ONCOLOGY

Key Enzymes of the Extracellular Matrix in Colorectal Cancer

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The role of extracellular matrix enzymes in the development of distant metastases of colorectal cancer was studied. The expression of types 2 and 9 matrix metalloproteinases was evaluated in primary colorectal adenocarcinomas (264 patients) and their metastases in the lymph nodes (127 patients) and liver (109 patients). Increased expression of matrix metalloproteinase-9 was associated with the probable progress of tumor process and with a low level of histological differentiation of the tumor (p=0.048), deeper invasion of the tumor in the intestinal wall (p=0.012), unfavorable outcome (p=0.037), high risk of metastases in the liver, and worse overall and uneventful survival of patients with colorectal cancer (p=0.001 and p=0.01, respectively). High expression of matrix metalloproteinase-2 was less significant for the invasive potential and prognosis of colorectal tumors. It is suggested that matrix metalloproteinase-9 is an important marker for analysis of the postoperative prognosis and risk of metastases in the liver in patients with colorectal cancer.

Key Words: *colorectal cancer; matrix metalloproteinases; metastases; extracellular matrix*

Proteolytic destruction of extracellular matrix proteins is one of the main mechanisms of human malignant tumor metastasizing. Among the factors involved in this process are matrix metalloproteinases (MMP), for example, representatives of the gelatinase group MMP-2 and MMP-9 [3,6,7]. These proteinases are involved in the specific hydrolysis of the basal membrane nonfibrillar collagen. Due to their ability to destroy basal membranes, MMP-2 and MMP-9 facilitate the invasion of tumor cells [1,4,6]. In addition, recent studies revealed that an important role of MMP in tumor metastasizing is due to their capacity to stimulate growth factors and angiogenesis [2,10].

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High expression and activity of MMP-2 and MMP-9, observed in human tumors of different location, as a rule correlate with a poor prognosis [5,7,9]. The enzyme levels in the tumor are intensely studied for predicting the disease course in colorectal cancer (CRC) patients [3,6,8,11]. However the impact of these factors for the clinical course, progress, and metastasizing of colorectal tumors remains little studied.

We analyzed the expression of MMP-2 and MMP-9 in the tumors of CRC patients with consideration for clinical picture and morphology, metastatic status, and prognosis.

MATERIALS AND METHODS

The postoperative material from 264 CRC patients was analyzed. By the moment of hospitalization 66

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(25%) patients had stages I-II and 198 (75%) stages III-IV tumor process.

The total group of CRC patients included 127 men (48.1%) and 137 women (51.9%) aged 34-79 (mean age 60.50±0.63 years; median 62 years); patients aged over 60 predominated (169/64%).

Local metastases were detected in 178 (67.4%) patients, distant metastases in 135 (51.1%), tumor invasion in adjacent organs and tissues (urinary bladder, uterus, small intestine) in 18 (6.8%) cases. Distant metastases in the liver were detected in 135 patients. Local relapses were detected in 34 patients. Metastases were removed surgically in 110 cases (metastatic group). No distant metastases and local relapses were detected during the 3-year period of observation in 127 patients (control group).

Histological study of the material revealed adenocarcinomas of different differentiation degrees in all patients.

Immunohistochemical staining using antibodies to MMP-2 and MMP-9 (Novocastra) was carried out on paraffin sections of tissues from the colorectal tumor (n=264), local (n=129) and distant metastases (n=110). For antigen decamouflage, paraffin sections were pretreated in the microwave mode at 650 Wt (2×5 min) using restoring solution (pH 6.0; Dako). The sections were incubated with the first antibodies during 18 h at 4°C. Standard LSAB$^+$ Kit (Dako) served as the second antibodies and the peroxidase complex. The reaction was visualized by diaminobenzidine solution (DAB$^+$; Dako). Cell nuclei were post-stained by Meyer's hematoxylin.

The reaction was evaluated by the semiquantitative method with consideration for staining intensity and count of antigen-positive cells (the sum of these factors determined the level of protein marker expression). Immunohistochemical reaction was evaluated as negative ("-"; no reaction), slightly positive ("+"; less than 10% stained cells), moderately positive ("++"; more than 10% cells with medium intense staining); and positive ("+++"; more than 10% cells with highly intense staining). Low (-/+) and high (++/+++) levels of the marker expression were distinguished for further comparison of immunohistochemical reaction and mathematical analysis. Specific reaction of the stromal cells was evaluated as positive or negative.

The data were statistically processed and the level of significance evaluated using χ^2 test (the differences were considered significant at $p<0.05$). Analysis of prognostic significance of the marker content and the survival curves were plotted by Kaplan-Meyer's method. A total of 264 CRC patients were followed up after surgical treatment. Overall survival was analyzed in 244, uneventful survival in 105 patients. The period of observation from the day of the operation till the last examination or death was 5-126 months. The significance of differences in survival was evaluated by the log-rank test; the risk of metastases in the liver was estimated.

RESULTS

The expression of MMP-2 and MMP-9 in the tumors of CRC patients was high during different stages of the disease. The enzyme accumulated in cancer cell cytoplasm and stromal components adjacent to tumor complexes (fibroblasts, macrophages, vascular walls, leukocytic elements). The expression of proteases in normal colorectal tissues was slightly positive.

High expression of MMP-2 in the cytoplasm of primary tumor cells was detected in 102 (38.6%) cases analyzed. The reaction was moderately positive (++) in 59 and positive (+++) in 43 cases. Slightly positive reaction (+) was noted in 73 (27.7%) cases, negative (-) in 89 (33.7%) cases. In addition, MMP-2 was detected in extracellular matrix components adjacent to the tumor complexes in 137 (51.9%) patients.

High accumulation of MMP-9 in primary tumor cell cytoplasm was detected in 133 (50.4%) cases. Moderately positive (++) reaction was noted in 45, positive (+++) reaction in 98 cases. Slightly positive cytoplasmatic expression of the enzyme was detected in 57 (21.6%), negative reaction in 74 (28.0%) cases. The presence of MMP-9 in the extracellular stroma was detected in 103 (39.0%) patients.

Highly intensive cytoplasmatic reaction was often observed in poorly and moderately differentiated adenocarcinomas, while reduced or focal expression of the markers was detected in the structures of well-differentiated tumors. The intensity of staining of extracellular matrix components depended on the type of stroma and intensity of desmoplastic reaction, but not on the degree of tumor differentiation.

Pronounced cytoplasmatic reaction (sometimes staining of extracellular matrix cells) was seen in the cells of the majority of local metastases. Accumulation of MMP-2 was observed in 45.7% studied metastases (in 58 of 127 cases) of CRC in the regional lymph nodes; MMP-9 was detected more often: in 73 (57.5%) cases.

Changes in the enzymes expression in distant metastases were similar to those in the primary tumor, but accumulation of proteases in extracellular

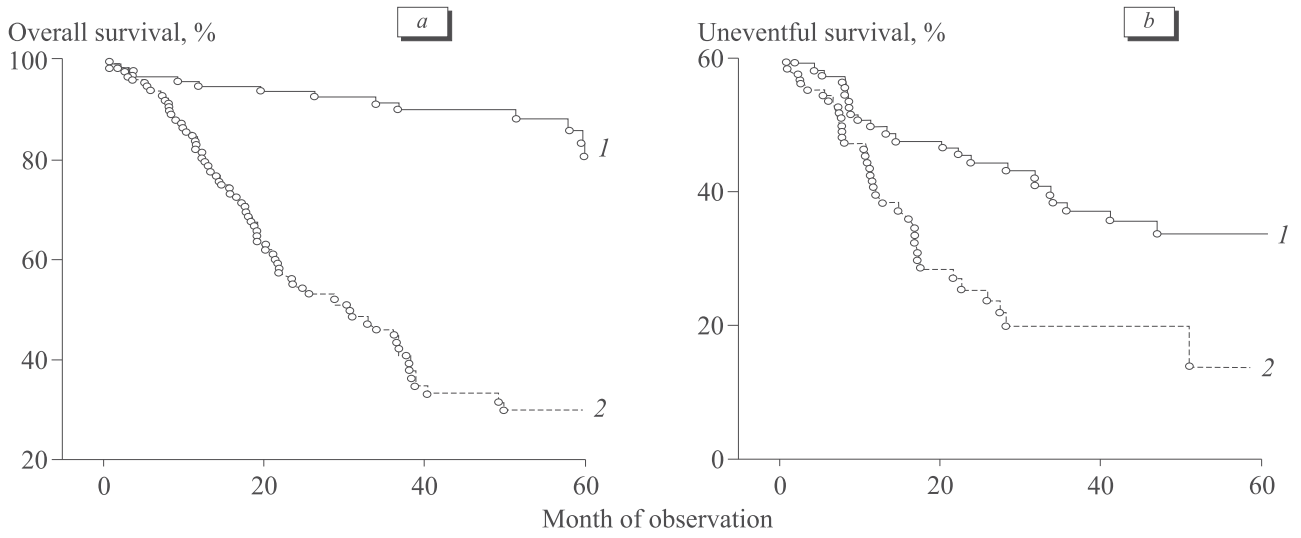

Fig. 1. Overall (*a*) and uneventful (*b*) survival of CRC patients depending on MMP-9 production in the tumor (groups of patients with high (*2*) and low (*1*) expression of MMP-9).

matrix components was less pronounced. High expression of MMP-2 in metastases in the liver was observed in 54 (49.1%) of 110 cases, of MMP-9 in 73 (66.4%). The enzymes accumulated in the metastatic cell cytoplasm irrespective of the tumor growth type, specific features of the invasive front-line, presence of lymphoid infiltration, desmoplastic reaction, and other morphological characteristics of secondary tumor foci.

The enzymes expression in the tumors of CRC patients depended on some clinical and morphological characteristics of the disease. The content of MMP in the tumors of patients with metastatic CRC was significantly higher. High expression of MMP-2 and MMP-9 was detected in 45.9% (62 of 135 cases) and 62.2% (84 cases) tumors in patients with distant metastases *vs.* 31% (40 of 129 cases) and 38% (49 cases) tumors in the group of patients without metastases in the liver (p=0.018 and p=0.0001, respectively). Increase of protease expression was associated with a low level of histological differentiation of the tumors and a greater depth of tumor invasion (disease stages according to Dukes'); significant differences were detected only for MMP-9 (p=0.048 and p=0.012, respectively). The level of enzymes expression was higher in cases with invasion of the vessels and with metastases in the lymph nodes, but the differences did not reach the level of significance.

Analysis of prognostic significance of enzyme levels in the tumor showed that high level of MMP-9 expression in tumor cell cytoplasm indicated an

TABLE 1. Relationship between Disease Outcome in CRC Patients after Surgical Removal of the Primary Tumor and Expression of MMP-2 and MMP-9

Expression			Favorable prognosis (n=155)	Unfavorable prognosis (n=89)	p
MMP-2					
	in cancer cells	high	54	44	0.286
		reduced	101	45	
	in tumor stroma	positive	58	40	0.988
		negative	97	49	
MMP-9					
	in cancer cells	high	55	71	0.037*
		reduced	100	18	
	in tumor stroma	positive	79	47	0.374
		negative	76	42	

Note. *p<0.05 *vs.* unfavorable prognosis.

unfavorable prognosis and was more often detected in CRC patients who died from the disease progress early after surgical treatment in comparison with patients with good prognosis (survival over 5 years; p=0.037; Table 1). Changes in the expression of MMP-2 associated with different disease outcomes were less pronounced.

High level of MMP-9 production is an important prognostic factor, increasing the risk of metastases in the liver 3.4 times during the first 3 years postoperation.

Overall and uneventful survival were significantly worse in patients with antigen-positive tumors in comparison with patients with antigen-negative tumors (p=0.0014 and p=0.01, respectively; Fig. 1).

Overall 5-year survival of patients without or with low level of MMP-9 expression in tumor cell cytoplasm was 73.7±4.5% (118 patients). This was significantly higher than in the MMP-9-positive groups, in which this value was 33.7±5.2% (126 patients). No significant differences in the relationship between uneventful survival and MMP-2 content in the tumor were detected.

The results confirm that high expression of MMP-2 and MMP-9 in cancer cells is characteristic of CRC with a high invasive and metastatic potential and is associated with the probable progress of tumor process. It was shown that high expression of MMP-9 was associated with a low level of histological differentiation of the tumor (p=0.048), deeper invasion of the tumor in the intestinal wall (p=0.012), and unfavorable outcome (p=0.037). High level of MMP-9 production is an unfavorable prognostic factor increasing 3.4 times the risk of CRC metastases in the liver during the first 3 years after the operation and deteriorating the overall and uneventful survival (p=0.001 and p=0.01, respectively). Analysis of these changes in the marker expression in the tumor is important for evaluation of the postoperative prognosis in CRC patients.

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