Stromal cells play a role in cervical cancer progression mediated by MMP-2 protein

T. Fernandes[1], L.A. de Angelo-Andrade[2], S.S. Morais[3], G.A. Pinto[3], C.A. Chagas[3], S.S. Maria-Engler[4], L.C. Zeferino[1]

[1]Department of Obstetrics and Gynecology, [2]Department of Pathology, [3]Woman's Hospital (CAISM), State University of Campinas, [4]Department of Clinical Chemistry and Toxicology, School of Pharmaceutical Sciences, University of São Paulo, Unicamp, Campinas, CP (Brazil)

Summary

Metalloproteinases, especially metalloproteinase-2 (MMP-2), are known for their role in the degradation of the extracellular matrix. Nevertheless, a thorough understanding of MMP-2 expression in neoplastic lesions of the uterine cervix has yet to be accomplished. This study aimed to analyze the MMP-2 expression in cervical intraepithelial neoplasia III (CIN3) and in cervical squamous cell carcinoma, in tumor cells and adjacent stromal cells. MMP-2 expression was assessed by an immunohistochemical technique. MMP-2 expression was greater in the stromal cells of invasive carcinomas than in CIN3 ($p < 0.0001$). MMP-2 expression in stromal cells correlates with the clinical stage, gradually increasing as the tumor progresses ($p = 0.04$). This study corroborates that stromal cells play an important role in tumor invasion and progression, mediated by the progressive enhancement of MMP-2 expression from CIN3 to advanced invasive tumor. The intense MMP-2 expression most probably is associated with poor tumor prognosis.

Key words: Matrix metalloproteinase 2; Cervix cancer; Cervical intraepithelial neoplasia; Stromal cells.

Introduction

Cervical carcinoma is the second most common neoplasia in women worldwide, corresponding annually to 16% of all cases of tumors in women [1]. Over the last ten years, many studies have demonstrated the unequivocal association between the human papilloma virus (HPV) and cervical carcinoma. The histological appearance of CIN3 is characterized by both cellular atypia and structural disorganization restricted to the squamous epithelium and limited by the basement membrane. For the occurrence of both invasion and tumor metastasis, degradation of the basement membrane and the extracellular matrix (ECM) must occur, since these structures act as a physical barrier to cell migration [3]. Such events suggest that during the progression of CIN, local phenomena occur that would result in the formation of genetically-modified cell clones capable of producing substances that would permit degradation of the basement membrane and initiate evolvement to invasive carcinoma [4].

ECM degradation is mediated by families of extracellular proteinases including serine proteases, cysteine proteases and matrix metalloproteinases (MMPs), which play a key role in the evolution of human malignant neoplasias through an increase in proteolysis mediated by these proteins [3, 5, 6]. The MMP family is composed of 20 zinc- and calcium-dependent proteolytic enzymes, subdivided into collagenases, gelatinases, elastases, stromelysins and MMPs according to the specificity of the substrate and the homology of domains [5, 7]. MMPs may be produced by malignant epithelium and by adjacent stroma, suggesting an important role of cell-to-cell interaction [8]. ECM degradation by MMPs would facilitate tumor invasion and progression of the cancer [5]. MMP-2 is a gelatinase and its potential to degrade type IV collagen in the ECM has been shown to be of great importance in facilitating stromal and vascular invasion by tumor cells [9]. *In vitro* studies have identified the cell-mediated MMP-2 activation mechanism [10].

Studies on several tissues such as those from the colon, pancreas, prostate, bladder and breast [11-15] have shown an increase in MMP-2 expression in human tumor cells and its relationship with an increase in metastases resulting from its ability to remodel ECM and degrade the basement membrane. Some studies on the uterine cervix [16, 17] have described MMP-2 expression in CIN lesions and in invasive carcinoma. Nevertheless, the significance of MMP-2 expression in cervical neoplastic lesions and its relationship with the processes of invasion and metastasis still remain to be established.

Therefore, the aim of this study was to evaluate variations in MMP-2 expression in tumor cells and in the cells of adjacent stroma, comparing cases of CIN3 with cases of invasive squamous cell carcinoma of the cervix. It was hoped that findings would contribute towards improving the current understanding of the role of MMP-2 protein in the invasive process of squamous cell carcinoma of the cervix. From a clinical viewpoint, it is also interesting to consider whether MMP-2 expression plays a role as a possible prognostic marker of invasion and metastasis.

Revised manuscript accepted for publication September 24, 2007

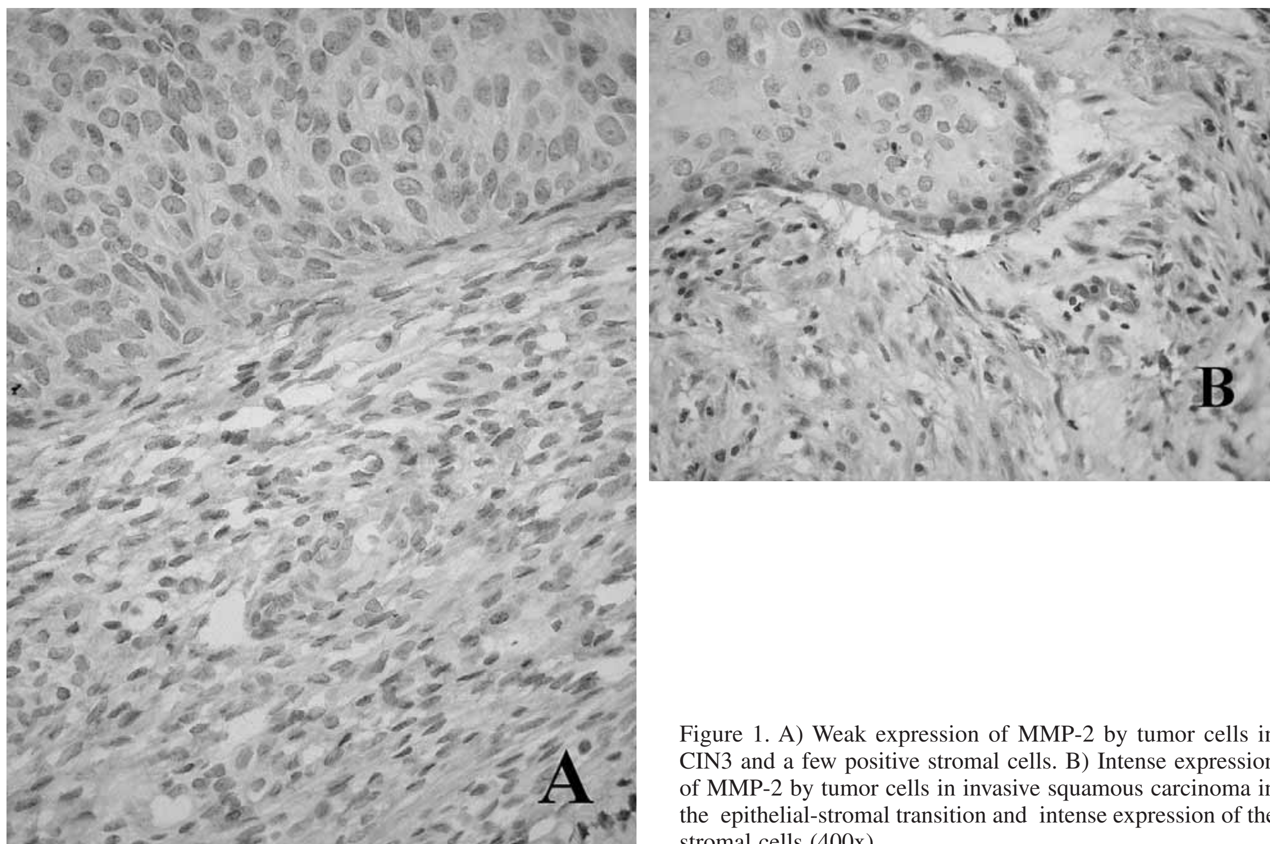

Figure 1. A) Weak expression of MMP-2 by tumor cells in CIN3 and a few positive stromal cells. B) Intense expression of MMP-2 by tumor cells in invasive squamous carcinoma in the epithelial-stromal transition and intense expression of the stromal cells (400x).

Materials and Methods

Samples

Following a review of clinical records, two groups were selected consisting of 45 women with a diagnosis of CIN3 and 45 with a diagnosis of invasive squamous carcinoma, all cases diagnosed by biopsy. Selection of cases for this study was carried out sequentially from January 2004 until the present among women receiving care at the Oncology Department of the Unicamp. The biopsy samples were reviewed for confirmation of diagnosis and those women with no other associated histological diagnosis and whose paraffin blocks contained sufficient material for immunohistochemical assays were included in this study.

Immunohistochemical analysis

Ninety blocks of paraffin-embedded material were cut into 5-μm thick sections using a microtome. Sections were deparaffinized in xylol and gradually rehydrated in an ethanol series. Following gradual hydration, nonspecific sites were blocked with a 10% hydrogen peroxide solution, after which antigen recovery was performed by immersion in a pH 8.9 buffer for 30 min at a temperature of 95°C. Next, the slides were incubated with primary monoclonal antibodies (matrix metalloproteinase 2, clone 17B11, code NCL-MMP2-507, Novocastra Laboratories, Newcastle, UK) in a chamber at 37°C for 30 min, and then incubated overnight. On the following day, the material was washed with PBS, and the LSAB peroxidase kit (LSAB/HRP, code K0690-1, Dakocytomation, Carpinteria, CA, USA) was used to detect the antigen-antibody reaction. After washing, the immunocomplex containing peroxidase was detected using 3,3-diaminobenzidine-HCL chromogen followed by hematoxylin counterstaining. For each reaction, a positive control was also evaluated, as recommended by the manufacturer. Negative controls were always evaluated and consisted of the same case as the positive control with the exclusion of the primary antibody in the reaction.

For each case, “hot-spot” reaction areas were selected and five photographs were taken that included the areas of epithelial-stromal transition. Photographs were taken at high magnification (400x) using a digital camera (Nikon, Coolpix, model 995). Images were transferred to a computer and analyzed using an imaging analyzer software package (Imagelab, 2000).

The final score was calculated by adding together the points received for intensity and percentage of staining. For evaluation of the percentage of positive cells, more than 1,000 tumor cells and more than 1,000 stromal cells were counted for each case. Stromal fibroblasts and inflammatory cells were considered stromal cells. Cases were grouped according to positivity and the score was classified as follows: (1) 0-10% stained cells, (2) 11-50% stained cells and (3) 51-100% stained cells. Semiquantitative evaluation of the slide took the intensity of staining and the percentage of positive cells into consideration. The intensity of staining in the tumor and stromal cells was evaluated as follows: (0) no staining, (1) weak staining, (2) moderate staining; and (3) intense staining.

Statistics

The software program used for the statistical analysis of MMP-2 was EPI-INFO. The association between the categorical variables was analyzed using Fisher’s exact test and the magnitude of the association was calculated using the odds ratios (OR) and their respective 95% confidence intervals (CI).

Results

Immunohistochemical reaction for MMP-2 was observed in the cytoplasm of tumor and stromal cells in the two study groups (Figures 1A-1B). Staining was also observed in some cases in the endothelial and glandular cells, although these were excluded from the analyses.

MMP-2 was found to be intensely positive in the stroma (score > 5) in 2% (1/45) of cases of CIN3 and in 40% (18/45) of cases of invasive carcinoma. With respect to the tumor cells, intense MMP-2 staining was found in 18% of cases (8/45) and in 24% (11/45) of cases, respectively, of CIN3 and invasive carcinoma. Expression of MMP-2 was greater in the stromal cells of cases of invasive carcinoma compared to cases of CIN3, and this difference was statistically significant ($p < 0.0001$). However, no statistically significant difference was found in MMP-2 expression in tumor cells between cases of CIN3 and invasive carcinoma ($p = 0.9423$) (Table 1).

Table 1. — *Comparison of MMP-2 protein expression (score) between the cases of CIN3 and invasive squamous carcinoma for stromal and tumor cells.*

Score	Stromal cells			Tumor cells		
	CIN 3	Invasive	CI 95%	CIN 3	Invasive	CI 95%
1-2	10	6	Reference	8	14	Reference
3-4	34	21	1.03 (0.33-3.25)	29	20	0.39 (0.14-1.11)
5-6	1	18	30.00 (3.15-285.71)	8	11	0.79 (0.22-2.77)
p*	< 0.0001			0.1524		

* Fisher's exact test (*score* 1-2; *score* 3-4; *score* 5-6).

With respect to CIN3, MMP-2 expression was significantly greater in epithelial tumor cells compared to stromal cells ($p = 0.05$), whereas in the cases of invasive carcinoma no statistically significant difference was found in MMP-2 expression between epithelial tumor cells and stromal cells ($p = 0.08$) (Table 2).

Table 2. — *Comparison of MMP-2 protein expression (score) between stromal and tumor cells in CIN3 and invasive squamous carcinoma.*

Score	Stromal cells			Tumor cells		
	CIN 3	Invasive	CI 95%	CIN 3	Invasive	CI 95%
1-2	10	8	Reference	6	14	Reference
3-4	34	29	1.07 (0.37-3.06)	21	20	0.41 (0.13-1.27)
5-6	1	8	10.00 (1.03-97.5)	18	11	0.26 (0.08-0.88)
p*	0.05			0.09		

* Fisher's exact test (*score* 1-2; *score* 3-4; *score* 5-6).

In accordance with the FIGO classification criteria for staging, 18 tumors were classified as Stages I-II, 26 as Stages III-IV and one case could not be classified due to the early death of the patient. A greater frequency of MMP-2 positivity with a score > 3 was found in the stromal cells in Stages III-IV compared to Stages I-II, and this difference was statistically significant ($p = 0.04$). No association was found between MMP-2 expression in tumor cells and staging (Table 3).

Table 3. — *MMP-2 expression (score) in stromal and epithelial tumor cells according to the clinical stage of invasive squamous carcinoma.*

Score	Stromal cells			Tumor cells		
	Stage		CI 95%	Stage		CI 95%
	I-II	III-IV		I-II	III-IV	
1-3	12	10	Reference	12	12	Reference
4-6	6	18	3.60 (1.03-12.54)	6	14	2.33 (0.67-8.12)
p*	0.0403			0.1791		

* Fisher's exact test (*score* 1-2; *score* 3-4; *score* 5-6).

Discussion

Up to the present time, we have been able to evaluate MMP-2 expression in 45 cases of CIN3 and in 45 cases of invasive squamous cell carcinoma of the cervix, using immunohistochemistry to compare the localization of protein expression in both the neoplastic epithelial cells and in the adjacent stromal cells. Our results suggest that MMP-2 expression in stromal cells is greater in cases of invasive carcinoma compared to cases of CIN3, and that it is also greater in more advanced stages of invasive carcinoma. Intense MMP-2 expression was more frequent in epithelial tumor cells than in stromal cells in cases of CIN3. In cases of invasive carcinoma, expression of MMP-2 was similar in both stromal and tumor cells.

Our results are in agreement with those of Davidson *et al*. [18], who studied a smaller number of cases and reported higher MMP-2 positivity in stromal cells adjacent to invasive carcinoma compared to cases of CIN. In the same study, no differences were found in MMP-2 expression in tumor cells between cases of CIN and invasive carcinoma. In addition, the authors quantified mRNA for MMP-2 in stromal and tumor cells, observing a greater amount of mRNA in tumor cells compared to stromal cells. These investigators hypothesized that tumor cells would be responsible for synthesizing MMP-2, while stroma cells would activate it, which would justify the intense stromal staining for MMP-2.

The importance of stromal cells in defining the role of MMP-2 has also been described in cases of non-small cell lung cancer and rectal cancer [19, 20]. Other studies have suggested that tumor cells may be responsible for inducing the production of proteolytic enzymes in neighboring stromal cells [16, 21, 22]. A study carried out by Sun *et al*. suggests that the presence of ECM metalloproteinase inducers (EMMPRIN) in tumor cells promotes latent MMP-2 production by fibroblasts [21]. The findings published by Sier *et al*. complement these observations, reporting that after being produced by the fibroblasts, MMP-2 would be activated on the surface of tumor cells in the tumor-stromal interface [22]. Irrespective of the localization of MMP-2 expression and activation, the above-mentioned investigators agree that the increase in MMP-2 expression is associated with a poorer prognosis in patients due to greater aggressivity of the tumor [19-22].

The presence of MMP-2 does not necessarily imply that there will be proteolytic activity, since the enzyme may be in its latent or active form, which cannot be dif-

ferentiated by immunohistochemistry. It is known that MT1-MMP (MMP-14) and TIMP-2 are necessary to activate MMP-2 [6]. Furthermore, in its active form, it may be inhibited by the presence of TIMP and RECK (reversion-inducing cysteine-rich protein with Kazal motifs) [23]. Thus, there are many possible control mechanisms in tissues, triggered by these proteins that try to reduce the process of degradation of the ECM that would lead to tumor invasion.

In an attempt to understand how the organism loses its ability to self-regulate for this protein, Smola-Hess *et al*. [23] recently demonstrated that the E7 protein from HPV type 16 was associated with an increase in MT1-MMP (MMP-14) expression in keratinocytes and consequently with an increase in MMP-2 activation. On the other hand, the E7 protein derived from HPV 1 was not able to induce MT-1 MMP expression. This result may explain the participation of high-risk HPV types in the imbalance of MMP-2 protein found in tumor invasion.

Conclusion

This study corroborates that stromal cells play an important role in tumor invasion and progression, mediated by the progressive enhancement of MMP-2 expression from CIN 3 to early invasive tumor, and from this stage to advanced invasive carcinoma. Intense MMP-2 expression most probably is associated with poor tumor prognosis.

References

[1] International Agency for Research on Cancer. Cancer Epidemiolgy Databases. Globocan 2002 http://www-depdb.iarc.fr/globocan/GLOBOframe.htm, 2007.

[2] Burd E.M.: "Human papillomavirus and cervical cancer". *Clin. Microbiol. Rev*., 2003, *16,* 1.

[3]Itoh Y., Nagase H.: "Matrix metalloproteinases in cancer". *Essays Biochem.,* 2002, *38*, 21.

[4] Zhou C.Y., Yao J.F., Chen X.D.: "Expression of matrix metalloproteinase-2, 9 and their inhibitor-TIMP 1,2 in human squamous cell carcinoma of uterine cervix". *Ai Zheng*, 2002, *2,* 735.

[5]Benaud C., Dickson R.B., Thompson E.W.: "Roles of the matrix metalloproteinases in mammary gland development and cancer". *Breast Cancer Res. Treat*., 1998, *50,* 97.

[6] Baker A.H., Edwards D.R., Murphy G.: "Metalloproteinase inhibitors: biological actions and therapeutic opportunities". *J. Cell. Sci,* 2002, *115,* 3719.

[7] Brummer O., Bohmer G., Hollwitz B., Flemming P., Petry K.U., Kuhnle H.: "MMP-1 and MMP-2 in the cervix uteri in different steps of malignant transformation-an immunohistochemical study". *Gynecol. Oncol.,* 2002, *84,* 222.

[8] Singer C.F., Kronsteiner N., Marton E., Kubista M., Cullen K.J., Hirtenlehner K. *et al*.: "MMP-2 and MMP-9 expression in breast cancer-derived human fibroblasts is differentially regulated by stromal-epithelial interactions". *Breast Cancer Res. Treat*., 2002, *72*, 69.

[9] Aznavoorian S., Murphy A.N., Stetler-Stevenson W.G., Liotta L.A.: "Molecular aspects of tumor cell invasion and metastasis". *Cancer*, 1993, *71,* 1368.

[10] Imai K., Ohuchi E., Aoki T., Nomura H., Fujii Y., Sato H. *et al*.: "Membrane-type matrix metalloproteinase 1 is a gelatinolytic enzyme and is secreted in a complex with tissue inhibitor of metalloproteinase 2". *Cancer Res.,* 1996, *56*, 2707.

[11] van der Jagt M.F., Sweep F.C., Waas E.T., Hendriks T., Ruers T.J., Merry A.H.: "Correlation of reversion-inducing cysteine-rich protein with kazal motifs (RECK) and extracellular matrix metalloproteinase inducer (EMMPRIN), with MMP-2, MMP-9, and survival in colorectal cancer". *Cancer Lett*., 2006, *237,* 289.

[12] Masui T., Doi R., Koshiba T., Fujimoto K., Tsuji S., Nakajima S., *et al*.: "RECK expression in pancreatic cancer: its correlation with lower invasiveness and better prognosis". *Clin. Cancer Res*., 2003, *9,* 1779.

[13] Boag A.H., Young I.D.: "Increased expression of the 72-kd type IV collagenase in prostatic adenocarcinoma. Demonstration by immunohistochemistry and in situ hybridization". *Am. J. Pathol*., 1994, *144*, 585.

[14] Davies B., Waxman J., Wasan H., Abel P., Williams G., Krausz T., *et al*.: "Levels of matrix metalloproteases in bladder cancer correlate with tumor grade and invasion". *Cancer Res*., 1993, *53*, 5365.

[15] Pellikainen J.M., Ropponen K.M., Kataja V.V., Kellokoski J.K., Eskelinen M.J., Kosma V.M.: "Expression of matrix metalloproteinase (MMP)-2 and MMP-9 in breast cancer with a special reference to activator protein-2, HER-2, and prognosis". *Clin. Cancer Res.,* 2004, *10,* 7621.

[16] Gaiotto M.A., Focchi J., Ribalta J., Stávale J.N., Baracat E.C., Lima G.R. *et al*.: "Comparative study of MMP-2 immune expression in normal uterine cervix, intraepithelial neoplasias, and squamous cells cervical carcinoma". *Am. J. Obstet. Gynecol*., 2004, *190*, 1278.

[17] Branca M., Ciotti M., Giorgi C., Santini D., Di Bonito L., Costa S. *et al*.: "Matrix metalloproteinase-2 (MMP-2) and its tissue inhibitor (TIMP-2) are prognostic factors in cervical cancer, related to invasive disease but to high-risk human papillomavirus (HPV) or virus persistence after treatment of CIN". *Anticancer Res.,* 2006, *26,* 1543.

[18] Davidson B., Goldberg I., Kopolovic J., Lener-Geva L., Gotlieb W.H., Ben-Baruch G. *et al*.: "MMP-2 and TIMP-2 expression correlates with poor prognosis in cervical carcinoma - a clinicopathologic study using immunohistochemistry and mRNA in situ hybridization". *Gynecol. Oncol.,* 1999, *73,* 372.

[19] Ishikawa S., Takenaka K., Yanagihara K., Miyahara R., Kawano Y., Otake Y. *et al*.: "Matrix metalloproteinase-2 status in stromal fibroblasts, not in tumor cells, is a significant prognostic factor in non-small-cell-lung cancer". *Clin. Cancer Res.,* 2004, *10,* 6579.

[20] Masaki T., Matsuoka H., Sugiyama M., Abe N., Izumisato Y., Goto A. *et al*.: "Laminin-5 gamma 2 chain and matrix metalloproteinase-2 may trigger colorectal carcinoma invasiveness through formation of budding tumor cells". *Anticancer Res.,* 2003, 23, 4113.

[21] Sun J., Hemler M.E.: "Regulation of MMP-1 and MMP-2 production through CD147/extracellular matrix metalloproteinase inducer interactions". *Cancer Res*., 2001, *61,* 2276.

[22] Sier C.F., Zuidwijk K., Zijlmans H.J., Hanemaaijer R., Mulder-Stapel A.A., Prins F.A. *et al*.: "EMMPRIN-induced MMP-2 activation cascade in human cervical squamous cell carcinoma". *Int. J. Cancer,* 2006, *118*, 2991.

[23] Smola-Hess S., Pahne J., Mauch C., Zigrino P., Smola H., Pfister H.: "Expression of membrane type 1 matrix metalloproteinase in papillomavirus-positive cell: role of the human papillomavirus (HPV) 16 and HPV8 E7 gene products". *J. Gen. Virol.,* 2005, 86, 1291.

Address reprint requests to:
L.C. ZEFERINO, M.D.
Hospital de Clínicas
Rua Alexander Fleming, 101
Cidade Universitária Zeferino Vaz
CEP 13083-970
Campinas, SP (Brazil)
e-mail: zeferino@hc.unicamp.br