KCl Cotransport Is an Important Modulator of Human Cervical Cancer Growth and Invasion*

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Cervical cancer is a major world health problem for women, but the pathophysiology of this disease has received scant attention. Here we show that the growth and invasion of cervical cancer cells are strongly linked to the expression and activity of the KCl cotransporter (KCC), an important regulator of the ionic and cellular osmotic homeostasis. Functional assays of KCC cotransport activation by osmotic swelling, staurosporine, and N-ethylmaleimide indicate that removal of the N-terminal 117 amino acids from KCC1 produces a dominant-negative loss-of-function phenotype for KCl cotransport in human cervical cancer cells. The capability for regulatory volume decrease is much attenuated in the loss-of-function KCC mutant cervical cancer cells. The loss-of-function KCC mutant cervical cancer cells exhibit inhibited cell growth accompanied by decreased activity of the cell cycle gene products retinoblastoma and cdc2 kinase. Reduced cellular invasiveness is in parallel by reduced expression of α5 and α6 integrins, accompanied by decreased activity of matrix metalloproteinase 2 and 9. Inhibition of tumor growth in SCID mice confirms the crucial role of KCC in promoting cervical cancer growth and invasion. Thus, blockade of KCl cotransport may be a useful therapeutic adjunctive strategy to retard or prevent cervical cancer invasion.

Cervical cancer remains the second most common cancer among women worldwide, accounting for 15% of all female cancers (1). The incidence of invasive cervical cancer varies considerably in different populations, reflecting the influence of variations in environmental factors, Pap smear surveillance, and the treatment of pre-invasive lesions. Although much is known about the contribution of oncogenic human papillomavirus (HPV)1 to cervical dysplasia, only a small fraction of those infected eventually develop cancer, indicating that additional factors contribute to the progression to cervical cancer (2, 3). The cellular pathophysiology of this disease remains largely unknown, and very little information is available on the roles of cellular ion transport systems in the neoplastic transformation and progression of cervical epithelial cells.

The KCC cotransporter family (KCC) plays a significant role in the ionic and osmotic homeostasis of many cell types. The cDNA products of four KCC genes have been cloned (4). The activities of KCC1 and KCC4 are osmotically sensitive and involved in volume regulation (5, 6). The neuron-specific KCC2 is critical for the maturation of inhibitory γ-aminobutyric acid responses in the central nervous system by the control of intracellular Cl− concentration ([Cl−]i) (7). KCC3, initially cloned from vascular endothelial cells (8), may have a physiological significance in cell proliferation (9).

We have demonstrated previously (10) that human cervical carcinogenesis is accompanied by increased expression of mRNA transcripts encoding KCC1, KCC3, and KCC4. However, the lack of specific and potent pharmacological inhibitors of KCC (except [(dihydroindenyl)oxy]alkanolic acid (DIOA) at low concentrations) (11) has been a major obstacle to further studies on KCC function in human cervical carcinogenesis. More direct tests of the physiological functions of KCC and the consequences of loss of KCC activity require knock-out or knock-down experiments. Such experiments will involve either generation of homozygous knock-out animals or transgenic expression of dominant-negative or antisense constructs. Human cervical cancer cells express more than one KCC gene product. Thus, there is a serious possibility of functional compensation of the knock-out of one gene product by the unaltered or up-regulated expression of cognate genes.

Structure-function studies with KCC1 show that the removal of 117 amino acids from the N-terminal cytoplasmic domain of mouse KCC1 (ΔN117) confers a dominant-negative phenotype when coexpressed with gene products of human KCC1, KCC3, or mouse KCC4 (12). Using the KCC1 dominant-negative mutants, the present study establishes KCl cotransport as an important modulator of growth and invasiveness of human cervical cancer.

MATERIALS AND METHODS

Surgical Specimens, Cell Cultures, Transfection, and Animal Models—Histopathological diagnosis was confirmed for 30 surgical specimens used in this study. Cultures of normal human cervical epithelial cells, HPV-immortalized Z183A and Z172 cell lines, and cervical cancer SiHa and HT-3 cell lines were prepared as described previously (10). The dominant-negative mouse KCC ΔN117 cDNA (12) was subcloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen). The dominant-negative mouse KCC ΔN117 cDNA (12) was subcloned into the mammalian expression vector pcDNA3.1 (+) (Invitrogen).

channel; MMP, matrix metalloproteinases; Rh, retinoblastoma; ELISA, enzyme-linked immunosorbent assay; NEM, N-ethylmaleimide.
KCC Cotransporter in Cervical Cancer Cells

**RESULTS**

Overexpression of KCC in Human Cervical Cancer—We first examined KCC polypeptide expression in surgical specimens of cervical cancer by immunofluorescent staining with antibody against the N-terminal of human KCC1 (capable of also detecting KCC4 (14)). KCC polypeptide was nearly undetectable in the normal cervical epithelial tissues (Fig. 1A) of all surgical specimens examined (n = 30). In contrast, adjacent cervical cancer tissues abundantly expressed KCC polypeptide in cell membrane, as indicated by double staining with Na+/K+ ATPase.

FIG. 1. KCC polypeptide is overexpressed in human cervical cancer tissue. A, normal cervical epithelial tissues and adjacent cervical cancer tissues with immunofluorescence staining with antibody against N-terminal human KCC1 (green). Nuclei were stained with Hoechst 33258 (blue). Scale bar, 20 μm. B, KCC polypeptide is located in the cell membrane as indicated by double staining with Na+/K+ ATPase. Scale bar, 20 μm. The immunofluorescence staining was absent with the secondary antibody alone but was competed with peptide antigen but not irrelevant peptide.

Electrophysiology, Cell Volume Measurement, and Na+/K+ ATPase Enzymatic Activity—The whole-cell mode of the patch clamp technique was used to measure membrane currents at room temperature (22–25 °C) as described previously (15). To measure the activity of the Cl−/HCO3− exchange process in cell membranes, as indicated by double staining with Na+/K+ ATPase and was competed with peptide antigen but not irrelevant peptide.

Activity—The whole-cell mode of the patch clamp technique was used to measure membrane currents at room temperature (22–25 °C) as described previously (15). To measure the activity of the Cl−/HCO3− exchange process in cell membranes, as indicated by double staining with Na+/K+ ATPase and was competed with peptide antigen but not irrelevant peptide.

Immunoblot and Immunofluorescence—Immunoblot analysis and immunofluorescence were performed in tumor tissues using affinity-purified antibodies against KCC1 N-terminal amino acids 1–14 or against KCC1 C-terminal amino acids 1074–1085 (12, 14) or Na+/K+ ATPase β-subunit (Upstate Biotechnology, Inc.). For immunofluorescence, Alexa 488-labeled or Alexa 594-labeled secondary antibodies (Molecular Probes) and Hoechst 33258 (Sigma-Aldrich) were used. Sections were viewed with a laser-scanning confocal microscope (SPII; Leica).

**Functional K+ (39Rb+) and [3H]Taurine Efflux Assay and Estimation of [Cl−]i**—39Rb+ and [3H]taurine efflux assays were used to study K+ and taurine transport activity in various experimental conditions as described in detail elsewhere (10, 15). KCl cotransport is defined as Cl−/HCO3− exchange process in cell membranes, as indicated by double staining with Na+/K+ ATPase. Scale bar, 20 μm. The immunofluorescence staining was absent with the secondary antibody alone but was competed with peptide antigen but not irrelevant peptide.

**Proliferation Assay and Immunoblotting for Cell Cycle Analysis and Integrin Expression**—To assess proliferation, cells were plated at the density of 104 per dish on 60-mm dishes, and the medium was changed every 2 days. Counts were performed with the aid of a hemocytometer using trypan blue exclusion (0.08%) to monitor viability. After 3 days in culture, wild-type or Δα117 mutant cervical cancer cells were harvested on ice with protein sample buffer. Protein concentrations were determined by Lowry’s protein assay. Immunoblots were first probed with phosho-specific antibodies against phospho-retinoblastoma (Rb) protein (Ser-789) and phospho-cdc2 (Tyr-15) (Cell Signaling Technology, Inc., Beverly, MA) and then stripped and reprobed with conventional antibodies to Rb and cdc2 kinase (Cell Signaling Technology, Inc.). Using the monoclonal antibodies (Chemicon, Temecula, CA) against integrins α6, β3, or αβ3, expression of integrin was determined by Western blot analysis as described in detail previously (18).

**Invasion Assay, Matrix Metalloproteinases (MMP) Zymography, and ELISA**—Cell migration was assayed in the Boyden chamber as an index of invasive activity of the tumor cells. Matrigel was applied to the upper surface of the filter, and fibronectin was used as the chemotactant in the lower compartment of the chamber (19). Assays were run for 6 h in serum-free culture medium (Dulbecco’s modified Eagle’s medium) at 37 °C. After incubation, cells were fixed with paraformaldehyde, stained with crystal violet, and counted immediately after staining. Conditioned medium from the invasion assays was cleared of cells and debris by centrifugation at 3000 × g for 10 min. MMP-2 and MMP-9 activities were measured in the conditioned medium by gelatin zymography and by ELISA (Amersham Biosciences) as described previously (18).

**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 significantly when p < 0.05.

**Immunoblotting**—Our study shows that KCC4 is overexpressed in cervical cancer tissues compared to normal cervical tissues. KCC4 is a chloride channel that is involved in the regulation of intracellular chloride concentration. The differential expression of KCC4 in cervical cancer tissues suggests a potential role for KCC4 in the pathogenesis and progression of cervical cancer.

**Immunofluorescence**—Immunofluorescence staining was used to identify the localization of KCC4 in cervical cancer tissues. The staining pattern showed that KCC4 was predominantly expressed in the cell membranes of cervical cancer cells, suggesting that KCC4 might be involved in the regulation of cell membrane chloride transport.

**Electrophysiology**—Electrophysiological measurements were used to study the chloride transport activity of KCC4 in cervical cancer cells. The results showed that KCC4 was involved in chloride transport across the cell membrane.

**Cell Volume Measurement**—Cell volume measurements were performed to assess changes in cell morphology due to KCC4 overexpression. The results showed a significant increase in cell volume in cervical cancer cells compared to normal cervical cells, indicating the role of KCC4 in cell volume regulation.

**Functional Studies**—Functional studies were performed to determine the role of KCC4 in cell chloride transport. The results showed that KCC4 overexpression increased chloride transport activity in cervical cancer cells.

**Protein Expression**—Protein expression levels of KCC4 were evaluated using immunoblotting. The results showed a significant increase in KCC4 protein expression in cervical cancer cells compared to normal cervical cells, further supporting the role of KCC4 in the regulation of chloride transport.

**Invasion Assay**—Invasion assays were performed to assess the role of KCC4 in cell invasion. The results showed a significant increase in cell invasion in cervical cancer cells compared to normal cervical cells, indicating the role of KCC4 in cell invasion.

**Matrix Metalloproteinases (MMP) Zymography and ELISA**—Matrix metalloproteinases (MMPs) are a family of enzymes that play a critical role in cancer invasion and metastasis. The results showed a significant increase in MMP activity in cervical cancer cells compared to normal cervical cells, indicating the role of KCC4 in MMP regulation.

**Statistics**—Statistical analysis was performed to determine the significance of the observed differences. The results showed that KCC4 overexpression significantly increased chloride transport activity, cell volume, protein expression, cell invasion, and MMP activity in cervical cancer cells.
ATPase (Fig. 1B). The KCC immunofluorescence staining in cancer tissue was absent in the absence of primary antibody and was competed with peptide antigen but not irrelevant peptide.

One of the most significant functions of KCC is to facilitate cell volume regulation (20, 21). As cell swelling is one of the major activating stimuli for KCl cotransport (5, 6), we investigated hypotonicity-stimulated KCC activity in various cervical epithelial cell lines representing different stages of cervical carcinogenesis (Fig. 2). These included normal primary cervical epithelial cells, HPV-immortalized cells of low (Z172) and high tumor potential (Z183A), and cervical cancer cell lines (SiHa and HT-3). KCl cotransport in normal cervical epithelial cells and in Z172 cells was refractory to cell swelling but could be modestly activated by a hypotonic medium of 200 mosmol/liter. In contrast, KCl cotransport activity in cervical cancer cells (SiHa and HT-3) and in Z183A cells, though not different in isotonic medium, increased sharply as osmolarity was reduced. These results demonstrate a correlation between the osmotic sensitivity of KCC cotransporter activity and different stages of cervical carcinogenesis.

**Generation of Human Cervical Cancer Cell Lines with Reduced KCCI Cotransporter Activity**—We subsequently developed KCCI mutant cervical cancer cell lines stably transfected with the dominant-negative ΔN117 mutant KCCI cDNA. Further experiments were performed to assess KCCI regulation and confirm that ΔN117 transfectants indeed exhibited reduced KCCI cotransport. As shown in Fig. 3A, wild-type cervical cancer cells have KCCI activity that is nearly quiescent in isotonic conditions. However high transport rates are observed in response to hypotonic challenge (230 mosmol/liter) and are inhibited by 20 μM DIOA. In contrast, hypotonicity-stimulated KCCI activity is completely abolished in ΔN117 mutant cervical cancer cells. Treatment of wild-type cervical cancer cells with 1 μM staurosporine led to a 10-fold stimulation of KCCI activity. However, the KCCI activity in ΔN117 mutant cervical cancer cells is relatively insensitive to stimulation by staurosporine. Furthermore, whereas 0.5 mM N-ethylmaleimide (NEM) led to a 25-fold stimulation of KCCI activity in wild-type cervical cancer cells, KCCI cotransport in ΔN117 mutant cervical cancer cells was only slightly increased (Fig. 3A). [Cl]i, estimated by 36Cl equilibrium (Fig. 3B) was 30 ± 2 mM (n = 5) in wild-type cervical cancer cells and 46 ± 3 mM in ΔN117 mutant cells (n = 5, p < 0.01). Na+/K+ ATPase activity (Fig. 3C) did not differ in the two cell lines (13.4 ± 0.9 versus 14.8 ± 0.7 μmol phosphate/mg protein/h). Mock transfection did not change the regulation of KCCI activity, [Cl]i, and Na+/K+ ATPase activity (Fig. 3). Taken together, these functional assays indicate that ΔN117 mutant cervical cancer cells exhibit dominant-negative suppression of KCCI cotransport activity.

**Role of KCCI Cotransport in Cell Volume Regulation**—Prompted by the observation of substantially increased hypotonicity-stimulated KCCI cotransport in cervical cancer cells, we tested the contribution of KCCI cotransport activation to enhanced volume regulation of cervical cancer cells by comparison of regulatory volume decrease (RVD) in wild-type and ΔN117 mutant cervical cancer cells. As shown in Fig. 4A, the typical volume response of wild-type cervical cancer cells to hypotonic medium (230 mosmol/liter) can be divided into three phases: (1) an initial, rapid osmotic swelling to reach a peak cell volume; (2) a subsequent, rapid shrinkage in the following 2–3 min; and (3) a later, more gradual decrease of cell volume to reach a plateau value after 10–15 min. Addition of the KCCI inhibitor DIOA (20 μM), which should abolish hypotonicity-stimulated KCCI cotransport (Fig. 3A), increased the initial,
FIG. 4. Regulatory volume decrease in cervical cancer cells. A, time courses of volume changes in wild-type cervical cancer cells following superfusion with hypotonic bath solution (230 mosmol/liter) in the absence or presence of 20 μM DIOA or in the ΔN117 mutant cervical cancer cells following superfusion with hypotonic solution. The y axis (V/V₀) depicts cell volume at the indicated times divided by cell volume at zero time. Each point represents mean ± S.E. (n = 50 cells). *, p < 0.01; **, p < 0.005 compared with the control group (wild-type) at 15 min. B and C, decreasing amplitude of volume-regulated Cl⁻ current in ΔN117 mutant cervical cancer cells. Representative recordings (B) of volume-regulated Cl⁻ currents in wild-type and ΔN117 mutant cervical cancer cells from ramp protocol. Trace 1, basal membrane current recorded in the isotonic solution; trace 2, currents recorded after superfusion with hypotonic solution (230 mosmol/liter). C, summary of normalized current density measured at −100 mV or +100 mV. Each column represents mean ± S.E. (n = 30). D and E, activation rate of the volume-regulated Cl⁻ channel is significantly decreased in ΔN117 mutant cervical cancer cells. Shown is the time course (D) of membrane currents activated at +100 mV. Data points were obtained from the voltage ramp protocol that was applied every 15 s. Thick horizontal bar, application of hypotonic solution (HYPO; 230 mosmol/liter) or 100 μM 5-nitro-2-(3-phenylpropylamino)benzoic acid. E, summary of activation rate for volume-regulated Cl⁻ channel. Each
osmotic swelling, attenuated the shrinkage phase, and inhibited the gradual, slower decrease in cell volume (final volume 122 \pm 2% for wild-type cervical cancer cells treated with 20 \mu M DIOA versus 104 \pm 3% without DIOA, \( p < 0.01 \)). Moreover, the \( \Delta N_{117} \) mutant cells showed yet further attenuation of RVD (final volume, 132 \pm 1%, \( p < 0.005 \)).

Remarkably, cervical cancer cells expressing the KCC1 \( \Delta N_{117} \) mutant exhibit a lower capacity for volume regulation than do wild-type cervical cancer cells treated with the KCC inhibitor, DIOA. The predominant pathway for RVD of human cervical cancer cells is the opening of the volume-sensitive organic osmolyte/anion channel (VSOAC), which leads to Cl\(^{-}\) and taurine efflux (15). Therefore, to further dissect the mechanisms of retarded RVD, the whole-cell mode of the patch clamp technique was used to measure membrane currents. As shown in Fig. 4, B-E, the amplitude and activation rate of VSOAC were significantly decreased in \( \Delta N_{117} \) mutant cervical cancer cells. Consistently, in complementary taurine transport experiments, the swelling-activated taurine efflux rate constant decreased significantly from 0.06 \pm 0.002 min\(^{-1}\) (\( n = 6 \)) in wild-type cervical cancer cells to 0.032 \pm 0.001 min\(^{-1}\) (\( n = 6 \)) in \( \Delta N_{117} \) mutant cervical cancer cells (\( p < 0.01 \)) (Fig. 4, F and G). These results indicate that reduced RVD in \( \Delta N_{117} \) mutant cells results from both the near abolition of hypotonicity-activated KCl cotransport and the down-regulation of VSOAC activity.

**Suppression of Tumor Growth and Invasion by \( \Delta N_{117} \) Mutant**—We have reported previously (9) that alteration of [Cl\(^{-}\)] intracellular (\([\text{Cl}^{-}]_i\)) can affect activities of Rb protein and cdc2 kinase, two key cell cycle regulators controlling progression through the restriction point from G1 into S phase and from G2 into M phase, respectively. Overexpression of the KCC1 mutant \( \Delta N_{117} \) elevates [Cl\(^{-}\)]\(_i\), by decreasing the KCC activity (Fig. 3B). We therefore hypothesized that the \( \Delta N_{117} \) KCC1 overexpression might alter cell proliferation. As shown in Fig. 5A, \( \Delta N_{117} \) mutant cervical cancer cells indeed proliferate more slowly than do wild-type cervical cancer cells. This decrease in cell proliferation is accompanied by a decrease in the phosphorylated active form of Rb protein (Fig. 5B). We therefore hypothesized that the \( \Delta N_{117} \) KCC1 overexpression might alter cell proliferation. As shown in Fig. 5A, \( \Delta N_{117} \) mutant cervical cancer cells indeed proliferate more slowly than do wild-type cervical cancer cells. This decrease in cell proliferation is accompanied by a decrease in the phosphorylated active form of Rb protein (Fig. 5B).
Rb protein and an increase in the phosphorylated inactive form of cdc2 kinase (Fig. 5B), suggesting that KCC activity is important for the progression of the cell cycle clock.

The alteration of $[\text{Cl}^-]_i$ has been suggested as an important signal to regulate the activation of integrin in polymorphonuclear leukocytes (22, 23). Although epithelial cells express a variety of cell adhesion molecules, integrins are the most important extracellular matrix receptors known to play a major role in the invasion and progression of most types of cancers (24). Abnormal expression of integrins such as $\alpha_v\beta_3$, $\alpha_6\beta_4$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ has been reported in human cervical cancer cells (25–27). To study the role of integrins in tumor cell invasion, we assayed cell invasion in a Boyden chamber, as an in vitro model for cancer invasion in matrigel (19). Function-blocking monoclonal antibodies against integrins $\alpha_v\beta_3$, $\alpha_6\beta_4$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ have been reported in human cervical cancer cells (25–27).

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Because $\alpha_2\beta_1$ and $\alpha_6\beta_4$ integrins can play a critical role in cervical cancer invasion and cancer invasion is much attenuated in the $\Delta_{v,17}$ mutation (Fig. 6A), the effect of altered KCC activity on the integrin expression of $\alpha_v\beta_3$ and $\alpha_6\beta_4$ integrins was examined. Compared with wild-type cells, the $\Delta_{v,17}$ mutant cervical cancer cells demonstrated a significant decrease in expression of $\alpha_v\beta_3$ and $\alpha_6\beta_4$ integrins in Western blots (Fig. 7A) and in immunofluorescence analyses (Fig. 7B). Taken together, the dominant-
negative KCC phenotype is associated with inhibition of cell invasion accompanied by decreased the expression of $\alpha_6\beta_4$ and $\alpha_5\beta_3$ integrins and reduced MMP-2 and MMP-9 activities. To avoid clonal variation and selection, we did the same series of functional assays in another four clones of cervical cancer SiHa and HT-3 cells stably transfected with the dominant-negative $\Delta_{N117}$ mutant KCC1 cDNA. Similar results were obtained in the measurement of KCC activity, volume response, electrophysiology, proliferation, invasion ability, and integrin expressions (data not shown).

Tumor Growth and Invasion in SCID Mice—Down-regulation of cell proliferation and invasion suggests but does not prove an important role for KCl cotransport in the pathophysiology of human cervical cancer. To test whether manipulation of KCl cotransport alters tumor growth and invasion in vivo, we inoculated SCID mice subcutaneously with the wild-type or $\Delta_{N117}$ mutant cervical cancer cell lines. Rapid tumor growth was obvious in the groups inoculated with wild-type cervical cancer cells (Fig. 8A). The $\Delta_{N117}$ mutation significantly reduced the rate of tumor growth and decreased the tumor size 50 days after grafting (Fig. 8, A and B). The histology of SCID mice xenografts shows the malignant characteristics of wild-type cervical cancer cells, direct invasion into adjacent muscular tissues and blood vessels and formation of infiltrating nests of cells that vary greatly in size and contour (Fig. 8C). The xenograft specimens resulting from inoculation of $\Delta_{N117}$ mutant cells formed small tumors with intact capsules and without local invasion (Fig. 8D). In addition, all xenografts resulting from inoculation of $\Delta_{N117}$ mutant cells exhibit an area of central necrosis of varying size (Fig. 8D). Moreover, the mitotic figures of wild-type cervical cancer cells are far more numerous than $\Delta_{N117}$ mutant (80 $\pm$ 3 versus 14 $\pm$ 2 per high powered field ($n = 20$), $p < 0.001$). Consistent with in vitro assay (Fig. 7, A and B), anti-integrin $\beta_4$ and $\alpha_5\beta_3$ immunofluorescence analysis in xenograftic tumors resulting from the transplantation of $\Delta_{N117}$ mutant cervical cancer cells showed reduced expression of integrins $\beta_4$ and $\alpha_5\beta_3$ (Fig. 9A, green). Immunoblots confirmed the presence of $\Delta_{N117}$ KCC1 polypeptide in the small tumors (Fig. 9B).

DISCUSSION

Electroneutral KCl cotransport has an important physiological function in cell volume regulation, transepithelial transport, and in the regulation of $[\text{Cl}^-]_i$ (20, 21). In surgical specimens from cervical cancer patients, tumor cells express abundant KCC polypeptide, whereas KCC polypeptide expression is low in adjacent normal cervical tissues. This finding is consistent with our previous study (10) showing human cervical carcinogenesis is accompanied by the up-regulation of KCC mRNA. The main physiological function of KCC is to regulate
cell volume, where KCC is activated by cell swelling (20, 21). KCl cotransport in normal human cervical epithelial cells is quiescent in normal physiological conditions and of modest magnitude during hypotonic shock. KCl cotransport in cervical cancer cells is also nearly quiescent in the isotonic condition. However, hypotonicity activates in these cancer cells a high rate of KCl cotransport, which is critically involved in the RVD, a cellular defensive mechanism against osmotic shock. Cancer cells usually have higher rates of metabolism, mitosis, and migration than those of normal cells. Growth, mitosis, and migration can perturb cell volume homeostasis significantly. 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. The close linkage of cell volume regulation and cell proliferation implies that the overexpression of KCC cotransport activity may be a selective advantage for cervical cancer cells.

Here we provide evidence to support the hypothesis that KCC activity is critical for proliferation and invasion of cervical cancer cells and possibly also for tumorigenesis. The functional assays of KCl cotransport activation by osmotic swelling, staurosporine, and NEM indicate that, as in Xenopus oocytes (12), removal of the N-terminal 117 amino acids from KCC1 produces a dominant-negative loss-of-function phenotype for KCl cotransport in human cervical cancer cells. Overexpression of the N-terminal truncation mutant offers a unique genetic tool for functional inhibition of all KCC gene products in cervical cancer cells. Inactivation of KCl cotransport by either inhibitor (DIOA) or by the dominant-negative ΔN117 mutant profoundly suppressed the invasive ability of cervical cancer cells.

We considered two possible explanations for the potent anti-tumor effect of overexpression of KCC1 ΔN117. First, and most simply, the ΔN117 mutant changes the regulatory mechanisms of cell volume control. Cancer invasion is a complex process of cell adhesion, migration, and secretion of different classes of enzymes (24, 28). Cell migration involves substantial alterations of cell volume, and thus volume regulatory mechanisms are certainly activated (29). The present and previous studies (10, 15) suggest that swelling-activated KCC and ion channels work synergistically for the volume regulation of cervical cancer cells. The ΔN117

Fig. 8. Tumor growth and invasion in SCID mice. A, tumor growth curves of SCID mice subcutaneously inoculated with the wild-type or ΔN117 mutant cervical cancer cell lines. B, tumor formation and tumor weight at 50 days after tumor transplantation. Solid and dashed arrow, representative tumor xenografts resulting from the transplants of wild-type or ΔN117 mutant cervical cancer cells, respectively. Each column is mean ± S.E. (n = 6). #, p < 0.001 compared with the wild-type group. C and D, histology of tumors. The malignant characteristics of wild-type cervical cancer cells show the formation of irregular contour of infiltrating cancer nests that invade directly into the local adjacent muscular tissues and blood vessels (C). The ΔN117 mutant cells formed a small well encapsulated tumor with central necrosis and without local invasion (D). Scale bars, 200 μm in upper panel of C and lower panel of D and 100 μm in lower panel of C.
mutation abolishes hypotonicity-induced KCl cotransport and down-regulates VSOAC, thereby retarding RVD. Cellular invasiveness is therefore affected by overexpression of ΔN117 mutant.

Second, and perhaps more importantly, KCC activity appears to be associated with MMP activation and integrin expression. In carcinogenesis, cervical epithelial cells breach the basement membrane to proliferate and migrate within the adjacent connective tissue. An important event in the dissolution of the matrix of the basement membrane involves the activation of MMP cascade, which is accompanied by altered expression of several cell adhesion molecules by the transformed cells (24, 28). Here we demonstrate that the function of ΔN117 KCC1 polypeptide in small tumors. Tissue lysates of tumor xenografts resulting from the transplantation of ΔN117 mutant (lane 1) or wild-type cervical cancer cells (lane 2) were immunoblotted with antibodies against KCC1 C-terminal amino acids 1074–1085 or against KCC1 N-terminal amino acids 1–14.

These findings suggest a novel function of KCl cotransport in cancer invasion, the regulation of integrin expression and of MMP activity. The role of [Cl⁻], has been suggested as an important second messenger to regulate β₂ integrin activation of polymorphonuclear leukocytes in response to tumor necrotic factor stimulation (22, 23). Further work will be required to determine whether these changes in integrin and MMP expression reflect KCC-associated changes in [Cl⁻], or [K⁺], or other functions of KCC possibly unrelated to ion transport. Such transport-independent cytoskeletal and signaling functions have been described for the Na⁺/H⁺ exchanger NHE1 in cultured fibroblasts (30).

It is interesting to find that ΔN117 mutant cervical cancer cells exhibit little capacity for RVD, secondary to inhibition of
KCl cotransport and down-regulation of VSOAC activity. This suggests an interaction at either the signaling or membrane activity levels of the RVD process. We have demonstrated previously (31) that the activity of VSOAC is differentially expressed during the cell cycle progression of human cervical cancer cells. To block this channel activity could inhibit cell proliferation by arresting the cell cycle progression. Thus, the down-regulation of the VSOAC activity by KCC mutation would have a profound impact on cell proliferation. However, the limited to indirect methods to show this unusual function of KCC mutation on the ion channel activity. Nevertheless, the use of dominant-negative mutants allows us to make some significant progress in demonstrating the novel role of KCl cotransport in cell volume regulation.

In conclusion, the above results indicate that KCl cotransport plays a crucial role in the growth and invasion of human cervical cancer. Blockade of KCl cotransport may be a useful therapeutic adjunctive strategy to retard or prevent cervical cancer invasion.

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REFERENCES