Insulin-like Growth Factor 1 Stimulates KCl Cotransport, Which Is Necessary for Invasion and Proliferation of Cervical Cancer and Ovarian Cancer Cells*

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The mechanisms by which insulin-like growth factor 1 (IGF-1) cooperates with membrane ion transport system to modulate epithelial cell motility and proliferation remain poorly understood. Here, we investigated the role of electroneutral KCl cotransport (KCC), in IGF-1-dependent invasiveness and proliferation of cervical and ovarian cancer cells. IGF-1 increased KCC activity and mRNA expression in a dose- and time-dependent manner in parallel with the enhancement of regulatory volume decrease. IGF-1 treatment triggers phosphatidylinositol 3-kinase and mitogen-activated protein kinase cascades leading to the activation of Akt and extracellular signal-regulated kinase1/2 (Erk1/2), respectively. The activated Erk1/2 mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways are differentially required for IGF-1-stimulated biosynthesis of KCC polypeptides. Specific reduction of Erk1/2 protein levels with small interference RNA abolishes IGF-1-stimulated KCC activity. Pharmacological inhibition and genetic modification of KCC activity demonstrate that KCC is necessary for IGF-1-induced cancer cell invasiveness and proliferation. IGF-1 and KCC co-localize in the surgical specimens of cervical cancer (n = 28) and ovarian cancer (n = 35), suggesting autocrine or paracrine IGF-1 stimulation of KCC production. Taken together, our results indicate that KCC activation by IGF-1 plays an important role in IGF-1 signaling to promote growth and spread of gynecological cancers.

The coupled electroneutral movement of K⁺ and Cl⁻ takes place at the membrane level via KCl cotransport (KCC). KCC activity plays an important role in cell volume regulation, epithelial transport, and ion homeostasis (1). Multiple activators and inhibitors regulate KCC activity through a putative kinase-phosphatase cascade (2). The cDNA products of four KCC genes have been cloned. The activities of KCC1, KCC3, and KCC4 are osmotically sensitive and involved in cell volume regulation (3–5). The neuron-specific KCC2 is critical for the maturation of inhibitory GABA responses in the central nervous system by the control of intracellular Cl⁻ concentration (6). KCC3, initially cloned from vascular endothelial cells (4), may have physiological significance in cell proliferation (7). Human cervical carcinogenesis is accompanied by increased expression of mRNA transcripts encoding KCC1, KCC3, and KCC4 (8). Furthermore, KCC is an important modulator of cervical cancer cell proliferation and invasiveness (9).

Specific growth factors significantly enhance the metastatic and invasive properties of cancer cells, which poses serious problems to the successful treatment of neoplastic disease. The insulin-like growth factor 1 (IGF-1) system performs multiple functions in the pathogenesis of most types of cancer (10). IGF-1 is the major mediator of growth hormone action. On the cellular level, IGF-1 has a strong influence on cell proliferation and potently inhibits apoptosis. Moreover, IGF-1 is also involved in angiogenesis. Each of these properties contributes to IGF-1-mediated maintenance and progression of cancer (11).

The mechanisms by which growth factor regulates plasma-membrane ion transport promotes cancer cell proliferation and invasiveness are little understood. This study tests the hypothesis that IGF-1 may promote cancer development and progression in part through its action on KCl cotransporter. The findings demonstrate that IGF-1 increases KCC expression and activity in a concentration- and time-dependent manner. KCC activity is necessary for tumor invasiveness and proliferation in vitro, and both KCC and IGF-1 expression levels correlate with tumor size in human clinical specimens.

MATERIALS AND METHODS

Cell Cultures and Creation of KCC Mutant Cell Lines—The human cervical cancer cell line SiHa and the human ovarian cancer cell line OVCAR-3 were obtained from the American Type Culture Collection (Manassas, VA). The removal of 117 amino acids from the N-terminal cytoplasmic domain of mouse KCC1 (KCC1ΔN117) confers a dominant-negative phenotype when co-expressed with wild-type KCC1, KCC3, or KCC4 polypeptides (9, 12). The dominant-negative mouse KCC1 ΔN117 cDNA was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen), transfected into SiHa and OVCAR-3 cell lines by lipofection, and stable lines were selected as described previously (9).

Functional K⁺ ("Rb⁺") Efflux Assays—Unidirectional K⁺ efflux was carried out at 37 °C as described in detail elsewhere (7, 8). In brief, cells...
were preincubated with isotonic culture medium loaded with 2 μCi/ml 86Rb+ for 2 h at 37 °C. After preincubation and washing, appropriate efflux medium (containing 0.1 mM ouabain and 0.01 mM bumetanide to inhibit the Na+/K+ pump and the Na+/K+2Cl− cotransporter, respectively) was added to the cells. The isotonic efflux medium contained 100 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.5 mM CaCl2, 10 mM glucose, 10 mM HEPES, and 80 mM mannitol, titrated to pH 7.4 with NaOH (200 ± 3 mosM). The components of the hypotonic medium are the same as those of the isotonic medium except mannitol was omitted, resulting in an osmolarity of 220 ± 3 mosM. Release of 86Rb+ from preloaded cells was measured in the efflux medium every 3 min within a 15-min duration. Cells were finally lysed with 0.5M NaOH to release remaining intra-cellular 86Rb+. 86Rb+ efflux rate constants were estimated from the negative slope of the graph of In[X(t)/X(t = 0)] versus time (t), where X(t = 0) denotes the total amount of 86Rb+ inside the cells at the beginning of the efflux time course and X(t) denotes the amount of 86Rb+ inside the cells at the time point t. To study the KCC activity, the Cl− dependence of K+− (86Rb+) efflux was examined by substituting NO3− for Cl− in the efflux medium. The Cl−−dependent K+− (86Rb+) flux was defined as the efflux difference between Cl− and NO3− media.

Measurements of Cell Volume—Cell volume was measured at 37 °C as described previously (9, 13). To monitor the change in cell size, the microscope was coupled to a video camera system, and the images were recorded in real time and stored on a video cassette recorder. Images were then analyzed by Image-Pro Express (version 4.0; Media Cybernetics, Silver Spring, MD). The majority of cells observed were spheroid, and the relative volume change (Vt/Vo) was calculated from the cross-sectional surface area at the beginning (S0) of experiment and during (S) the experiments from the relation: Vt = (S/So)3/2 (13−15). Data were presented as the percentage of starting isotonic volume (Vt/Vo), as a function of time.

Reverse Transcriptase (RT)-Polymerase Chain Reaction—Total RNAs were isolated by RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. After annealing 5 μg of total RNA with oligo(dT), cDNA was prepared with Superscript II (Invitrogen) following the manufacturer’s instructions. Oligonucleotide primers regions specific for KCC1, KCC3, and KCC4 cDNAs (8) were synthesized as follows. For KCC1, forward (5′−TGGGACATTCTTCGTGACC−3′) and reverse (5′−CATGTTCTGACGAGCT−3′) primers were used to amplify a 421-bp fragment from KCC1 (hKCC1: nt 762–1182). For KCC3, forward (5′−CATGCCCTTACCTGGAGTGC−3′) and reverse (5′−CATGCCTTCTCCACGCTAC−3′) primers were used to amplify a 597-bp fragment from KCC3 (hKCC3: nt 200–796). For KCC4, forward (5′−GACTCGTGTTCGGGCAAACCT−3′) and reverse (5′−AGGATTTACCCGAAAGTGGT−3′) primers were used to amplify a 783-bp fragment from KCC4 (hKCC4: nt 2498–3280). PCR was carried out as described in detail elsewhere (8). In brief, after heating the reaction contents for 3 min at 94 °C, 30 cycles of PCR were performed (each consisting of incubation of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C). To assess the relative changes in KCC mRNA content by IGF-1 treatment, we used semiquantitative RT-PCR with GAPDH as the internal standard.

Invasion and Proliferation Assays—Cell migration was assayed in the Boyden chamber as an index of invasive activity of tumor cells (16). Cells were incubated with or without 50 ng/ml IGF-1 for 24 h before the performance of the invasion assay. Invasion assays were conducted for 12 h in serum-free culture media (Dulbecco’s modified Eagle’s medium) at 37 °C. Cells were then fixed with paraformaldehyde, stained with crystal violet, and counted immediately after staining. To assess proliferation,
cells were plated on 60-mm dishes, and the medium was changed every 2 days. Cell counts were performed with the aid of a hemocytometer using trypan blue exclusion (0.08%) to monitor viability.

**Immunoblotting and Immunofluorescence Analysis of Cultured Cells**—After serum starvation for 24 h, the culture medium was replaced with medium containing 50 ng/ml IGF-1; then, after incubation for the indicated time periods, cells were harvested with ice-cold lysis solution. Protein concentrations of cleared lysates were determined by Bradford assay (Bio-Rad). Equal protein loads were separated by 10% SDS-polyacrylamide gel electrophoresis, then transferred to polyvinylidene difluoride membranes (Stratagene, La Jolla, CA). All immunoblots were first probed with phosphospecific antibodies, then stripped and reprobed with antibodies against the corresponding proteins. Phosphorylated levels were analyzed by scanning densitometry and normalized to the total protein bands. The corresponding proteins were identified using affinity-purified antibodies against phosphorylated MAPK or p38 MAPK (Thr-180/Tyr-182), anti-phospho-SAPK/JNK (Thr-183/Tyr-185), and anti-phospho-Akt (Ser-473) (Cell Signaling Technology, Beverly, MA). All immunoblots were analyzed for histology, IGF-1 expression, and KCC expression. Two investigators trained in gynecological pathology blindly examined each section. IGF-1 and KCC staining was evaluated manually or by image analysis with the use of a CoolSnap-Pro color digital camera (Roper Scientific, Trenton, NJ) and Image-Pro Plus 4.1 software (Media Cybernetics) over 15 to 20 high-power fields. The intensity and distribution of immunofluorescent stains were graded as 1, 2, 3, or 4 for each field.

**Statistics**—All values in the present study were reported as mean ± S.E. Student’s paired or unpaired t test was used for statistical analyses. Differences between values were considered significant when p < 0.05.

**RESULTS**

**IGF-1 Stimulated KCC Activity and Enhanced KCC-dependent Cell Volume Regulation**—Because cell swelling is one of the major activating stimuli for KCC activity (1, 2), we investigated IGF-1 stimulation of swelling-activated KCC activity in 24-h serum-deprived cervical cancer SiHAs cells. Fig. 1A shows that 50 ng/ml IGF-1 increased swelling-activated K+ (86Rb+/H+) efflux in a time-dependent manner, when measured in Cl−-containing medium. In contrast, swelling-activated Cl− (36Cl−) efflux was independent of IGF-1 stimulation when NO3− was substituted for Cl− in the efflux medium. KCC activity is taken as the Cl−-dependent K+ (86Rb+) flux, defined as the difference between efflux into Cl− and NO3− media. However, in the presence of the KCC inhibitor DIOA (50 μM), the swelling-activated...
K\(^+\)\(^{(86\text{Rb}^-)}\) efflux was insensitive to IGF-1 stimulation whether efflux was measured in Cl\(^-\) or NO\(_3\)-containing medium (Fig. 1B). Moreover, functional-blocking monoclonal antibody against the IGF-1 receptor α-subunit, but not IgG or monoclonal antibody to insulin receptor, completely inhibited the swelling-activated KCC activity enhanced by IGF-1 (Fig. 1C).

**Fig. 3.** IGF-1 triggers MAPK cascades leading to the activation of Erk1/2. A, IGF-1 (50 ng/ml) treatment increases the phosphorylation of Erk1/2 MAPK (p-Erk1/2), which can be abolished by 50 μM PD98059. Whole-cell extracts were separated by SDS-PAGE (50 μg/lane) and transferred to polyvinylidene difluoride. Western blots first probed with anti-phospho-p44/42 (Thr-202/Tyr-204) MAPK were stripped and reprobed with anti-p44/42 MAPK. Phosphorylated and total Erk1/2 levels were analyzed by scanning densitometry, and the results are expressed as arbitrary units. Each column represents mean ± S.E. (n = 3 independent experiments). B, immunofluorescent stainings show that IGF-1 treatment results in the strong activation of Erk1/2 and increases the nuclear localization of phosphorylated Erk1/2, which is abolished by 50 μM PD98059. Scale bar, 10 μm. C, the phosphorylation of p38 and JNK are insensitive to IGF-1 stimulation. Whole-cell extracts were separated by SDS-PAGE (50 μg/lane) and transferred to polyvinylidene difluoride. Western blots first probed with anti-phospho-p38 MAPK (Thr-180/Tyr-182) or anti-phospho-stress-activated protein kinase (SAPK)/JNK (Thr-183/Tyr-185), were stripped and reprobed with anti-p38 MAPK or anti-stress-activated protein kinase/JNK. The immunoblot is a representative of three similar experiments.

**Fig. 4.** A, IGF-1 (50 ng/ml) stimulates Akt phosphorylation, which is abolished by LY294002 (20 μM) or wortmannin (50 nM). The immunoblot is a representative of three similar experiments. B, immunofluorescent stainings show that IGF-1 treatment results in the strong activation of Akt and increases the nuclear localization of phosphorylated Akt, which is abolished by 20 μM LY294002. Scale bar, 10 μm.
These results indicate that IGF-1 stimulates the swelling-activated KCC mediated K⁺ efflux via IGF-1 receptor signaling.

Our previous studies suggested that the swelling-activated KCC works in concert with ion channels to mediate regulatory volume decrease of cervical cancer cells (8, 9). Prompted by the observation of substantially increased swelling-activated KCC by IGF-1, we tested the hypothesis that IGF-1 might similarly enhance cell volume regulation. As depicted in Fig. 1D, the typical volume changes induced by hypotonic stress in serum-deprived SiHa cells (Control) exhibited three phases: 1) a rapid, initial osmotic swelling, reaching a peak cell volume (1.35 ± 0.08 of original cell size) at 3 min after exposure to hypotonic shock; 2) a rapid shrinkage in the 5 min thereafter; and 3) a more gradual cell volume decrease that within 10 min reached a value 18% above the original cell size. The rate and extent of regulatory volume decrease was significantly enhanced by IGF-1 treatment as a function of time (Fig. 1D), consistent with the time course of IGF-1 stimulation of KCC activity.

**IGF-1 Increased KCC mRNA Levels in a Concentration- and Time-dependent Manner—**IGF-1 might increase KCC activity either by direct stimulation of signaling pathways underlying activation of KCC activity, or by regulation of KCC gene expression or RNA stability. The relatively slow time course of KCC activation by IGF-1 (Fig. 1A) suggests regulation of gene expression or RNA stability as more likely mechanisms. Fig. 2 shows by semiquantitative RT-PCR that IGF-1 stimulated mRNA levels of KCC1, KCC3, and KCC4 in a concentration- and time-dependent manner. After 6-h treatment exposure to 10 ng/ml IGF-1, KCC1, KCC3, and KCC4 mRNA levels increased 20, 35, and 10%, respectively, above control levels (Fig. 2, A and B). These values increased to 45, 130, and 35% after 6-h exposure to 50 ng/ml IGF-1 (Fig. 2). Thus, increased KCC mRNA levels accompanied IGF-1-stimulated KCC activity.

**Molecular Mechanisms by Which IGF-1 Regulated KCC Expression—**IGF-1 elicits its actions on cells by binding to the IGF-1 receptor and activating its intrinsic receptor tyrosine kinase activity (18). The activated IGF-1 receptor phosphorylates docking proteins, which in turn activate downstream signaling proteins leading to diverse biological responses. PI3K and MAPK are two key enzymes that are activated by IGF-1; each represents a distinct signaling pathway that mediates the biological functions of IGF-1 (19). We therefore investigated the roles of PI3K and MAPK pathways in KCC production in response to IGF-1 stimulation. As shown in Fig. 3, A and B, IGF-1 treatment resulted in the strong activation of Erk1/2 and increased the nuclear localization of phosphorylated Erk1/2. PD98059 (50 μM) abolished Erk1/2 phosphorylation and nuclear translocation induced by IGF-1 stimulation. In contrast, the phosphorylation status of other MAPKs, such as p38 and JNK, was insensitive to IGF-1 stimulation (Fig. 3C). IGF-1 treatment also resulted in Akt phosphorylation and increased the nuclear localization of phosphorylated Akt (Fig. 4). Two structurally distinct inhibitors of PI3K (LY294002 and wortmannin) significantly decreased IGF-1 stimulation of Akt phosphorylation and nuclear translocation.

Further experiments addressed the possible contribution of the Erk1/2 and PI3K pathways to IGF-1 stimulation of KCC levels. Treatment with either PD98059 or LY294002 differentially decreased KCC mRNA levels in response to stimulation by 50 ng/ml IGF-1 (Fig. 5). For example, abolition of IGF-1-induced Akt phosphorylation by 20 μM LY294002 was accompanied by 15, 10, and 50% inhibition of IGF-1-stimulated mRNA levels of KCC1, KCC3, and KCC4 (Fig. 5A). Abolition of IGF-1-induced Erk1/2 phosphorylation by 50 μM PD98059 was also inhibited IGF-1-stimulated KCC1 and KCC4 levels by 85 and 50%, respectively. PD98059 further inhibited the basal level of KCC3 mRNA production (Fig. 5A). Immunoblots confirmed that IGF-1 increased KCC polypeptide levels and that PD98059 and LY294002 decreased IGF-1 stimulation of KCC polypeptide level to different extents (Fig. 5B).

To test more definitively the crucial importance of Erk1/2 signaling pathways for IGF-1 stimulation of KCC levels, we used siRNA to knock down Erk1/2 protein levels in SiHa cervical cancer cells. Endogenous Erk1/2 protein levels were specifically reduced in the presence of a MAPK1-specific double-stranded RNA oligomer (Fig. 6A). As a control, we used an siRNA containing a two-nucleotide mutation in the MAPK1 sequence. This control siRNA did not alter Erk1/2 protein levels (Fig. 6A). In addition, reduction of Erk1/2 protein levels with siRNA modestly reduced basal swelling-activated KCC activity and almost abolished IGF-1 stimulation of swelling-activated KCC activity (Fig. 6B).

**KCC Is Necessary for IGF-1-stimulated Cellular Invasion and Proliferation—**The invasion assays were performed in cervical cancer SiHa cells pretreated for 24 h with 50 ng/ml IGF-1
Fig. 6. Reduction of Erk1/2 protein levels with siRNA abolishes the IGF-1-stimulating KCC activity. A, endogenous Erk1/2 protein levels were efficiently and specifically reduced in the presence of a MAPK1-specific double-stranded RNA oligomer. For siRNA knockdown of Erk1/2 MAPK, SiHa cells were transfected with 50 nM of either MAPK1 siRNA or a scrambled siRNA negative control. Protein was extracted from the cells at 36 h, and the knockdown activity was measured by Western immunoblots using antibodies against Erk1/2 or β-actin. The knockdown is the relative amount of protein remaining compared with mock transfection. Erk1/2 levels were analyzed by scanning densitometry, and the results are expressed as arbitrary units. Each column represents mean ± S.E. (n = 3 independent experiments). B, reduction of Erk1/2 protein levels with siRNA modestly reduced the endogenous swelling-activated KCC activity and almost abolished the stimulatory effect of IGF-1 on the KCC activity. Each column represents mean ± S.E. (n = 3).

or with serum starvation. As shown in Fig. 7, A and B, IGF-1 increased the invasive migration of SiHa cells (1.8 ± 0.3-fold increase compared with absence of IGF-1), with fibronectin as chemotactic in the lower Boyden chamber. Endogenous invasiveness was much attenuated in the KCC dominant-negative mutant cervical cancer cells (ΔN117), and their residual migration was insensitive to IGF-1 stimulation. Furthermore, SiHa cervical cancer cell growth was stimulated by IGF-1 in a concentration-dependent manner, the growth of KCC dominant-negative mutant cervical cancer cells (ΔN117) was IGF-1 insensitive. The KCC inhibitor DIOA (50 μM) inhibits basal invasive and proliferative ability (Fig. 7, B and C). Moreover, DIOA (50 μM) abolished IGF-1-stimulated cellular invasion and proliferation (Fig. 7, B and C).

Expression Patterns of IGF-1 and KCC in Surgical Specimens of Cervical Carcinoma—Studies in cell culture systems have revealed that IGF-1 and KCC are important modulators of cervical cancer cell proliferation and invasion. To evaluate the in vivo condition, IGF-1, KCC, and nuclear DNA were simultaneously immunostained in surgical specimens (n = 28) of cervical cancer. As shown in Fig. 8, A–C, IGF-1 and KCC colocalize in the cervical cancer tissues, suggesting likely autocrine or paracrine IGF-1 stimulation of KCC production. Moreover, importantly, expression of IGF-1 and KCC in the same surgical specimens shows linear correlation (r = 0.98) (Fig. 8D). This correlation in turn corresponds well to tumor size (Fig. 8E); i.e., tumors of larger size exhibit higher intensity IGF-1 staining and higher intensity KCC staining. It is interesting that IGF-1 and KCC proteins were nearly undetectable in normal or noncancerous cervical tissues of all surgical specimens examined (n = 28).

KCC Is Stimulated by IGF-1 and Is Required for Invasiveness and Proliferation of Ovarian Cancer Cells—We also studied the effect of IGF-1 on KCC activity in ovarian cancer OVCAR-3 cells. As shown in Fig. 9A, IGF-1 (50 ng/ml) enhances swelling-activated KCC activity via IGF-1 receptor signaling. IGF-1 (50 ng/ml) increases KCC1, KCC3, and KCC4 mRNA levels to plateau values over a period of 3–6 h (Fig. 9B). Pharmacologic inhibition of Erk1/2 MAPK and PI3K signaling pathways differentially inhibit IGF-1-stimulated increase in mRNA levels of KCC1, KCC3, and KCC4 (Fig. 9C). IGF-1-stimulated cellular invasiveness and proliferation are abolished by the KCC inhibitor DIOA (50 μM; Fig. 9, D and E). KCC dominant-negative mutant ovarian cancer cells (ΔN117) exhibit severely attenuated IGF-1-insensitive invasiveness and proliferation (Fig. 9, D and E). In addition, IGF-1 and KCC colocalize in ovarian cancer tissues (Fig. 9F, n = 35).

DISCUSSION

The present study shows that KCC activity is necessary for IGF-1-dependent proliferation and for IGF-1-stimulated chemotaxis through Matrigel, which may reflect in situ invasion. This conclusion is supported by the following findings: 1) IGF-1 stimulated KCC-mediated K⁺ efflux via IGF-1 receptor signaling. 2) IGF-1 stimulated KCC activity enhances the cellular capability for volume regulation, a fundamental mechanism for maintaining structural integrity and constancy of the intracellular milieu. 3) IGF-1 stimulated cellular invasion and proliferation in a concentration-dependent manner. Both of these activities were almost completely blocked by the KCC inhibitor, DIOA. 4) In addition, both invasiveness and proliferation of KCC dominant-negative mutant cells were insensitive to IGF-1.
Fig. 7. **KCC is necessary for IGF-1 dependent cellular invasion and proliferation.** *A*, invasive migration was assayed in the Boyden chamber as an index of invasive activity of tumor cells. SiHa (wild-type) or KCC dominant-negative mutant SiHa (ΔN117) cells have been incubated with or without 50 ng/ml IGF-1 for 24 h before the performance of invasion assay. Invasion assays were run for 12 h in serum-free culture media (Dulbecco’s modified Eagle’s medium) at 37 °C. After invasion assay, cells were fixed with paraformaldehyde, stained with crystal violet, and counted immediately after staining. *B*, summary of invasion assay for SiHAs (wild-type) and KCC dominant-negative mutant SiHa (ΔN117). Each column represents mean ± S.E. from six independent experiments. *C*, the growth of wild-type SiHa cells was stimulated dose-dependently by IGF-1, whereas that of ΔN117 cells was not. Each point represents mean ± S.E. (n = 6). The KCC inhibitor (50 μM DIOA) inhibited the basal invasive and proliferative ability of SiHa cells. It also abolished the IGF-1 stimulated cellular invasion and proliferation. FCS, fetal calf serum.

Fig. 8. **Patterns of IGF-1 and KCC expression in cervical cancer tissues.** *A–C*, the triple-immunofluorescent stain technique was used to identify IGF-1 (red), KCC (green), and nucleus (blue) in a pelvic lymph node metastasized by cervical cancer cells. Scale bar, 20 μm. *D*, a linear correlation (r = 0.98) of IGF-1 and KCC expression in the same surgical specimen of cervical cancer tissues. The case number is indicated in parentheses beside each point. *E*, the relationship between IGF-1 expression, KCC expression, and tumor size in the same surgical specimen of cervical cancer tissues (n = 28).
stimulation. 5) Immunofluorescent staining of sections from surgical specimens revealed colocalization of IGF-1 and KCC in cervical cancer and ovarian cancer tissues, suggesting possible autocrine or paracrine IGF-1 stimulation of KCC production in vivo.

A main physiological function of KCC is to regulate cell volume (1, 2). In this study, we demonstrated that swelling-activated regulatory volume decrease was significantly enhanced by IGF-1 treatment as a function of time, consistent with the time-dependence of KCC activity stimulated by IGF-1. Cancer cells usually have higher rates of metabolism, mitosis, and migration than those of normal cells. Growth, mitosis, and migration each can perturb cell volume homeostasis significantly (20). The maintenance of cell volume homeostasis is a fundamental property of mammalian cells, and most cells possess mechanisms to regulate their volume during osmotic challenge. This close linkage of cell volume regulation and cell proliferation suggests the possibility that overexpression of KCC activity may serve as a selective advantage for cancer cells. The 3–6-h time course of KCC stimulation by IGF-1 makes unlikely the possibility that IGF-1 receptor signals directly to the short-term regulatory mechanisms of KCC activation. Furthermore, IGF-1 increases KCC1, KCC3, and KCC4 mRNA levels in a similar time- and concentration-dependent manner. Thus, it is very likely that IGF-1 stimulates KCC activity via regulation of gene transcription or of mRNA stability. Immunoblot analysis revealed activation of Erk1/2 and Akt by IGF-1 stimulation. Immunofluorescent staining further demonstrated translocation of activated Erk1/2 and Akt into the cancer cell nucleus. This implies that the activated Erk1/2 and Akt pathways are probably involved in gene regulation by IGF-1. Specific inhibition of MEK suppressed Erk1/2 activation and markedly reduced stimulation of KCC by IGF-1. In contrast, blockade of Akt activation by a PI3K inhibitor modestly inhibited the stimulatory effect of IGF-1 on KCC level and activity. Therefore, Erk1/2 MAPK and PI3K signaling pathways are differentially required for IGF-1 stimulation of KCC activity and polypeptide levels. Most importantly, specific reduction of Erk1/2 protein levels with siRNA abolished IGF-1-stimulated KCC activity, indicating a crucial role for the Erk1/2 signal pathway in the IGF-1 stimulation of KCC activity and protein level.

It has been reported that increased KCC activity and expression is associated with the stimulation of growth factors and...
cytokines. For example, platelet-derived growth factor increased KCC1 and decreased KCC3 mRNA expression in a time-dependent manner in vascular smooth muscle cells (21). IGF-1 also up-regulated KCC3 expression and stimulated proliferation of NIH/3T3 cells stably transfected with human KCC3, whereas tumor necrosis factor-α down-regulated KCC3 expression and caused growth arrest in the same cell lines (7). The KCC3 mRNA level in endothelial cells increased after treatment with vascular endothelial cell growth factor and decreased with tumor necrosis factor-α, whereas KCC1 mRNA levels remained unchanged (4). Brain-derived neurotrophic factor could raise the expression of KCC2, which is responsible for the maturational conversion of neuronal GABA responses from depolarizing to inhibitory, through the control of the equilibrium Cl⁻/H⁻ exchanger NHE1 in cultured fibroblasts (24). Taken together, IGF-1 elicits its actions on KCC by binding to IGF-1 receptor and activating PI3K and Erk1/2 MAPK. The Erk1/2 MAPK and PI3K signaling pathways are differentially required for IGF-1 stimulation of activity and expression level of distinct KCC gene products (summarized in Fig. 10). Blockade of KCC activity or expression may provide novel strategies for the treatment of IGF-1 dependent cellular invasion and proliferation of gynecological malignancy.

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**FIG. 10. Schematic diagram of IGF-1 receptor signals which lead to regulate KCC production.** IGF-1 elicits its actions on KCC by binding to IGF-1 receptor and activating PI3K and Erk1/2 MAPK. Erk1/2 MAPK and PI3K signaling pathways are differentially required for IGF-1 stimulating production of KCC isoforms. The KCC activity is necessary for IGF-1-dependent cellular invasion and proliferation.
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