METALLOPROTEINASE 2 AND 9 ACTIVITY IN THE DEVELOPMENT OF PANCREATIC CANCER*

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Pancreatic adenocarcinoma is the fourth most common cancer occurring in both women and men. In Poland, within the past ten years the number of deaths from pancreatic cancer increased by 29%. The aim of the study was to determine the correlation between the activity of metalloproteinase (MMP) 2 and 9 and progression and aggressiveness of pancreatic cancer.

Material and methods. Tissue samples were collected from 36 patients with diagnosed pancreatic adenocarcinoma who underwent Whipple resection. Tumor tissues were analyzed by gel zymography, zymography in situ and immunohistochemistry.

Results. The activity of MMPs was found mainly in cancer cells. Active form of MMP2 (62 kDa) was present in 88% of cases and MMP9 (83 kDa) in 38% of cases. By contrast, immunohistochemical staining revealed the presence of metalloproteinase 9 in all studied tissues. MMP activity was assessed against histological grade of the tumor. In the case of group G1 there was no activity of matrix metalloproteinase 9. By comparing the activity we concluded that the activity of MMPs in tumors with the highest degree of differentiation is significantly lower than in G2 and G3. Metalloproteinase 9 expression analysis revealed no significant differences between the groups of various degrees of histological maturity. The level of expression did not differ between the groups N0 and N1.

Conclusion. Lack of metalloproteinase 9 activity in group G1 may indicate that MMP9 is activated only in higher tumor grades. We have shown that an active form of MMP2 is found in all histological grades, which supports its involvement in the development of pancreatic cancer. Metalloproteinases are attractive target of anticancer therapy but not only the level of expression of metalloproteinases should be taken into account but also their level of activity and factors associated with their activation.

Key words: pancreatic cancer, metalloproteinases, MMP, pancreatic tumor

Pancreatic adenocarcinoma is the fourth most common cancer occurring in both women and men. In Poland, within the past ten years the number of deaths from pancreatic cancer increased by 29% (1). Average 5-year survival is only 4% and has not changed over the past 20 years despite progress in diagnostic methods. Previous studies have clarified many processes related to this tumor, however reliable markers that would enable detection of

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pancreatic cancer at an early stage of development are still lacking (2).

Poor survival in the pancreatic cancer is related to an aggressive phenotype that is characterized by early invasiveness and formation of metastases. Microenvironment in which tumor cells live, plays a particularly important role in the development of the pancreatic cancer. Fibroblasts, cells related to anti-inflammatory response and endothelial cells that are part of this microenvironment, interact with each other. They form specific conditions for the tumor development and for the formation of metastases by secreting multiple growth factors and proteolytic enzymes (3).

Metalloproteinases (MMP) are a group of 20 proteases and include 4 classes: collagenases, gelatinases, stromelysins and Membrane-type MMP. They participate in the remodeling of extracellular matrix and thus are a significant player in such processes as angiogenesis, cellular migration and formation of metastases. Metalloproteinases also participate in the activation of multiple proteins and thus take part in regulation of cellular proliferation and apoptosis (4).

Metalloproteinases 2 and 9 belong to the group of gelatinases and are particularly important, because collagen IV that is a component of the basal membrane, is their substrate. Its degradation enables and facilitates migration of the tumor cells. Their increased expression was found in multiple tumors. In breast cancer, increased MMP9 expression was demonstrated in advanced tumors with distant metastases. Increased MMP2 expression was also found, but no clear correlation with prognostic factors was found (5).

Metalloproteinases 2 and 9 were also fund to play an import an role in an invasiveness of the esophageal tumor. MMP2 was found in 78% of cases, while MMP9 in 70% of cases (6). MMP2 activity was found to correlate with metastases to lymph nodes and lymphatic and vascular invasion, while MMP9 correlated only with vascular invasion.

Increases metalloproteinase expression was also demonstrated in the pancreatic cancer. In a study conducted with 6 cell lines, increased MMP2 expression and activity were found to be related to higher invasiveness potential of the pancreatic cancer cells (7). Furthermore, increased MMP2 and MMP9 expression, both at the mRNA and protein level, was also confirmed in a study conducted with pancreatic tumors (8).

Undoubtedly active MMP2 and MMP9 participate in the remodeling of the microenvironment in the pancreatic cancer.

The aim of this study was to determine the correlation between the activity of these enzymes and progression and aggressiveness of pancreatic cancer, which could be of importance for the individualization of anticancer therapy.

MATERIAL AND METHODS

Patients and tissue collection

Tissue samples were collected from 36 patients with diagnosed nonendocrine pancreatic adenocarcinoma who underwent Whipple resection. The tissue collection protocol was approved by the Bioethics Committee of Medical University of Warsaw. Histopathological assessment of the tumors was done at the Department of Pathology Central Clinical Hospital of Ministry of Internal Affairs and Administration (tab. 1).

Tissue collected from the primary tumor of the pancreatic cancer were examined. To isolate the proteins, tumor fragments were stored at −20°C until use. 5 x 5 x 5 mm specimens were snap frozen for 45 seconds in acetone on dry ice to –70ºC and stored at –80ºC. Specimens intended for further examinations were cut to 5 µm sections using a cryostat (Leica CM1850 UV, Nussloch, Germany).

Gel zymogrpahy

Proteins were isolated from the tumor tissues using Protein Extraction Kit (Milipore, Billerica, USA), according to manufacturer’s instructions. Concentration of obtained isolates was assessed using the Bradford assay, with Nano Drop spectrophotometer. 10µg of total mixture of isolated proteins was diluted in Zymogram Sample Buffer (Bio-Rad Laboratories, Hercules, USA), in 2:1 ratio and separated on 7.5% polyacrylamide gel containing 0.1% gelatin (Sigma-Aldrich, St. Louis, USA). Then the gel was washed for 30 minutes, twice, using 2.5% Triton X-100 solution to remove SDS. Another stage involved incubation in an
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activating buffer (50 mm pH 7.5 TRIS, 5 mM CaCl2, 0.2 M NaCl) for 20 hours at 37°C. Gelatinase activity was demonstrated by gel staining with 0.5% Commasie blue for 1 hour and destaining with 40% methanol solution and 10% acetic acid. After destaining, the gel was washed with water. To assess activity, the stained gel was photographed and analyzed using MicroImage (Olympus, Japan).

Zymography in situ

Analysis was done on tissue sections of 5 μm thickness. After drying at room temperature, the tissue sections were washed with PBS solution (3 x 5 min).

Immunohistochemical staining

Analysis was done on tissue sections of 5 μm thickness. A secondary antibody conjugated with biotin and streptavidin – alkaline phosphatase (LSAB 2 Kit AP, DAKO A/S, Denmark) was used for the staining using goat antibodies. After drying at room temperature, the tissue sections were fixed in acetone (10 minutes). Then the tissues were incubated with Dual Endogenous Enzyme Block (Dako, Glostrup, Denmark) for 10 minutes and with swine serum for 20 minutes. Incubation with an antibody (MMP9 (C-20) – SantaCruz Biotechnology) lasted for 30 minutes. Then the procedure was conducted according to manufacturer’s protocol. Result of staining was expressed as positive (+) or negative (-).

Statistics

Results of metalloproteinase activity measurements were subjected to statistical analysis using Kruskal-Wallis test (for three groups), using Statistica 6.0 software. Statistical significance level was p = 0.05.

RESULTS

Zymography in situ

Location of metalloproteinase activity area was determined using zymography in situ

In 30 cases metalloproteinase activity area was identified as a clear field formed by digestion of fluorescein-labeled gelatin. Gelatinolytic activity was found in all cases. Fig. 1 dem-

![Fig. 1. Zymography in situ in the pancreatic tumor](image-url)
onstrates typical image after the analysis. Metalloproteinase activity was fund mainly in the tumor cells. Contrary to immunohistochemical staining, not all MMP9-positive cells exhibited lytic activity.

Gel zymography

Gelatinolytic activity was assessed in the tissue isolates from 30 pancreatic tumors. Active form of MMP2 (62 kDa) was present in 88% of cases, while MMP9 (83 kDa) in 38% of cases. In 6 cases we were unable to determine whether there was metalloproteinase activity due to poor differentiation of a digested area on the gel.

Analysis of results according to the tumor differentiation indicated that metalloproteinase 9 activity was not found in G1 group. MMP9 activity was found in group G2 in 7 (of 9) cases, ain in group G3 only in 4 (of 11) cases. Active form of MMP3 was found in 3 (of 4) cases in group G1, in all cases in group G2 and in 10 (of 11) cases in group G3.

When we compared densitometric values of metalloproteinase 2 activity, it was significantly lower in the group of tumors with the highest differentiation degree than in groups G2 and G3 (p = 0.0403). MMP2 activity was: G1: 3.27±3.6; G2: 16.57±13.9; G3: 13.6±12.2. MMP9 activity was not found in G1 group, while for the other groups these values were – G2:18±13.9; G3: 38.2±22.3.

The staining demonstrated metalloproteinase 9 in any of the tested specimens (20). Its presence was found mainly in the tumor cells as well as in the ducts with intraepithelial neoplasia. MMP9 expression was also found in sporadic stromal cells.

DISCUSSION

We studied metalloproteinase 2 and 9 activity in our study. Analysis of isolates from 30 pancreatic tumors demonstrated that the metalloproteinase activity is smallest in the group of tumors with the highest differentiation grade. No metalloproteinase 9 activity was found in G1 group. When MMP9 expression was studied using immunochemistry, we found that it was present in the tumor cells, while positive reaction was found only in sporadic stromal cells and no significant differences were found between the study groups. This indicates that the expression alone is an imperfect marker of the role of metalloproteinases.

Metalloproteinase 2 and 9 overexpression has been previously shown both at the mRNA
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and protein level. Pryczynicz at al. also did not find any significant differences in MMP9 expression between various histological differentiation grades (8). However, they did demonstrate that MMP9 overexpression was associated with involvement of lymph nodes and distant metastases. We did not find any association between MMP9 expression or activity and metastases in lymph nodes in our study; this may be related to a small group size. Increased MMP9 expression has also been associated with the angiogenesis process in the pancreatic tumors and presence of macrophagal infiltrates (9). Metalloproteinase 9 activity was demonstrated in the pancreatic cancer (10). Lack of activity of this metalloproteinase in G1 group can indicate that MMP9 undergoes activation only in highly advanced tumors.

As we have shown, active MMP2 is present in all histological grades which indicates its involvement with the development of the pancreatic cancer. Previous studies did not demonstrate increased metalloproteinase 2 expression in the tissue specimens of the pancreatic cancer and no correlation between its expression and any of the histological criteria were demonstrated (8). However studies conducted in cell lines of the pancreatic cancer (IMIM-PC1; IMIM-PC2; PANC-1) demonstrated good correlation between MMP2 activity and tumor invasive potential. These cells demonstrated better mobility in Matrigel than cells that did not exhibit increased MMP2 expression or activity (7). MMP2 activity was also studied in the pancreatic juice and that study demonstrated that such measurement may be useful in the diagnosis of the pancreatic cancer (11). Similarly our studies indicate utility of metalloproteinase activity measurements for the determination of the tumor grade.

CONCLUSIONS

Metalloproteinases 2 and 9 are important part of the microenvironment of the pancreatic cancer and contribute to the development of this tumor. Previously tested metalloproteinase inhibitors proved too toxic or inadequately effective (12), and recently requirement for individualized therapy was also emphasized (13, 14). This was based on heterogeneity of the pancreatic tumors. Our results support this theory. Individual approach may prove more effective in the treatment of the pancreatic cancer. However, not only expression level of metalloproteinases, but also level of their activity and factors related to their activation need to be considered.

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