行政院國家科學委員會專題研究計畫　期中進度報告

子宮頸癌細胞外介質胸與腫瘤免疫抑制反應之相關性研究

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行政院國家科學委員會研究計畫成果報告

計畫名稱:子宮頸癌細胞外介質胺與腫瘤免疫抑制反應之相關性研究

Study on the Immunosuppressive Role of Cancer-derived Extracellular Matrix Enzymes in Human Cervical Cancer

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中華民國 **91** 年 **5** 月 **15** 日

In our second year project, we investigate the gelatinolytic activity of MMPsexpressions in human cervical neoplastic tissues. We utilized microdissection to evaluate the kinetic expression of MMPs in this model.

Materials and Methods

Case recruitment. Primary cases of cervical neoplasia are enrolled in this study. Tissue specimens will be obtained after radical hysterectomy. Cervical tissues from cases of uterine fibroids are included as normal controls. Each case of SCC is evaluated for clinical parameters including grade, lymphatic or vascular permeation, lymph node metastatic status, and clinical stage. Histologic grades of SCC included well, moderately, and poorly differentiated, based on the degree of differentiation toward keratinization with the formation of squamous pearls. Clinical staging of each patient is defined according to the 1995 modification of the International Federation of Gynecologists and Obstetricians (FIGO) staging of carcinoma of the cervix uteri.

Expression of MMPs, MT-MMPs, and TIMPs in CC tissue Five-micron sections of paraffin-embedded tissue are dewaxed and rehydrated before being transferred to 0.5% H_2O_2 for blocking of endogenous peroxidase prior to ishing with phosphate-buffered saline (PBS) buffer. An avidin-biotin-peroxidase complex method (Vector Laboratory, Burlingame, CA) is used for immunostaining. Monoclonal antibodies against MMP-1, -2, -3, -7, -8, -9, -13, -14, and -15, as well as TIMP-1, -2, and –3, purchased from Chemicon Inc., are used. Quantitative evaluation is performed by counting of the percentage of positively stained cells by use of a 10×10 square grid placed over the eyepiece of the microscope. Immunostaining results for MMPs/TIMPs will be scored by two independent observers.

Microdissection. Fresh tissue fragments were obtained from specimens after conization, simple or radical hysterectomy. Fresh uterine cervical tissues obtained from patients who underwent hysterectomy for myoma were included as controls. The samples were embedded without fixation in Tissue-Tek OCT compound (Miles Scientific, Naperville, Illinois) and snap-frozen. The remaining tissues were formalin-fixed and evaluated immunohistochemically for MMPs, and the findings were correlated with the results of zymography.

Tumor cell nests were carefully separated and scraped from underlying stroma along contiguous margins with a fine needle ($30G^{1/2}$) from frozen sections. The removed tissues were homogenized with sample lysis buffer containing glycerol (10%), Triton X-100 (1%), sodium pyrophosphate (1 mM), NaCl (137 mM), EDTA (5 mM), sodium orthovanadate (1 mM), NaF (10 mM), and Tris, pH 7.9 (20 mM). The cell lysates were centrifuged, and protein concentrations were measured by a dye-binding method according to the manufacturer's instructions (Bio-Rad Inc., Hercules, CA, USA).

Gelatin zymography. The gelatinolytic activity was determined by gelatin-substrate gel electrophoresis. With this method, we also detected the inactive proforms of gelatinases because SDS causes activation of the enzymes without proteolytic cleavage of the N-terminal sequence (12). Aliquots of tissue lysates (15 μg

protein/lane) were applied without heating or reduction to a 10% SDS-polyacrylamide gel containing 1 mg/ml of gelatin. After electrophoresis, gels were washed for 1 hr at room temperature in a 2% Triton X-100 solution before being transferred to a reaction buffer containing 50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, and 10 mM $CaCl_2$, and incubated at 37°C for overnight. The gel was stained with 0.1% Coomassie blue in 50% methanol/10% acetic acid and de-stained in 20% methanol/10% acetic acid. Clear zones of gelatin lysis against a blue background stain indicated the presence of enzyme. Quantitative analysis of gelatinolytic activity was achieved by scanning densitometry of the zymograms (IS-1000 Digital Imaging System, Alpha Innotech Corp., San Leandro, CA) as previously described (13). Values were expressed as lysis per μg protein. Each sample was processed in triplicate, and the mean levels were recorded.

Statistical analysis. Data were expressed as mean ± SD unless stated otherwise. Data analysis was performed with SPSS software (R.9.0.1, SPSS Inc., Chicago, IL, USA). One-way ANOVA with a multiple comparison test (Bonferroni *t*-test) was used for data analysis. Correlation and linear regression analyses were utilized for identifying potentially causal associations between variables. Statistical significance was defined by a P value of < 0.05.

Results

The clinical stages of the SCC group included 5 case in stage Ia, 13 cases in stage Ib, 7 cases in stage IIa, 2 cases in stage IIb, and 4 cases in stage III. The mean ages of the patients diagnosed with SCC was 50.8±13.5 years, compared to 41.7±12.8 and 38.2±10.8 years in the HSIL and LSIL patient groups, respectively. All patients treated for HSIL and LSIL are currently free of disease for at least 2 years' follow-up. In the SCC group, 21 cases (stage Ia to IIa) underwent radical hysterectomy and pelvic lymphaednectomy. The remaining 10 cases (stage IIa to III) had radiotherapy. Seven patients have recurrent/persistent cancer disease after treatments during 2 years follow-up. Histologic grading in SCC group showed well-differentiated, moderately differentiated, and poorly differentiated grades in 11(35.5%), 14 (45.2%), and 6 (19.4%) cases, respectively.

Immunohistochemical staining of tumor cells

Both MMP-9 and MMP-2 were found in the great majority of cases of SCC and HSIL. MMP-9 was immunoreactive in tumor cells in 30 of 31 cases (97%) of SCC and all cases (100%) of HSIL. The percentage of tumor cells positive for MMP-9 varied from 50% to 95%. MMP-2 was detected in 90% cases (28 in 31) of

SCC, and weak to moderate staining of MMP-2 was also noted in 82% cases of HSIL. The percentage of MMP-2^{+} tumor cells in HSIL was significantly less than that in SCC (P=0.006). The staining intensity of cells was similar in most cases, but appeared enhanced at the invasive edges of SCC or the basal layers of the tumor in some cases of HSIL. Both MMP-2 and MMP-9 were absent or minimally expressed in LSIL and normal cervical epithelium.

Carcinoma cells stained for MMP-3, -7, -8, and -13 were found in various portions of cases with SCC (10-35%) and HSIL (4-8%), but not in LSIL. The staining intensity was not as strong as that for MMP-2 and -9. TIMP-1 and TIMP-2 were weakly expressed in 10 (32%) and 19 (61%) cases of SCC, and infrequently in HSIL (8%). The staining was diffusely weak in most cases of SCC and HSIL. Only 3 of the 31 cases of SCC showed weak staining for TIMP-3.

Gelatin zymography

Gelatinase activity was assessed in 31 cases of SCC and 11 cases of HSIL. Five cases of normal cervical squamous epithelial tissues were also used as controls. The 92 kDa form of MMP-9 appeared as a broad band in all cases of SCC. The 84 kDa activated products of MMP-9 were not clearly distinguished in the acrylamide gel, as has been reported previously. Mean levels of total MMP-9 in SCC and HSIL groups were approximately 30-fold higher than those in normal squamous epithelium

(Table 2). In contrast to MMP-9, the gelatinolytic bands of proform MMP-2 (72 kDa) and active-form MMP-2 (66 kDa) were clearly separated in these gels (Fig.2). The levels of total MMP-2 (proform + active form) in HSIL and SCC were about 4 to 6-fold higher than those in normal controls. The levels of active-form MMP-2 were significantly higher in stage II and III SCC (approximately 14 times those of normal tissues) than stage I SCC and HSIL (2-4 times those of normal tissues). Gelatinolytic bands at 200 kDa and 140 kDa were also observed and may represent dimer forms of MMP-9 and MMP-2, respectively.