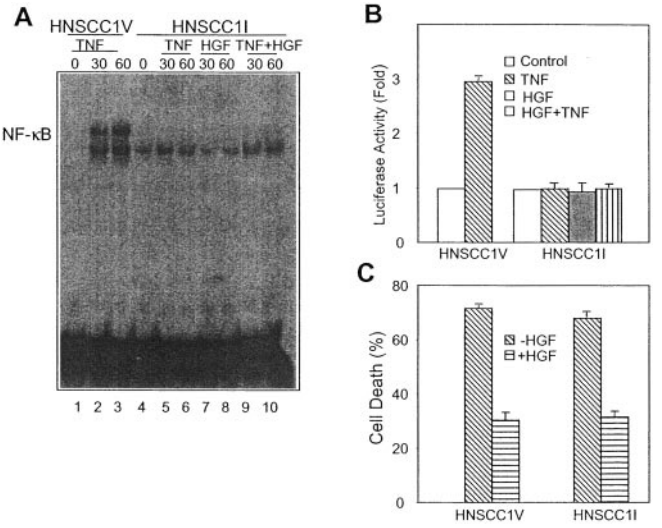

FIG. 4. **Inhibition of anoikis by HGF is independent of NFκB activation.** *A*, HGF or TNF did not induce the nuclear translocation of NFκB in HNSCC1I cells. Cells were treated with TNF (20 ng/ml), HGF (40 ng/ml), or TNF plus HGF for the indicated times. The nuclear extracts were prepared, and 5-μg aliquots of proteins were incubated with ^{32}P-labeled NFκB DNA probe. The binding complexes were resolved by 5% polyacrylamide gel electrophoresis and exposed to film. *B*, HGF did not activate NFκB transcription in HNSCC1I cells. Cells were transfected with a κB-dependent luciferase reporter with LipofectAMINE. 24 h after transfection, cells were stimulated with HGF (40 ng/ml), TNF (20 ng/ml), or TNF plus HGF for 24 h. Luciferase activities were determined as described under "Experimental Procedures." *C*, HGF inhibited anoikis independent of NF-κB. The induction of anoikis and HGF stimulation in both HNSCC1V and HNSCC1I was performed as described in Fig. 1*A*. The assays were performed in duplicate, and the results present average values from three independent experiments. The *error bars* represent the mean ± S.D.

regulated by NFκB, the expression of SR-IκBα significantly reduced the expression of endogenous IκBα (Fig. 3*C*). Consistent with our previous studies (40, 44), HNSCC1I cells were sensitive to TNF killing (Fig. 3*D*), suggesting that SR-IκBα functioned in HNSCC1 cells. Interestingly, the pretreatment with HGF significantly blocked TNF killing in HNSCC1I cells, indicating that HGF-mediated protection against TNF killing was independent of NFκB (Fig. 3*D*).

To demonstrate that HGF or TNF was unable to activate NFκB in HNSCC1I cells, EMSA was performed. As shown in Fig. 4*A*, TNF rapidly induced the nuclear translocation of NFκB in control cells (HNSCC1V) but not in HNSCC1I cells (compare *lanes 1–3* with *lanes 4–6*). EMSA supershifts confirmed the specificity of the NFκB-containing complex (data not shown). HGF or HGF plus TNF stimulation failed to induce the nuclear translocation of NFκB in UM-SCC1I cells (*lanes 7–10*). Moreover, the NFκB-dependent luciferase reporter assay verified that HGF, TNF, or TNF plus HGF did not activate NFκB transcription in HNSCC1I cells (Fig. 4*B*). Finally, we examined whether HGF could inhibit anoikis in HNSCC1I cells. As shown in Fig. 4*C*, HGF stimulation suppressed anoikis in both HNSCC1V and HNSCC1I cells at the similar level. Taken together, these results suggest that the inhibition of anoikis by HGF was independent of NFκB activation.

HGF Activated Both ERK and Akt Signaling Pathways to Suppress Anoikis—We next examined whether the ERK or PI3K/Akt signaling pathway was involved in HGF-mediated inhibition of anoikis in HNSCC cells, because HGF activated ERK and Akt in several human cancer cell lines including HNSCC cell lines (8–10, 20). To determine whether ERK was activated, HNSCC1 cells were treated with HGF for different periods. Whole cell extracts were prepared and immunoblotted with antibodies specifically recognizing the phosphorylated and active forms of ERK1/2. As shown in Fig. 5*A* (*top panel*, *lanes 1–5*), HGF stimulation rapidly activated the phosphorylation of ERK1/2 as detected by phospho-specific antibodies against ERK1/2, whereas total ERK proteins remained unchanged (*middle panel*, *lanes 1–5*). To determine whether ERK activation played a role in HGF-mediated inhibition of anoikis, we utilized a specific MEK1/2 inhibitor, U0126, to block the activation of ERK. As shown in Fig. 5*A* (*top panel*, *lanes 11–15*), pretreatment with U0216 totally abolished HGF-induced ERK phosphorylation, whereas a PI3K/Akt inhibitor LY294002 had no effects on it (*lanes 6–10*). To ensure that U0126 functioned in cells, anoikis was induced for only 48 h. As shown in Fig. 5*B*, HNSCC1 cells were resistant to anoikis following HGF stimulation. Approximately 47% cells were dead after being deprived of matrix contact. In comparison, only 22% cells treated with HGF were dead. However, when the cells were pretreated with U0126, HGF-induced protection was abolished (Fig. 5*B*). These results suggest that HGF-mediated anoikis resistance was dependent on ERK activation.

To determine whether HGF activated the PI3K/Akt signaling pathway in HNSCC1 cells, we examined Akt phosphorylation with phospho-specific Akt antibodies. As shown in Fig. 6*A* (*top panel*, *lanes 1–4*), Akt phosphorylation was induced following HGF stimulation. Pretreatment with LY29400 but not U0126 suppressed HGF-induced Akt phosphorylation (Fig. 6*A*, compare *lanes 5–8* with *lane 9–12*). Interestingly, as shown in Fig. 6*B*, pretreatment with LY294002 also significantly rendered SCC1 cells sensitive to anoikis despite HGF stimulation,

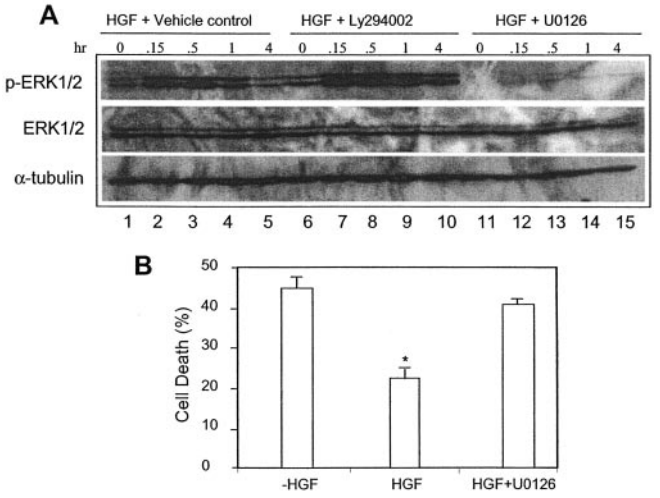

FIG. 5. **HGF inhibits anoikis by activation of ERK.** *A*, HGF induced ERK phosphorylation in HNSCC1 cells. HNSCC1 cells were treated with HGF (40 ng/ml) for the indicated times. To assess the specificity of chemical inhibitors, cells were pretreated with U0126, LY29000, or vehicle control for 30 min and then treated with HGF for the indicated times. Cells were harvested, and the whole cell extracts were prepared with radioimmune precipitation buffer. 50-μg aliquots of protein extracts were separated on a 12% SDS-PAGE gel. The blots were probed with polyclonal antibodies against phospho-specific ERK1/2 or non-phosphorylated ERK1/2. For internal control, the blots were stripped and reprobed with an anti-α-tubulin monoclonal antibody. *B*, inhibition of ERK activation abolished HGF-mediated anoikis resistance. HNSCC1 cells were pretreated with U0126 inhibitor or vehicle control for 30 min and then treated with HGF (40 ng/ml) for 48 h. Cell viability was determined with the trypan blue exclusion assay. The assays were performed in duplicate, and the results represent average values from three independent experiments. Statistical differences between each group were determined by the Student's *t* test. *, $p < 0.01$.

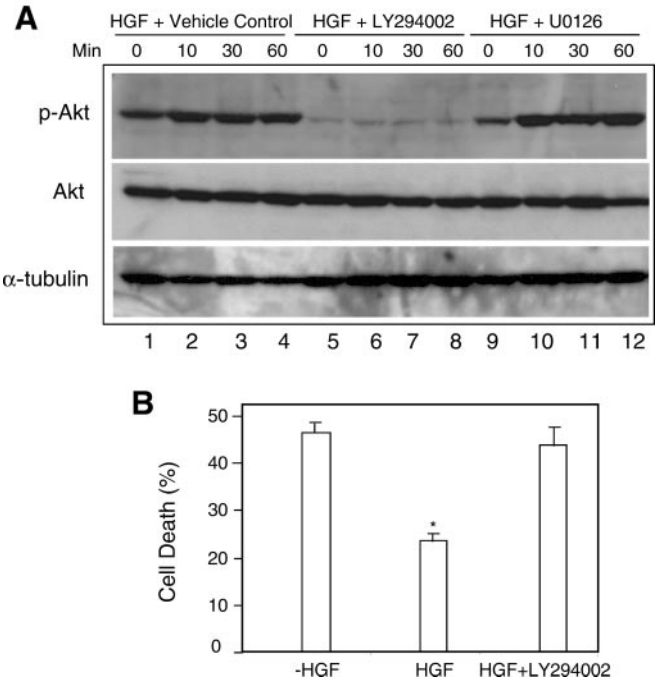

FIG. 6. **HGF inhibits anoikis by activation of Akt.** *A*, HNSCC1 cells were pretreated with the chemical inhibitor U0126, LY29000, or vehicle control for 30 min and then treated with HGF (40 ng/ml) for the indicated times. The blots were probed with polyclonal antibodies against phospho-specific Akt or non-phosphorylated Akt. For internal control, the blots were stripped and reprobed with an anti-α-tubulin monoclonal antibody. *B*, inhibition of Akt abolished HGF-mediated anoikis resistance. HNSCC1 cells were pretreated with LY294002 (10 μM) or vehicle control for 30 min and then treated with HGF for 48 h. Cell viability was determined with the trypan blue exclusion assay. The assays were performed in duplicate, and the results represent average values from three independent experiments. Statistical differences between each group were determined by the Student's *t* test. *, $p < 0.01$.

indicating that HGF-mediated anoikis resistance was also required for the PI3K/Akt signaling pathway. Taken together, these results suggest that HGF activates both ERK and Akt signaling pathways to provide protection against anoikis.

DISCUSSION

SCC is the most common epithelial tumor occurring in the head, neck, and oral cavity (49–53). It is widely accepted that the presence of lymph node metastases is the most important prognostic factor in head and neck squamous cell carcinoma (25, 26, 49, 50). The evasion of apoptosis appears to play a critical role in cell transformation and tumor development. During the late stage of human cancer development, primary tumor cells invade adjacent tissues, and some tumor cells travel to distant organs and lymph nodes where they may give rise to new tumors (54). In this regard, anoikis resistance plays an important role in promoting the survival of circulating tumor cells, which lose adhesion and matrix contact during metastasis (28). Our results suggest that in addition to invasion, HGF may promote metastasis of HNSCC by inhibiting anoikis, providing a new explanation for c-Met-HGF-mediated metastasis of HNSCC. Moreover, we have characterized the downstream signaling pathways that were involved in HGF-induced anoikis resistance. We found that HGF-mediated anoikis resistance is dependent on ERK and Akt activation but independent of NFκB transcription. These findings have important implications in intervening HNSCC metastasis.

Given the fact that several studies have demonstrated that HGF activates NFκB transcription (16), it is surprising that we found that HGF could not induce IκB kinase activity or stimulate NFκB transcription in HNSCC cells. Recently, we and others (48) have demonstrated that Akt signaling stimulated NFκB transcription by targeting the transactivation domain of the NFκB subunit RelA/p65 in several cell types. Interestingly, although HGF induced Akt activation, we could not find that HGF stimulated NFκB transcription in HNSCC cells. Currently, the underlying mechanisms responsible for this difference are unknown. The results may reflect the importance of cell context in signal transduction. It is possible that some key adaptors that link Akt to the transactivation domain of RelA/p65 might be missing in HNSCC cells. To further support our results that HGF-mediated survival is independent of NF-κB, our unpublished observation also found that NFκB-induced anti-apoptotic genes including *A1* and c-*FLIP* (46, 47) were not induced by HGF stimulation in HNSCC cells.

The ERK signaling pathway has been shown to be required for HGF-mediated scattering and proliferation (4, 9). ERK activation provides protection against apoptosis induced by growth factor and serum withdrawal (12). The role of ERK activation in the HGF-mediated anti-apoptotic function is controversial (23, 55). On one hand, HGF-induced ERK activation is required for the inhibition of apoptosis induced by chemotherapeutic drugs and UV irradiation (3, 55). In contrast, studies by Nakagami *et al.* (55) demonstrated that HGF-elicited anti-apoptotic function is independent of the ERK activation. However, in their experimental approaches, Nakagami *et al.* (55) utilized the PD098059 inhibitor, which may not completely inhibit ERK activation. In our studies, we found that ERK was markedly activated by HGF stimulation in HNSCC cells and that inhibition of ERK activation with the specific chemical inhibitor U0126 suppressed HGF-mediated anoikis resistance, supporting the fact that HGF-induced ERK activation is an important anti-apoptotic signal.

PI3K/Akt signaling is involved in diverse mechanisms to promote cell survival (10, 11, 15, 28, 48). The ectopic expression of a constitutively active form of PI3K or Akt suppresses apoptosis of epithelial cells deprived of matrix contact (28). Akt can exert its anti-apoptotic effects by phosphorylation of Bad and caspase-9. Consistent with our studies, Khwaja *et al.* (XXX)-showed that oncogenic Ras activates Akt to suppress anoikis in Madin-Darby canine kidney epithelial cells (56). In contrast, McFall *et al.* (33) found that Ras did not activate Akt in rat intestinal epithelial cells and that constitutive activation of Akt also could not provide protection against anoikis. They suggested that a PI3K- and RalGEF-independent Ras effector cooperates with ERK signaling to confer anoikis resistance (33). These results underscore the importance of cell context and stimuli. Of interest, we found that both ERK and Akt signaling pathways play an important role in anoikis resistance in HNSCC cells, which were different in immobilized epithelial cells by the studies discussed above. The inhibition of either ERK or Akt abolished HGF-mediated cell survival, indicating that ERK and Akt alone may not be enough to provide anoikis resistance in HNSCC cells. Currently, we do not know how the activation of ERK and Akt cooperatively suppress anoikis in HNSCC cells. It is possible that the ERK and Akt kinase cascade may directly modify the apoptosis machinery by inactivation of pro-apoptotic proteins through phosphorylation. Another possibility is that HGF may rescue HNSCC cells from anoikis by induction of new gene products that are dependent on both ERK and Akt signaling pathways. Although the cell receptor signaling pathway has been implicated in the regulation of anoikis, our preliminary studies found that NF-κB-induced anti-apoptotic gene products such as *A1*, c-*FLIP*, and c-*IAP2* were not regulated by HGF. Our results suggest that HGF-induced ERK and Akt signaling may regulate a distinct set of gene products to suppress anoikis. In the future, it will be interesting to identify these gene products by the gene array technique.

It is generally considered that transformed cells or cell lines derived from human cancers are anchorage-independent and therefore can survive under loss of adhesion (28, 33, 54). However, we found that in contrast to Ras-transformed cell lines or other human cancer cell lines, many types of human HNSCC cell lines underwent apoptosis upon loss of an appropriate matrix adhesion *in vitro*. Although our culture conditions might not have been optimized for the maintenance of HNSCC anchorage-independent growth *in vitro*, the results suggested that tumor environment and paracrine and autocrine factors produced by stromal cells and/or tumor cells *in vivo* may be critical for the survival and progression of HNSCC. Our findings presented here indicate that HGF might be such a survival factor for HNSCC. Most importantly, HGF has been found to be associated with HNSCC cells invasion *in vitro* and SCC metastasis *in vivo* (19–22, 24–27). Our results suggest that HGF probably enhances metastasis of human HNSCC not only by promoting invasion and angiogenesis (20, 24) but also by providing an anchorage-independent survival function for circulating tumor cells that have lost matrix contact during metastasis.

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