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The serine protease stratum corneum chymotryptic enzyme (kallikrein 7) is highly overexpressed in squamous cervical cancer cells

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Abstract

Objective. To determine whether the Stratum Corneum Chymotryptic Enzyme (SCCE), a novel serine protease known to contribute to the cell shedding process by catalyzing the degradation of intercellular cohesive structures at the skin surface, is overexpressed in human cervical tumors.

Methods. SCCE expression was evaluated in 18 cervical cancer cell lines (i.e., 10 primary and 8 established cell lines) as well as in 8 normal cervical keratinocyte cultures by RT-PCR. In addition, SCCE expression was evaluated by immunohistochemistry on paraffin-embedded tumor tissue.

Results. Normal cervical keratinocytes did not express SCCE. In contrast, 50% of the primary and 50% of the established cervical cancer cell lines expressed SCCE by RT-PCR. Eighty percent (i.e., four of five) of primary squamous cervical tumors and 20% (i.e., one of five) of primary adenocarcinomas expressed SCCE. Five out of five (100%) of the patients harboring SCCE-positive tumors were found to have metastatic involvement of the pelvic tumor draining lymph nodes. Immunohistochemistry staining of paraffin-embedded cervical cancer specimens confirmed SCCE expression in tumor cells and its absence on normal cervical epithelial cells.

Conclusion. Squamous cervical cancer expressed high levels of SCCE, suggesting that this protease may play an important role in invasion and metastasis. Because SCCE appears only in abundance in tumor tissue and contains a secretion signal sequence, suggesting that SCCE is secreted, it may prove to be a useful diagnostic/prognostic tool for the detection of metastatic or recurrent disease or as a novel molecular target for cervical cancer therapy.

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Keywords: Cervical cancer; SCCE; Serine protease

Introduction

Tumor invasion and metastatic spread is a multistep process that begins with the shedding of malignant cells from the primary site, transport through lymphatic and blood vessels, and seeding at distant organs, resulting in tumor-associated metastasis [1–3]. Serine proteases are a family of protein-degrading enzymes that play crucial roles in numerous biological processes including activation of complement, blood coagulation, degradation of extracellular matrix component, and activation of growth and angiogenic factors [4,5]. To assess the value of secreted proteases as markers for tumor detection and targets for therapeutic intervention, our group has recently developed a screening strategy using redundant primers to the conserved catalytic triad domain of the serine protease family. Using this approach to display serine protease transcripts found in

Abbreviations: SCCE, Stratum Corneum Chymotryptic Enzyme; CVX, squamous cervical cancer; ADX, cervical adenocarcinoma; KRT, normal keratinocytes.

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abundance in gynecologic tumor tissues, with little or no expression in normal tissues, we recently reported the identification and cloning of several transmembrane serine proteases, including Stratum Corneum Chymotryptic Enzyme (SCCE/kallikrein 7), in ovarian cancer [6–10]. Originally characterized by Hansson et al. [11] and Brattsand and Egelrud [12], SCCE represents a serine protease expressed in the upper spinous and granular layers of the epidermis playing a crucial role during keratinocyte cell shedding (i.e., desquamation). Because of our previously reported association between SCCE expression and tumor growth and metastasis in ovarian cancer [7] and the fact that SCCE appears to catalyze the degradation of intercellular cohesive structures in the outermost cornified layer of the skin, in this study, we have evaluated the extent and frequency of expression of the SCCE gene in several primary and established cervical cancer cell lines. We report, to our knowledge for the first time, expression of SCCE on the majority of squamous cervical tumors evaluated and a possible association between SCCE expression and metastatic involvement of tumor draining lymph nodes. These findings may have important implications for the use of SCCE protease as a diagnostic/prognostic tool or as a molecular target for cervical cancer therapy.

Materials and methods

Primary and established cervical cancer cell lines

Eighteen cervical cancer cell lines (i.e., 10 primary and 8 established) were evaluated for SCCE expression by RT-PCR. Commercially available established cervical cancer cell lines (i.e., CaSki, SiHa, MS-751, HT-3, C33a, Me180, HeLa, and C4-I) were purchased from the American Type Culture Collection. Tumors derived from primary specimens were staged according to the FIGO operative staging system. Fresh tumor biopsies from patients diagnosed with frankly invasive stage IB–IIA cervical cancer were obtained at the time of surgery through the Gynecologic Oncology Division and the Pathology Department, UAMS, under approval of the Institutional Review Board. Patient characteristics are described in Table 1. Radical abdominal hysterectomy and lymph node dissection for invasive cervical cancer were performed in all patients. Eight normal keratinocyte (KRT) control samples (i.e., four primary keratinocyte cultures and four flash-frozen biopsies) were obtained from cervical biopsies of hysterectomy specimens from women diagnosed with benign disease and with a previous report of a normal cytological evaluation. Cervical cancer and normal keratinocyte cell lines were established following previously reported standard tissue culture techniques [13]. We studied 10 primary cervical cancer and 4 primary keratinocyte cell lines, some of which had been cultured for 3–4 weeks and others over a much longer period of time. Purity of fresh tumor cultures was tested by morphology, immunohistochemistry staining, and/or flow cytometry with antibodies against cytokeratins. Only cell lines containing more than 99% tumor cells were evaluated. Because tissue digestion and prolonged in vitro cell culture may potentially alter antigen expression, four adjunctive normal cervical keratinocyte biopsies were also evaluated for SCCE expression before enzymatic digestion and/or in vitro culture (i.e., snap frozen in liquid nitrogen immediately after collection).

Table 1
Characteristics of the patients

Patient	Age	Race	Stage	Treatment	Lymph nodes status
CVX 1	40	White	I B	surgery	–
CVX 2	26	White	I B	surgery	+
CVX 3	38	Afro-American	I B	surgery	+
CVX 4	40	White	IIA	surgery	+
CVX 5	42	White	I B	surgery	+
ADX 1	33	White	I B	surgery	+
ADX 2	33	Afro-American	I B	surgery	–
ADX 3	27	White	I B	surgery	–
ADX 4	50	Afro-American	I B	surgery	–
ADX 5	46	White	I B	surgery	–

RNA isolation and cDNA synthesis

RNA isolation from different cell lines was performed using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. To verify integrity, 4 μg of RNA from each sample was run in 1% agarose gel using 18S + 28S Ribosomal RNA (Sigma) as positive control. First-strand cDNA was synthesized using 5 μg of total RNA, 1 × RT-PCR buffer, 5 mM $MgCl_2$, 1 mM dNTPs, 2.5 μM random hexamers, 1 U/μl Rnase Inhibitor, and 2.5 U/μl of MuLV Reverse transcriptase (Gene Amp RNA PCR kit; Applied Biosystems) in a total volume of 20 μl. RNA extracted from CaOV3 serous papillary ovarian cancer cell line, previously reported to express SCCE [7], was used as a positive control.

PCRs

The PCR reaction mixture consisted of cDNA, 0.4 μM of sense and antisense primers, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.025 U/μl Taq polymerase (Gene Amp RNA PCR kit; Applied Biosystems) with reaction buffer in a total volume of 50 μl. The target sequences were amplified in parallel with β-tubulin gene. Thirty-five cycles of PCR were performed in a Gene Amp PCR System 2700 (Applied Biosystems). Each PCR cycle included 30 s of denaturation at 95°C, 30 s of annealing at 58°C, and 30 s of extension at 72°C. The sequences of the SCCE specific primers that produce the 339-bp product were as follows: 5′-AGATGAATGAGTACACCGTG-3′ forward and 5′-CCAGTAAGTCCTTGTAAACC-3′ reverse. The sequences of β-tubulin primers that produce the 454-bp product were as follows: 5′-CGCATCAACGTGTACTA-

CAA-3′ forward and 5′-TACGAGCTGGTGGACT-GAGA-3′ reverse. These primers span an intron such that the PCR products generated from pure cDNA can be distinguished from cDNA contaminated with genomic DNA.

Antibody production and immunohistochemistry

Polyclonal antibodies were generated by immunization of white New Zealand rabbits with one of two poly-lysine-linked multiple antigen peptides derived from the amino acid sequence of SCCE as previously reported [7]. These sequences are PLQILLLSLALE and SFRHPGYSTQTH. Formalin-fixed, paraffin-embedded tissue blocks from 8 normal uterine cervix (i.e., controls) and 10 invasive cervical carcinomas of the uterus (5 squamous carcinomas and 5 adenocarcinomas) from which primary cell lines were established were retrieved from the surgical pathology files of the University of Arkansas for Medical Sciences (UAMS) and evaluated for SCCE expression. Study blocks were selected after histopathologic review. The most representative hematoxylin and eosin-stained block sections were used for each specimen. Immunohistochemical staining was performed using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA). Briefly, formalin-fixed and paraffin-embedded specimens were routinely deparaffinized and processed using microwave heat treatment in 0.01 M sodium citrate buffer (pH 6.0). The specimens were incubated in methanol with 0.3% H_2O_2 for 30 min at room temperature and then incubated with normal goat serum for 30 min. The samples were incubated with anti-SCCE peptide-derived polyclonal antibody for 1 h at room temperature in a moisture chamber, followed by incubation with biotinylated anti-rabbit IgG for 30 min, and then incubated with ABC reagent (Vector Laboratories) for 30 min. The final products were visualized using the AEC substrate system (DAKO, Carpenteria, CA), and sections were counterstained with hematoxylin before mounting. Negative controls were performed by using normal serum instead of the primary antibody.

Statistical analysis

Data were analyzed using the two-tailed t test, and Student's t test for paired data. In all tests, the difference was considered significant when P values were less than 0.05.

Results

SCCE expression in primary and established cervical cancer cell lines

To characterize the frequency of expression of the SCCE gene in cervical tumors, we used RT-PCR with cDNA derived from primary and established squamous and adenocarcinoma cervical cancer cell lines as a template. Cultures of normal cervical keratinocytes were used as controls. PCR primers that amplify a SCCE-specific 339-bp product were synthesized and used in reactions. Primers that produce a specific 454-bp PCR product for β-tubulin were also used as internal controls. Of the 18 cervical carcinomas studied, 9 (50%) were found to express SCCE by RT-PCR. Fig. 1 shows an ethidium bromide-stained agarose gel with the separated RT-PCR products for normal cervical keratinocytes and primary cervical cancer cell lines, while Fig. 2 shows the RT-PCR results for the established cervical cancer cell lines. Both are representative of the typical results observed. Flash-frozen samples or cultures of normal cervical keratinocytes did not express SCCE (Figs. 1 and 2). In contrast, SCCE was found to be expressed in 50% (i.e., 5 of 10) of primary cervical cancer cell lines ($P < 0.01$) and in 50% (i.e., 4 of 8) of established cervical cancer cell lines ($P < 0.01$). Importantly, when individual histological subtypes of primary tumors were evaluated, SCCE expression was found in four out of five (80%) of the primary squamous cervical tumors and one out of five (20%) of the adenocarcinomas.

Five out of ten of the early-stage cervical cancer patients from which primary cell lines were successfully established in vitro presented with metastatic involvement of the pelvic lymph nodes (Table 1). Strikingly, all patients for whom the

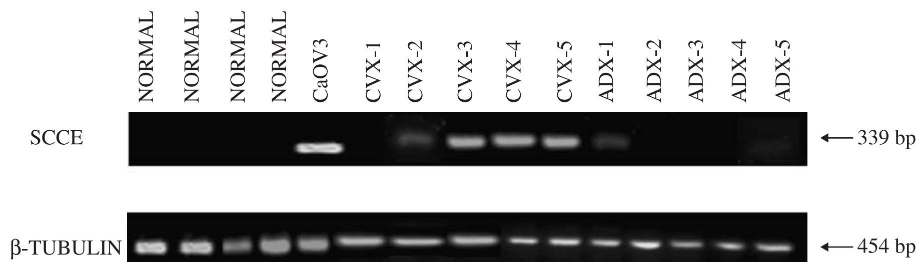

Fig. 1. SCCE expression by RT-PCR on primary squamous cell carcinoma (CVX-1-5), primary adenocarcinoma (ADX1-5), and normal cervical keratinocyte primary cell lines. The reaction products were electrophoresed through a 2% agarose gel and stained with ethidium bromide. The 454-bp band represents the β-tubulin product, and the 339-bp band represents the SCCE product. SCCE mRNA expression was not detected in any of the four normal cervical keratinocyte cell lines tested. In contrast, 80% (i.e., four out of five) of the squamous cell and 20% (i.e., one out of five) of the cervical adenocarcinomas expressed SCCE.

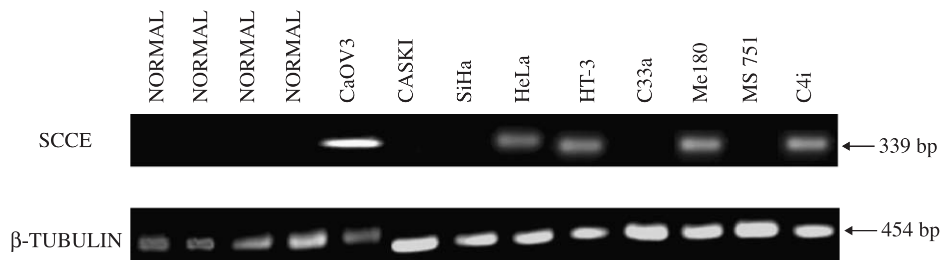

Fig. 2. SCCE expression by RT-PCR on established cervical cancer cell lines and normal keratinocyte flash-frozen biopsies. The reaction products were electrophoresed through a 2% agarose gel and stained with ethidium bromide. The 454-bp band represents the β-tubulin product, and the 339-bp band represents the SCCE product. SCCE mRNA expression was not detected in any of the four normal cervical keratinocyte samples tested. In contrast, 50% (i.e., four out of eight) of the established cell lines expressed SCCE.

primary tumors expressed SCCE (i.e., CVX-2, CVX-3, CVX-4, CVX-5, and ADX-1 = 100%) were found to harbor metastatic disease in the tumor draining lymph nodes.

Immunohistochemistry staining

To determine whether SCCE expression detected by RT-PCR in primary cervical cancer cell lines is the result of a selection of a subpopulation of cancer cells present in the original tumor, or whether in vitro expansion conditions may have modified gene expression, we performed immunohistochemical analysis of SCCE protein expression on formalin-fixed paraffin-embedded tumor tissue from all primary tumors from which primary cell lines were derived. As shown in Table 2, immunohistochemical staining supported the data obtained by RT-PCR. Using a SCCE peptide-directed antibody, we observed no significant staining with normal cervical keratinocytes samples (Fig. 3A). In contrast, intense staining was associated with cervical tumor cells of both adenocarcinoma and squamous histological subtypes (Figs. 3C and D). Both adenocarcinoma and squamous cell carcinoma specimens (Figs. 3C and D, respectively), similarly to ovarian cancer cells (i.e., positive control, Fig. 3B), showed diffuse cytoplasmic and cell membrane staining for SCCE.

Table 2
SCCE expression in squamous and adenocarcinoma cervical cancers and normal keratinocytes by immunohistochemistry

Tissue type	SCCE expression
CVX 1	−
CVX 2	+
CVX 3	+
CVX 4	+
CVX 5	+
ADX 1	+
ADX 2	−
ADX 3	−
ADX 4	−
ADX 5	−
KRT 1	−
KRT 2	−
KRT 3	−
KRT 4	−
KRT 5	−
KRT 6	−
KRT 7	−
KRT 8	−

Discussion

In the process of studying proteolytic enzymes playing a key role in tumor metastatic processes, we have recently identified overexpression of SCCE as well as other serine proteases in a high percentage of ovarian cancers [6–10]. Because SCCE is known to catalyze the degradation of intercellular cohesive structures at the skin surface and thus may play a role during the metastatic process of cervical cancer, in this work we have analyzed the expression of the novel serine protease SCCE in several primary cultures as well as established cervical cancer cell lines. We chose this approach to evaluate differential SCCE gene expression in highly enriched populations of cervical tumor-derived epithelial cells. We report that SCCE is highly expressed in 80% of the primary squamous cervical cancer cell lines studied whereas it is undetectable in normal cervical keratinocytes. These data therefore support the view of SCCE as a highly differentially expressed serine protease in squamous cervical tumors when compared to normal control cells (i.e., cervical keratinocytes). In contrast, only 20% of the cervical adenocarcinoma were found to express SCCE.

To validate gene expression data at protein level, SCCE expression was evaluated by immunohistochemistry on paraffin-embedded tissue from which all primary tumor cell lines were established. All primary uncultured tumor specimens from which SCCE expression was detected by RT-PCR expressed SCCE by immunohistochemistry. These included four out of five (80%) primary squamous carcinoma and one out of five (20%) primary adenocarcinoma, confirming that SCCE protein expression is detectable on SCCE RNA-positive cervical cancer cells. Because the biological aggressiveness of neoplastic cells lies in their ability to proliferate abnormally, invade normal host tissues and metastasize to distant sites, and proteases may provide a variety of services that assist in the process of tumor

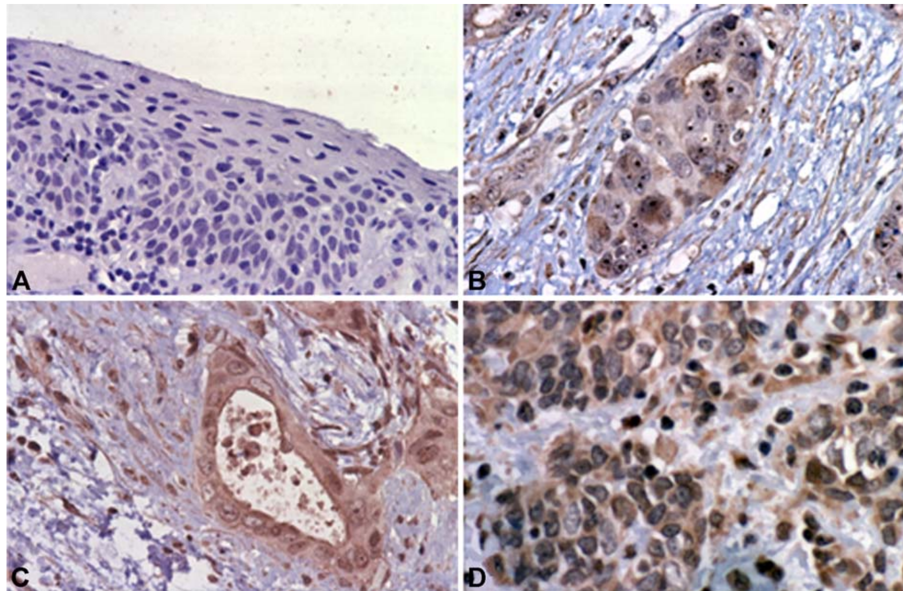

Fig. 3. Immunohistochemical analysis of SCCE expression in cervical tumors. Representative immunoreactivity of SCCE in normal epithelial cells (A) (×200), ovarian serous papillary carcinomas (positive control) (B) (×400), adenocarcinoma ADX-1 (C) (×400), and cervical squamous cell carcinoma CVX-3 (D) (×400). While no staining is observed in normal cervical keratinocytes, in both squamous cell carcinoma and adenocarcinomas, SCCE shows diffuse staining throughout all tumor cells.

progression, we hypothesized that a high expression of SCCE on cervical tumor cell surfaces may play an important role during cervical cancer metastatic spread. Consistent with this view, we found that all cervical cancer patients overexpressing SCCE on their tumors (i.e., four out of four of the squamous cell carcinoma, and one out one of the adenocarcinoma, respectively) were harboring metastatic disease in their pelvic lymph nodes. Thus, although our study population of 10 early-stage cervical cancer patients is small and further studies will be necessary to validate our findings, nevertheless, these data seem to suggest that SCCE overexpression in cervical cancer may identify a group of aggressive tumors characterized by the ability to metastasize early to the regional lymph nodes. Taken together, these data suggest that future research assessing the clinical usefulness of SCCE as a marker for early identification of cervical cancer patients with high risk of lymphatic involvement might be worthwhile.

Because SCCE appears in abundance in a significant percentage of squamous cell carcinoma and SCCE can be released, as suggested by immunohistochemical data [7,11,12], this protease may prove to be a useful tool for monitoring response to primary treatment and/or the early detection of recurrent/persistent cervical cancer after standard therapy. Indeed, a serum tumor marker that accurately reflects the active tumor status of the majority of patients with cervical cancer would be particularly useful in the latter setting where the distinction between radiation fibrosis and disease recurrence may be extremely difficult. Consistent with this view, PSA and kallikrein 2 have already been validated as potential tumor markers for the early diagnosis or recurrence of prostate cancer due to their abnormal prevalence in the peripheral blood of patients [14]. Furthermore, other secreted members of the serine proteases family including kallikrein 5, 10, 11, and 14 are in the process of being validated as novel cancer biomarkers in other human solid tumors [15–20]. To validate this hypothesis, an ELISA detection kit for quantification of SCCE in the circulation of ovarian and cervical cancer patients is under development in our laboratory.

The recognition of tumor antigen loaded dendritic cells (DC) as one of the most promising approaches to induce a tumor specific immune response in vivo has recently generated widespread interest in the use of these “natural adjuvants” for the therapy of several human malignancies refractory to standard treatment modalities [21]. The identification and cloning of a group of preferentially expressed serine proteases including SCCE as novel tumor-associated antigens may therefore offer the opportunity to use these targets to assess the potential for therapeutic DC vaccination for the treatment of human cancer patients refractory to standard treatment modalities [22]. Indeed, the lack of SCCE transcript presence in all normal adult and fetal tissues examined so far, with the exception of the desquamating stratum corneum of the skin [7], support the potential of this secreted extracellular enzyme as a useful therapeutic marker for ovarian and cervical carcinoma. Supporting this view, our laboratory has recently identified cytotoxic T lymphocyte (CTL) epitopes within SCCE [22]. These data, combined with the recently reported immunodominant role played by other serine proteases (i.e., PSA) in the prostate cancer-specific immune response induced by DC transfected with amplified tumor RNA [23] or loaded with killed prostate cancer cells [24], further suggest that

peptide epitopes derived from SCCE may become selective targets for DC-driven CD8+ CTL against cervical cancer.

In conclusion, we have shown that squamous cervical cancer cell lines, but not normal cervical epithelial cells, frequently overexpress the SCCE gene, and we have further demonstrated that cervical tumors expressed this secreted/membrane-associated serine protease. In addition, we have reported evidence that SCCE expression may identify a group of cervical tumors endowed with the ability to metastasize early during disease progression to tumor draining lymph nodes. On the basis of these findings, we suggest that SCCE has the potential to become a useful diagnostic/prognostic tool for identifying biologically aggressive cervical tumors, monitoring response to therapy as well as serve as a novel target antigen for the treatment of cervical tumors refractory to standard treatment modalities. The future design and implementation of clinical trials will ultimately determine the validity of this approach.

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