

Original Article

Trypsin proteolytic activity in cervical cancer and precursor lesions

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Abstract: The aim of the present study was to study the expression and activity of trypsin in tissues from patients with cervical cancer and precursor lesions. Immunohistochemistry assays were used to analyze trypsin expression in fixed paraffin-embedded tumor tissues obtained from patients with squamous cervical carcinomas (large-cell keratinizing and non-keratinizing types) and squamous intraepithelial lesions. Proteolytic activity was evaluated in tissue extracts by zymography. Human papillomavirus (HPV) genotyping was performed using PCR. We describe for the first time that trypsin is expressed in invasive squamous cervical carcinomas. Both trypsin expression and proteolytic activity were linked to tumor progression. Our data suggest that trypsin, directly or indirectly, plays a key role in cervical cancer progression.

Keywords: Trypsin, cervical cancer, serine proteases

Introduction

Worldwide, cervical cancer is the second most common malignant neoplasm in women. It has been established that infection with human papillomavirus (HPV) is a necessary but not unique factor for the development of cervical cancer. Co-factors that increase the risk for cervical cancer among HPV DNA positive women include the use of oral contraceptives, smoking, high parity, a history of previous sexually transmitted disease and immunodeficiency [1-4]. Cervical carcinogenesis implies HPV infection, viral persistence, progression and invasion [5]. Proteases play a key role in cancer invasion and metastasis [6]. Studies have found a direct correlation between proteolytic activity and malignant progression from precursor lesions to invasive cervical carcinoma [7, 8].

New insights in cancer research have revealed a crucial role of new serine-proteases, including Type II Transmembrane Serine Protease, Trypsin-like and Trypsin proteinases [9-11]. Adv-

anced ovarian cancer has been associated with tumor expression of trypsinogen-1, trypsinogen-2, tumor-associated trypsin inhibitor and high trypsin serum levels [12].

A strong association between trypsin and PAR-2 expression in five different cervical cancer cell lines has been reported. PAR-2 expression was found to correlate with tumor proliferation upon trypsin or PAR-2-agonist stimuli [13]. However, the expression of trypsin in tissues from patients with cervical cancer and precursor lesions as of yet remains unknown. The aim of the present study was to evaluate the expression and proteolytic activity of trypsin in tissues obtained from patients with cervical cancer and precursor lesions.

Materials and methods

Patients and tissue samples

Cervical samples were obtained from 20 patients with invasive squamous cervical carcino-

mas (large-cell keratinizing and non-keratinizing types) and from patients with squamous intraepithelial lesions. Women without a history of abnormal Pap smears, who planned to undergo a hysterectomy due to non-malignant conditions, were included as controls (normal epithelium confirmed by histopathology). All women (age range 36-65 years) were patients of the OPD Hospital Civil de Guadalajara, Mexico. Two different pathologists independently confirmed the diagnoses for each specimen. The protocol was approved by Biomedical Sciences and Ethics Committees according to the latest guidelines of the World Medical Association Declaration of Helsinki (Fortaleza, Brazil, October 2013).

Tissue extracts

After removing necrotic and bloody areas, the samples were frozen at -70°C until extraction. Extracts were prepared from 100-150 mg (wet weight) tissue samples as previously described [7]. The specimens were homogenized in a high-speed mixer-homogenizer (Polytron PT 3000, Kinematica AG, Brinkmann, Switzerland) for 5 min at 15000 rpm, in 4 mL of serum-free AIM-v® medium (GIBCO Laboratories, Grand Island, NY) at 4°C. After three freeze-thaw cycles, each homogenate was sonicated (Sonic Dismembrator Brand, U.S.A.) twice at 21 kilocycles/sec for 1 min at 4°C. The homogenate was centrifuged at 8000 g for 10 min at 4°C to obtain the supernatant. The protein concentrations of the extracts were determined using the Bradford assay [14, 15]. The supernatants (1.5 mg/mL protein) were divided into aliquots and stored at -70°C until they were analyzed.

Immunohistochemistry

Paraffin-embedded tissues were cut into 5 µm sections and transferred to silanized slides and then treated with xylene (J.T. Baker; Xalostoc, Mexico) to remove the paraffin. Tissues were rehydrated through graded alcohol (Sigma-Aldrich Corp., St. Louis, MO, USA) and heated in a steamer for 30 min in citrate buffer (10 mM, pH 6.0) for antigen retrieval. Slides were rinsed in Tris-buffered saline (TBS: 50 mmol/L Tris, 150 mmol/L NaCl, pH 7.4) and treated with Dako Peroxidase Block (Dako; Carpinteria, CA) for 5 min at room temperature to quench endogenous peroxidase activity. Sections were incubated with an anti-trypsin (human specific)

monoclonal antibody (Chemicon®) for 2 h. The two-step EnVision system (Dako; Carpinteria, CA) was used for color development. Application of the primary antibody was followed by incubation with a polymeric conjugate [secondary anti-mouse antibodies bound to a dextran backbone containing horseradish peroxidase (HRP)] for 30 min at room temperature. The highly sensitive 3-amino-9-ethylcarbazol plus (AEC1) chromogen (Dako) was used as substrate for the EnVision HRP enzymes. All sections were counterstained with Mayer's Hematoxylin and mounted in a gelatin-glycerin-based medium (Glycergel, Dako Faramount; Dako), and examined by light microscopy using a grid eyepiece. Negative controls included sections negative for the anti-trypsin and stained with nonspecific mouse IgG at the same protein concentration as the primary anti-trypsin antibody. Pancreatic tissue was used as a positive control in each staining run.

Evaluation of immunohistochemical staining

Two pathologists performed histological and immunohistochemical evaluations independently. For each sample, at least 3000 cells were evaluated for trypsin expression. The staining reaction was evaluated using the immunoreactive score (IRS) reported by Ikeda et al. [16]. $IRS = SI \text{ (staining intensity)} \times PP \text{ (percentage of positive cells)}$. SI was defined as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong). PP was defined as 0 (negative), 1 (1-10% positive cells), 2 (11-50% positive cells), 3 (51-75% positive cells) and 4 (76-100% positive cells).

Zymography

Gelatin zymography was carried out on 12.5% polyacrylamide gels containing 1 mg/mL gelatin at 4°C. After electrophoresis, gels were incubated for 20 h in 50 mM Tris-HCl (pH 7.5) with 0.1 M NaCl and 2.5% (v/v) Triton X-100 in the presence of 5 mmol/L ethylenediaminetetraacetic acid (EDTA) to inhibit metalloproteinase activity. The gels were stained with Coomassie Brilliant Blue and rinsed with methanol-acetic acid-water for 60 min [17].

DNA extraction

Tissue regions of interest, including invasive cervical tissue, were defined by morphological

Table 1. Primer sequences of different types of HVP virus

VIRUS	Primer Sequences (5'-3')	Amplimer length (base pair)
HVP (Cpl/CpII)	(DNA)-CGTCCAAGAGGATACTGATC 1F (DNA)-GCACAGGGTCATAATAATGG 1R	188
HVP6/11	(DNA)-CTCTGCCGGTGGTCAGTGCAT 1F (DNA)-ATGCCTCCACGTCTGCAAC 1R	120
HVP16	(DNA)-CTGCACATGGGTGTGTGC 1F (DNA)-GCAGCTCTGTGCATAAC 1R	229
HVP18	(DNA)-GAATCACTCTATGTGCAG 1F (DNA)-TAGTTGTTGCCTGTAGGTG 1R	221
HVP31	(DNA)-TTCAAAAATCCTGCAGAAAG 1F (DNA)-CTTTGACACGTTATACACCT 1R	320
HVP33	(DNA)-ACCTTTGCAACGATCTGAGG 1F (DNA)-GAACCGCAAACACAGTTTAC 1R	108

for 30 s, 51°C for 30 s, 72°C for 60 s for 40 cycles; HPV16: 92°C for 120 s, 48°C for 90 s, 72°C for 60 s for 40 cycles; HPV6/11 and HPV18: 92°C for 120 s, 48°C for 90 s, 72°C for 60 s for 38 cycles; and HPV31 and HPV33: 94°C for 60 s, 45°C for 60 s, 72°C for 60 s for 45 cycles. Amplification products were electrophoresed on 1.8% agarose gels and visualized using ethidium bromide staining under UV light.

Statistical analysis

criteria. Tissue regions were outlined and excised from the paraffin block using a small scalpel (3 mm × 3 mm). Fragments were collected in autoclaved plastic microtubes (1.5 mL). The paraffin was dissolved twice in xylene (1 mL) for 10 min. Then, 0.5 mL of 100% ethanol was added and mixed for 5 min followed by centrifugation at 10,000 g for 3 min with two changes. After ethanol evaporation at 37°C, 200 µg/mL of proteinase K (Sigma Chemical, St. Louis, MO, USA) was added for 36 h at 37°C. Proteinase K was inactivated at 94°C for 10 min. Aqueous supernatants were transferred to another fresh microtube. The DNA was precipitated by adding 100% ethanol and 20 µg/mL glycogen (Sigma Chemical, St. Louis, MO, USA) for 30 min at -20°C. The pellet was washed twice with 70% ethanol, dried and resuspended in 20 µL of distilled water and measured spectrophotometrically.

PCR assay

HPV genotyping was performed using specific primers (Table 1). All PCR reactions were performed in a total volume of 50 µL. The PCR mixture contained 75 mM Tris-HCl pH 8.8, 20 mM (NH₄)₂SO₄, 0.01% Tween 20, 2 mM MgCl₂, 0.2 mM dNTPs, 0.6 µM of each primer, 1.25 U of recombinant Taq DNA polymerase (Fermentas) and 100 ng DNA. Genomic DNAs from SiHa (HPV16), HeLa (HPV18) and tissue samples with known HPV infection for HPV6/11, HPV31 and HPV33 were used as positive controls. Genomic DNA from C33-A (HPV negative) cervical cancer cells was used as the negative control. The cycling protocol for CPI/CPII was 94°C

Statistical analysis was performed using the SPSS software package (version 10.0; SPSS Inc., Chicago, IL). Measures of central tendency and dispersion were determined. Trypsin expression in normal cervical tissues, squamous intraepithelial lesions and cervical cancer were compared using the ANOVA test. Pearson's correlation was used to correlate trypsin expression or activity with HPV infection. Trypsin expression and proteolytic activity were correlated with clinical stage. Differences were considered significant at P< 0.05.

Results

We included negative controls (tissue sections not stained with the anti-trypsin antibody or stained with a nonspecific mouse antibody) and positive controls (pancreas sections) to ensure the specificity of the immunohistochemical staining. Immunohistochemical analysis showed that trypsin was not expressed in normal cervical tissues. Neither ectocervical nor endocervical normal epithelia from the 10 normal women reacted to the anti-trypsin antibody. Trypsin was poorly expressed in 3/10 squamous intraepithelial lesions, with an immunoreactive score (IRS) of 1. In contrast, 70% of invasive cervical tumors were positive for trypsin, with IRS ranging from 2 to 4; these samples exhibited a cytoplasmic staining pattern (Figures 1 and 2). Trypsin expression was stronger on the invasive fronts of the tumors. Using Pearson's correlation, we observed a significant positive correlation between clinical stage (FIGO) and IRS (r = 0.65, P = 0.04).

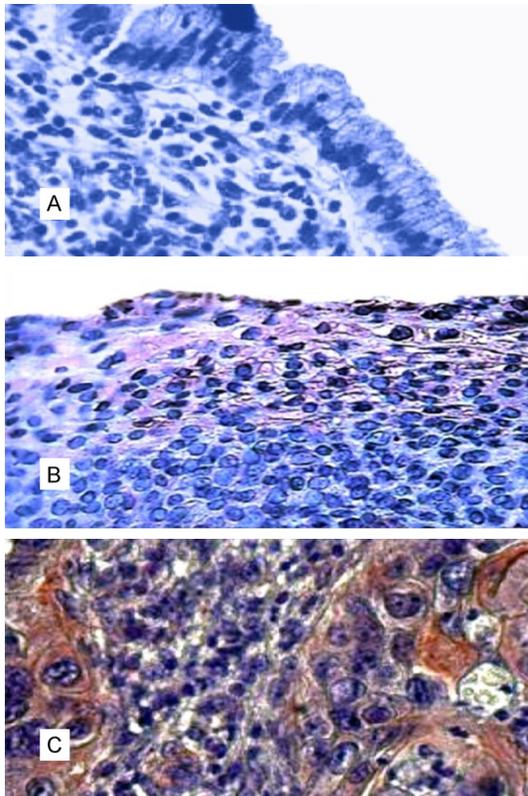


Figure 1. Trypsin immunoreactivity in cervical tissues. Trypsin expression in normal, SIL and malignant cervical tissues was detected by immunohistochemical assay and analyzed by an immunoreactive score (IRS) ranging from 0 to 4. The figure shows representative samples from all study groups: A: Control section which did not reveal any staining reaction; B: SIL section which showed only weak trypsin immunoreactivity; C: Malignant section with a strong trypsin signal at the invasive front of the tumor (magnification 400 ×).

None of the normal or intraepithelial lesion extracts showed evidence of trypsin activity as demonstrated by zymography. In contrast, six out of ten tissue extracts obtained from invasive cervical cancer showed a characteristic trypsin band with an apparent molecular weight of 23 kDa (**Figure 3**). All tumor tissues with zymographic evidence of trypsin activity were also positive for the presence of trypsin when tested in the immunohistochemistry assay (with IRS ranging from 2 to 4, as previously mentioned). One tumor sample that was positive for trypsin using immunohistochemistry (with an IRS of 2) was negative for trypsin activity. Using Pearson's correlation, we observed a significant positive correlation between immunoreactive score (IRS) and proteolytic activity

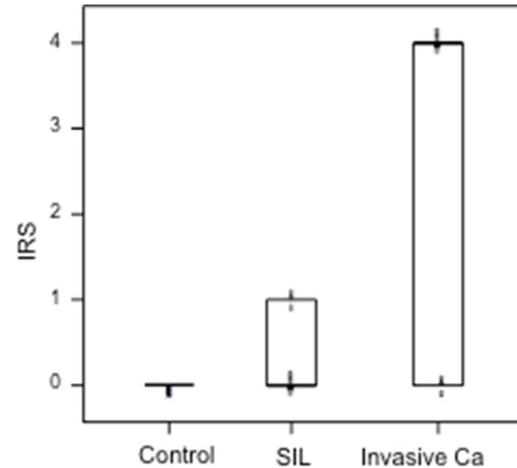


Figure 2. Immunoreactive trypsin is mainly detected in samples derived from patients with cervical cancer. Trypsin expression in normal, SIL and malignant cervical tissues was detected by immunohistochemical assays and analyzed by an immunoreactive score (IRS) ranging from 0 to 4. A strong trypsin immunoreactivity was observed in those samples obtained from patients with cervical cancer. Only a weak reaction was detected in two SIL samples, while all the normal tissues were completely negative for the presence of trypsin. A significant positive Pearson's correlation was seen between clinical stage (FIGO) and IRS.

index (PAI) ($r = 0.74$, $P = 0.01$). Interestingly, the proteolytic activity increased according to tumor progression. Tumor tissues from patients with stage I, stage II and stage III-IV showed a median proteolytic activity index of 50, 100 and 175, respectively (**Figure 4**). Pearson's correlation analysis between stage and proteolytic activity showed a significant positive correlation ($r = 0.66$, $P = 0.03$). We did not find a significant correlation between trypsin expression (IRS) or proteolytic activity and HPV infection with high risk or low risk types.

Discussion

Tumor interaction with its microenvironment is a key element for tumor progression, invasion and metastasis. Proteases produced by tumor or stromal cells induce extracellular matrix (ECM) degradation and changes in cell-cell or cell-ECM interactions, generating signals for survival, proliferation, cell behavior, motility or death [18].

Trypsin, a potent serine proteinase expressed in different malignant tumors, has been associ-

Trypsin in cervical cancer

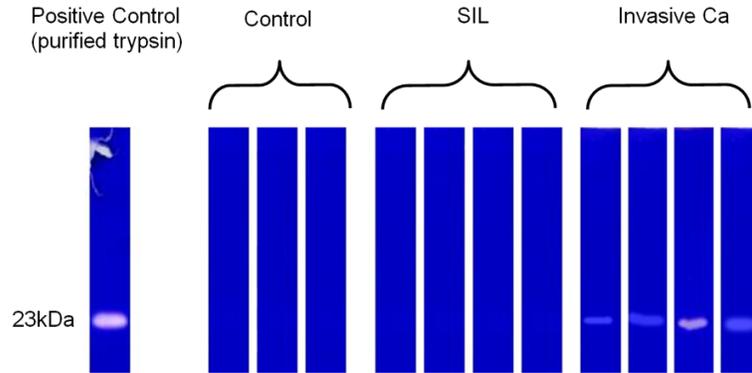


Figure 3. Trypsin activity is present in cervical cancer homogenates. Trypsin activity in extracts from normal, SIL and malignant cervical tissues was determined through zimography. A band on the gel corresponding to the molecular mass of trypsin (23 kDa) was detected in more than half of the cancer extracts. Conversely, extracts from normal and SIL tissues did not reveal any bands. All malignant tissues showing proteolytic activity were strongly immunoreactive to trypsin (IRS ranging from 2 to 4, **Figure 2**).

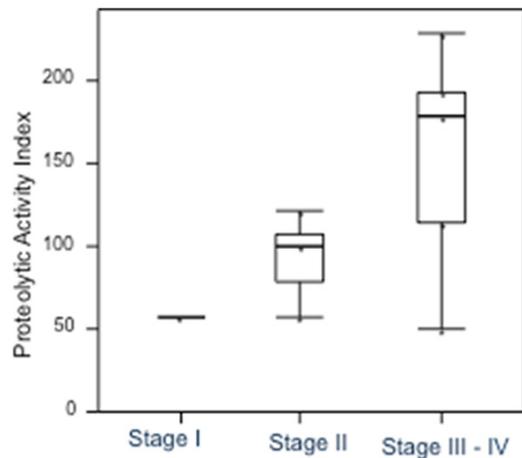


Figure 4. Proteolytic activity increases according to the tumor progression. The malignant homogenates were stratified following the FIGO System of Clinical Staging as stage I, II, and III-IV. The highest proteolytic activity index was found in those patients with more extensive tumors. The box plots represent each tumor stage. Medians are represented as thick horizontal lines, 25th and 75th percentiles as boxes and 10th and 90th percentiles as whiskers.

ated with tumor progression and poor prognosis [10, 12, 19]. In this work, we describe for the first time that trypsin is expressed in invasive squamous cervical carcinomas and can be linked to tumor progression. We demonstrated both the presence of trypsin and its activity in cervical cancer tissues. A significant correlation between trypsin expression or proteolytic activity and FIGO stage was observed. Trypsin

expression is a late event, associated with advanced clinical stages but unrelated to HPV infection.

It has been reported that trypsin activates metalloproteinase (MMPs), which in turn facilitate invasion and metastasis. Patients with trypsin-positive colorectal tumors have a poor prognosis and shorter disease-free survival. Reported data suggest that trypsin acts both directly by ECM digestion and indirectly through activation of other proteinases such as MMP-2, MMP-7, MMP-9 and matrilysin or PAR-2 activation [19,

20]. Previous studies confirmed a key role for MMPs in cervical cancer progression mediated by complex interactions with serine and cysteine proteases [7, 8, 21, 22].

Trypsin and other serine proteases such as trypsin, plasmin and thrombin regulate cell signaling by proteinase-activated receptors. Human PAR-1, PAR-2 and PAR-4 can be activated by trypsin and generates a 'tethered' receptor-triggering ligand during receptor cleavage. It has been reported that both PAR-1 and PAR-2 play a central role in epithelial tumors, promoting invasion through interactions with proteins such as Akt/PKB, Etk/Bmx and Vav3, via pleckstrin homology domains [23].

We previously demonstrated a strong correlation between PAR-2 and trypsin in five different cervical cancer cell lines. Exposure to trypsin or a PAR-2-agonist enhanced PAR-2 dependent tumor proliferation as measured by flow cytometry [13]. The biological and clinical relevance of these previous findings is supported by our present results that show a significant increase in trypsin and proteolytic activity in the later clinical stages of cervical cancer.

Unfortunately, the progress of the treatment for advanced cervical cancer has been restricted to palliative management. Basic research and clinical trials are needed to discover new targets for effective treatments [24]. The research of protease and protease-inhibitors, including

the PAR pathways, represent an important therapeutic window for cancer treatment [6]. Taken together our current results in the context of previous reports, suggest that trypsin and the PAR systems may be biomarkers for cervical cancer prognosis and targets for treatment.

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Disclosure of conflict of interest

None.

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