Original Article

Matrix Metalloproteinase-2 and -9, Lactate, and Malate Dehydrogenase and Lipid Peroxides in Sera of Patients with Colorectal Carcinoma

(colorectal carcinoma / matrix metalloproteinase-2 / malondialdehyde / matrix metalloproteinase-9 / lactate dehydrogenase / malate dehydrogenase)

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Abstract. Matrix metalloproteinases (MMPs) are involved in tumour invasion and metastasis of colorectal carcinoma. Oxidative stress represents one of the possible mechanisms that activate inactive MMPs. Oxidative stress increases lipid peroxidation, which causes impaired membrane permeability and leakage of lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) into circulation. Our aim was to assess the activity of MMP-2 and MMP-9 and its relation to the parameters of oxidative stress and membrane damage markers in patients with different TNM (tumour, lymph nodes, metastasis) stages of colorectal carcinoma. MMP-2 and -9 activities were evaluated by gelatin zymography. Oxidative stress was examined by quantifying serum malondialdehyde (MDA) concentration. LDH and MDH activities were determined spectrophotometrically. The activities of MMP-2 and -9 were significantly higher in the sera of colorectal carcinoma patients when compared to healthy subjects. There was a stage-dependent increase in relative MMP-2 activity compared to the overall serum gelatinolytic activity. The activity of MMP-9 was the highest in TNM III. The MDA concentration and the LDH and MDH activities were significantly higher in colorectal carcinoma patients than in controls, while LDH and MDH activities were stage dependent. There was significant correlation between serum MMP-2 and LDH activity in TNM II, III and IV patients. A stage-dependent increase of LDH and MDH activity was observed. We highlight here that MMP-9 could be a 100% sensitive marker of TNM stage III of colorectal carcinogenesis. In this study it was shown for the first time that gelatinolytic activity in colorectal carcinoma is associated with redox imbalance.

Introduction

Colorectal carcinoma (CC) is among the leading causes of cancer-associated death worldwide and despite the substantial advances in diagnosis and treatment, the mortality remains to be alarmingly high (Xie et al., 2016). Dashwood (1999) stressed that 90 % of patients can be cured by surgery if the cancer is detected at an early stage, which directly implies that early diagnosis is crucial for the proper management of CC. Early detection, diagnosis and defining treatment targets rely on the understanding of the molecular mechanism of CC development (Alessandro et al., 2005). Several human solid tumours have been reported to have increased serum and tissue levels of proteolytic enzymes, strongly suggesting that they may be important in tumour invasion and metastasis. With respect to the gastrointestinal tract, it was previously demonstrated that proteolytic enzymes may have a role not only in the process of gastric or colorectal cancer invasion, but also in the progression of gastrointestinal precancerous changes into cancer (Herszenyi et al., 2007).

Matrix metalloproteinases (MMPs) are a unique family of zinc-binding endoproteinases that can degrade the

components of ECM. MMP-2 (gelatinase A) and MMP-9 (gelatinase B), which can degrade denatured collagen and type IV, V, VII, IX, and X collagen, are now also thought to be involved in cell differentiation, apoptosis, angiogenesis, immune response, and cancer cell growth. Since MMP-2 and -9 can degrade collagen type IV, which is a major component in the basement membrane, it is considered that they are essential in invasive growth and metastasis (Cho et al., 2007). Elevated levels of serum or plasma MMP-2 and-9 have been found in colon cancer and they are associated with tumour stage and/or prognosis. MMPs are secreted in latent form (proMMPs) and are activated by partial proteolytic cleavage (Roy et al., 2009). Among many other factors, reactive oxygen metabolites produced during oxidative stress can activate MMPs by reacting with the cysteine switch in the propeptide (Nagase et al., 2006). Reactive oxygen species (ROS) are formed in excess during colorectal carcinogenesis, but the precise mechanism underlying oxidative stress in cancer cells and molecular pathogenesis of CC remains to be understood. It has been suggested that ROS and lipid peroxidation (LPx) products such as malondialdehyde (MDA) contribute to both onset and progression of different types of cancer including CC (Kim et al., 2012). Increased LPx in tumour tissue leads to the impairment of membrane permeability, which can cause leakage of different compounds that are normally located intracellularly. Leakage of cytosolic enzymes such as lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) correlates with cellular viability, and hence it is a useful indicator of membrane damage (Bi et al., 2006; Sarkar and Sil, 2010). Activities of LDH and MDH reflect the bioenergetic status and disturbances in metabolic pathways. They have a potential for the design of marker panels to assist in early diagnostics and therapeutic strategies in CC (Roblick et al., 2004).

The aim of our study was to evaluate MMP-2 and MMP-9 profiles in the sera of patients with TNM II, III and IV stages of CC and to determine the presence of oxidative stress by assaying the whole amount of LPx in the sera (malondialdehyde-MDA). The effect of LPx on the membrane permeability was examined by quantifying activities of LDH and MDH. All selected markers have a role in evaluation of possible connections between active circulating MMP-2 and -9 in the presence of oxidative stress among CC patients.

Material and Methods

Patients and sera

Four experimental groups were defined. The first group consisted of 39 patients (21 men and 18 women with median age of 63, ranging from 41 to 83) with primary colorectal adenocarcinoma in TNM II stage. The second group comprised 44 patients (25 men and 19 women with median age of 58, ranging from 37 to 85 years) with primary colorectal adenocarcinoma in TNM III stage. The third group included 41 patients (23 men and 18 women with median age 60, ranging from 42 to 86 years) with primary colorectal adenocarcinoma in TNM IV stage. Preoperative histopathological diagnosis was established for all patients by two independent pathologists. Pathological diagnosis, which included the depth of the tumour invasion, vascular invasion, lymphatic permeation and lymph node metastasis, was made according to the general recommendations for colorectal carcinoma outlined by the Japanese Research Society for Cancer of the Colon and Rectum. Tumour stage was determined according to the TNM Classification System of the International Union against Cancer.

Preoperatively, serum was collected from patients who underwent neither previous radiotherapy nor chemotherapy. Considering the location of primary tumour, in the TNM II, III and IV stages the locations were the following: 25 in the colon and 14 in the rectum, 23 in the colon and 21 in the rectum, and 25 in the colon and 19 in the rectum, respectively. The fourth experimental group served as a control and included 47 healthy individuals (26 men and 21 women with median age of 59, ranging from 39 to 85) who were matched by age and gender with the CC patient group. These individuals had no previous neoplastic disease, neither metabolic nor any other disease that would interfere with the parameters of interest. Serum was derived from peripheral venous blood, stored at –20 °C and incubated for 15 min at 37 °C before each biochemical analysis.

This study was carried out after fulfilling all ethical standards according to The Declaration of Helsinki. The study protocol was approved by the local ethics committee and informed consent was obtained from each patient.

Gelatin zymography

Activities of MMP-2 and -9 were determined by SDS-PAGE reverse electrophoretic zymography according to the method of La Rocca et al. (2004). For each sample, equal total serum protein was loaded, after protein concentration determination according to the Bradford method (Bradford, 1976). After 72 h renaturation, gels were stained with Coomassie brilliant blue G-250 (CBB G-250) dye, and MMP-2 and -9 appeared as regions of transparency on the blue background. In order to verify that the clear zones represent the activity of gelatinases, 5 mmol/l EDTA was added to the samples before incubation to inhibit gelatinolytic activities in gelatin zymography. As a standard, human recombinant MMP-2 and -9 were used.

Lipid peroxides

Lipid peroxidation as evidenced by formation of thiobarbituric acid-reactive substances (TBARS) was assayed in the sera by the method of Varshney and Kale. The pink-coloured chromogen absorbance formed by the reaction of 2-thiobarbituric acid with the breakdown products of lipid peroxidation was read at 535 nm. Absorbances obtained from the standard curve were transformed to units of molar MDA concentration (Varshney and Kale, 1990).

Enzyme assays

Serum LDH activities were determined according to the method of Buhl et al. (1977). The activities were determined spectrophotometrically, measuring the absorbance drops during NADH oxidation at 340 nm. One unit (U) catalyses transformation of 1 μmol of NADH per one min of reaction. The method of Frieden and Fernandez (1975) was used to quantify serum MDH activities. The absorbance drops were measured during the oxidation of NADH at 340 nm. One unit (U) catalyses transformation of 1 μmol of NADH per one min of reaction.

Statistics

All biochemical measurements were run in triplicates and data related to MDA concentration, LDH and MDH activities were expressed as mean ± SEM. Distributions of gelatinases are expressed as relative gelatinolytic activities of each enzyme. Data distribution was examined using the Kolmogorov-Smirnov test. Evaluation of statistical significance was assessed using χ^2 test to estimate the difference between MMP activities, while Student's t-test and Mann Whitney U test were used to estimate the difference between LDH and MDH activities. Correlation exploration was performed with Pearson's and Spearman's correlation tests. For all analyses, P values at the level of 0.05 and less were considered statistically significant. All statistical analyses were performed using SPSS18.0 software package (IBM Corp., Armonk, NY).

Results

MMP-2 and MMP-9

Increased MMP-2 and -9 activities in the sera were detected by electrophoretic zymography in all examined CC groups, while all individuals from the control group had MMP-2 and -9 activities below the detection threshold. A representative zymogram of serum MMP activities is presented in Fig. 1. TNM II, III and IV patient groups had different distribution of the two MMP activities. The frequencies of MMP activities are presented in Table 1. It is clear that more than half of the patients in TNM II and III stages had increased serum MMP-2 activity, while in the TNM IV stage, the increased serum MMP-2 activity was manifested by a vast majority of the patients. The TNM II and III groups expressed the same activities of serum MMP-2, while both groups differed highly significantly (P < 0.01) when compared to the TNM IV group. Similarly to MMP-2, a different distribution of MMP-9 activities was also demonstrated. The most remarkable finding can be observed in the TNM III group, where all examined patients had increased serum MMP-9 activity. TNM II was characterized by a greater proportion of patients with increased MMP-9 activity, while the same number of patients in the TNM IV group had increased and normal values of MMP-9 activity in the sera. These groups differ highly significantly (P < 0.01) in the distribution of patients with elevated and normal serum MMP-9 activities.

Lipid peroxides

Production of free oxygen radicals was indirectly estimated by assaying the amount of serum MDA as a marker of the extent of lipid peroxidation caused by increased peroxide production. In the sera of CC patients, increased lipid peroxidation was revealed in all stages when compared to the control individuals. Fig. 2 shows the MDA concentration in both patients and controls. There is a highly significant difference in the serum MDA levels between all CC groups and control. The extent of lipid peroxidation, reflected in the amount of se-
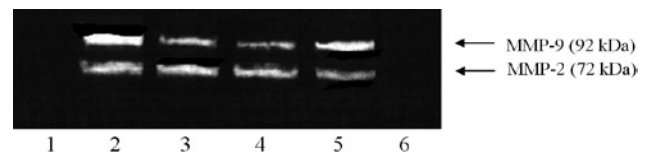

Fig. 1. Representative activities of MMP-2 and -9 in the sera of CC patients and controls. Position **1** represents a negative control in which standard MMP-2 and -9 enzymes were inhibited by EDTA. There are no visible activities either for MMP-2 or for MMP-9. Position **2** shows a positive control with visible activities of both standard human recombinant MMP-2 and -9. Positions **3**, **4** and **5** represent sera of CC patients with TNM II, III and IV stage, respectively. Position **6** shows the absence of MMP-2 and -9 activities in the serum of control group individuals.

Table 1. Percentage of patients with increased MMP-2 and MMP-9 activities

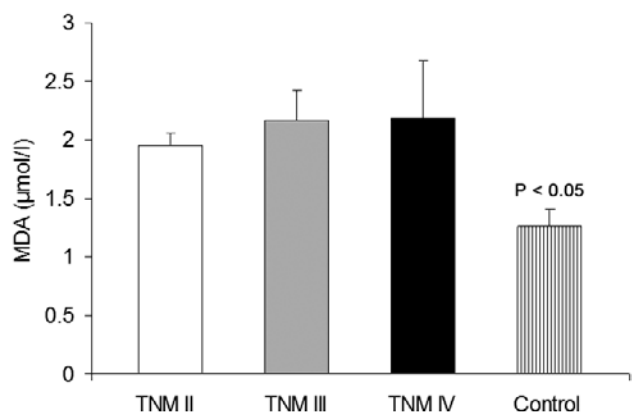| Experimental group	Activity of MMP-2 (%)	Activity of MMP-9 (%)
Control	0	0
TNM II stage	60	80
TNM III stage	60	100
TNM IV stage	88	50
Statistical difference	TNM II vs. TNM IV P < 0.01	TNM II vs. TNM IV P < 0.01
	TNM III vs. TNM IV P < 0.01	

Fig. 2. Total serum amount of MDA in different stages of CC and in the control group. There is a significantly lower amount of lipid peroxides, indicated by MDA concentration, in the sera of control group individuals than in any other CC group.

Fig. 3. Activities of LDH in the sera of patients with different CC stages and in the control group. Serum LDH activities are significantly higher (P < 0.01) in all examined groups of CC patients when compared to the control. The evolution of CC is followed by an increase in LDH activity, evident between TNM II and TNM III stages as well as TNM II and TNM IV stages. A noticeable increase in LDH activity exists between TNM III and TNM IV, but with no statistically significant difference.

rum MDA, rises with the tumour progression; however, not statistically significantly.

LDH and MDH activities

Serum activities of LDH and MDH, assayed as indicators of membrane damage and carbohydrate metabolism, were detected in substantially higher values among the CC patients than in healthy controls. Fig. 3 shows the serum activities of LDH in different stages of CC and in the control group. CC evolution is followed by an increase in the serum LDH activity, according to the highly significant differences (P < 0.01) between the control group and all TNM groups. Also, a sizeable rise in LDH activity occurred between the TNM II and TNM III stages, while the TNM IV stage expressed the greatest LDH activity of all CC stages. A slight increase in LDH activity could be observed between TNM III and TNM IV, but with no statistically significant difference. Correlation analyses showed significant positive correlations between the MMP-2 profile and LDH activity in the sera of CC patients in TNM II, III and IV stages (ρ = 0.492, P < 0.05; ρ = 0.568, P < 0.05; ρ = 0.423, P < 0.05), respectively.

The serum MDH activities were found to be considerably different between CC patients in all stages of the disease and control group individuals. Fig. 4 shows the serum activities of MDH in different stages of CC and in the control group. CC evolution is also characterized by an increase in serum MDH activity, according to the highly significant differences (P < 0.01) between the control group and all TNM groups. When patients in different stages of CC were analysed, it was evident that the lowest MDH activities were present in the sera of TNM II patients compared to TNM III and finally the TNM IV stage. This statistically significant linear increase in the serum MDH activities follows the CC progression on the way to the later stages of the disease.
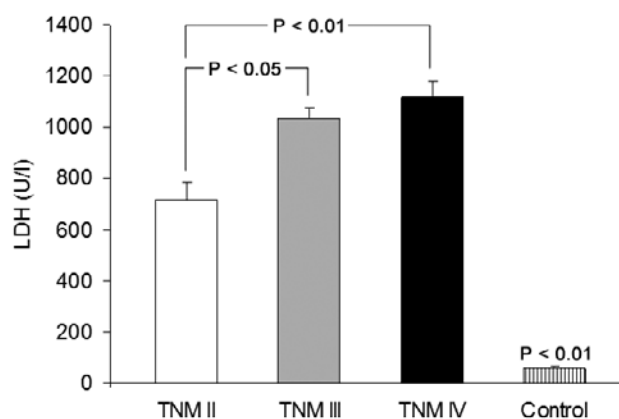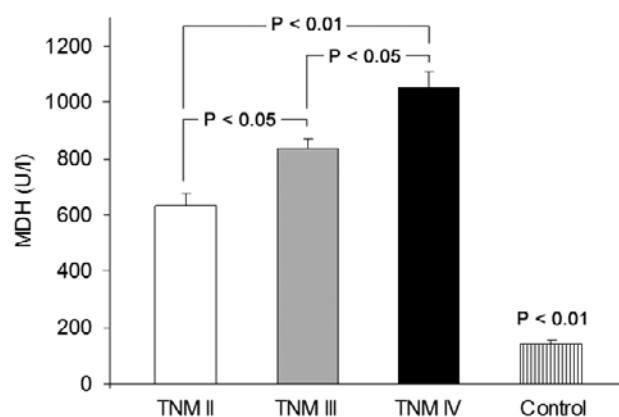

Fig. 4. Activities of MDH in the sera of patients with different CC stages and in the control group. Serum MDH activities are significantly higher (P < 0.01) in all examined groups of CC patients when compared to the control. The evolution of CC is followed by an extensive increase in MDH activity, evident between TNM II and TNM III stages, TNM III and TNM IV stages, as well as TNM II and TNM IV stages.

Influence of gender and tumour localization on the study parameters

All the presented data were also analysed by grouping based on gender and tumour localization, but no statistically significant differences and correlations were found in any experimental group considering any of the examined parameters. These findings suggest that the gender and tumour localization do not play an important role in

the regulation and distribution of any evaluated parameter included in this study.

Discussion

The roles of MMPs in cancer growth and metastasis are various and they include degradation of extracellular matrices, cancer cell adhesion, migration and secretion of growth factors, cytokines and factors of angiogenesis. The process of extracellular matrix remodelling is an integral part of normal tissue growth and differentiation, but unregulated proteolysis may lead to an advantage during carcinogenesis (Hong et al., 2011). Studies investigating MMPs have been performed among many different tumours, including breast cancer, pancreatic cancer, lung cancer, ovarian cancer, prostate cancer, brain cancer, as well as colorectal carcinoma (Roy et al., 2009). A subgroup of MMPs, gelatinases (MMP-2 and -9), have been particularly implicated in progression, angiogenesis and metastasis of various cancer types including CC. They are associated with degradation of collagen IV and loss of the basement membrane integrity, which correlates with increased probability of distant metastasis and poor prognosis (Turpeenniemi-Hujanen, 2005).

This study included patients in different TNM stages of CC in order to observe gelatinolytic activity during different phases of tumour growth. Both MMP-2 and -9 had been detected in the sera of CC patients, but their activity remained undetected in the sera of all healthy individuals, corresponding to the physiologically low circulating MMP amounts. The increased circulation amount of both MMPs was present in the sera of patients in all examined stages of CC. It has been demonstrated that all stages of tumour progression are characterized by augmented MMP activity, which provides the malignant potential to the tumour tissue. Especially interesting was the finding that 100 % of patients in TNM III stage expressed increased MMP-9 activity. Overexpression of MMP-2 and -9 and its relation with more frequent metastatic potential and poor prognosis was shown in several studies (Heslin et al., 2001; Ishida et al., 2003; Langers et al., 2008; Hong et al., 2011).

It seems that overexpression of MMPs is not a result of tumour cell activity. Tumour stromal fibroblasts produce MMPs at high intensity as a result of various stimulating factors that include paracrine tumour signalling by cytokines and presence of oxidative stress (Cirri and Chiarugi, 2011; Kolář et al., 2012). Extensive ROS production was indirectly detected by using estimation of the circulating LPx levels, and it was detected in a highly significant rate in all CC groups. The patients in more advanced stages of CC have higher circulating levels of MDA. In our previous study, it has been shown that advancement in CC pathogenesis is accompanied by disequilibrium in antioxidant defence mechanisms, leading to oxidative stress, which further destabilizes and leads the tumour toward progression (Gopčević et al., 2013). However, the precise mechanism of carcinogenesis promoted by oxidative stress is still not fully understood (Rainis et al., 2007). A study by Skrzydlewska et al. (2005) demonstrated an increased rate of LPx in CC, pointing out that colorectal carcinogenesis is associated with serious oxidative stress.

The consequences of LPx can also be observed in the light of increased membrane permeability, causing leakage of some compounds into the circulation. LDH and MDH serum activities can be used as biomarkers of membrane damage and as an insight into the energetic metabolism status. Activities of these two enzymes were significantly increased in the sera of all examined CC patients, following the linear trend of rising activity parallel to the tumour progression. LDH represents an enzyme that is included in glycolysis, converting lactate into pyruvate (and back). Lactate is produced in a large excess in tumours and constitutes an alternative metabolic fuel for cancer cells. Tumour cells with oxidative metabolism can use lactate instead of (or in addition to) glucose, which can, in turn, diffuse deeper into the tumour to provide fuel for hypoxic cells located farther away from tumour blood vessels (Draoui and Feron, 2011).

MDH is involved in the Krebs cycle, converting malate into oxaloacetate, and it can be a useful marker of aerobic metabolism conditions. It was found that poorly differentiated tumour cells have higher anaerobic metabolism, followed by an increase in MDH activity that utilizes the excess of metabolites produced in glycolysis (Danis and Farkas, 2009). No valuable literature data exist to explain the link between MDH activities and colorectal carcinogenesis. The MDH level in the serum was the same as that of LDH. Colorectal carcinogenesis causes increasing MDH activity as the tumour progresses. We propose that the increased LDH and MDH activities in the sera of CC patients can be dually observed. First, the increase in both enzyme activities is a reflection of higher catabolism during carcinogenesis, which is a consequence of increased tumour demands for glucose and energy. Second, the high rate of LPx damages the membranes of tumour and nearby cells, causing leakage of LDH and MDH into the circulation and leading to the overall increase in their activity. Therefore, these two different mechanisms synergistically participate in high LDH and MDH serum activity in the CC patients, but this study could not determine which effect is stronger.

The significant positive correlation that exists between MMP-2 and LDH activities can be explained by a direct relation of gelatinolytic activity augmentation and increase in oxidative/energetic imbalance of the tumour tissue, supporting the hypothesis that oxidative stress is one of the factors activating MMPs. The high correlation between the MMP-2 profile and LDH activity in the sera of patients who have a primary tumour localized in the colon also suggests that this anatomical location is specifically stimulating for MMP-2 activation in the state of present oxidative/energetic imbalance (Said et al., 2014). Since there are no data in the literature about

this phenomenon, it will remain unclear until further investigation is performed, which we believe is strongly warranted. The evaluation of circulating MMP activities can be a reliable surrogate non-invasive parameter for the tumour expression of these enzymes (Tutton et al., 2003). To establish a link between the gelatinolytic profile and rate of oxidative stress and the membrane damage markers, additional parameters of the redox status should be involved in such investigations. Many clinical studies of CC have shown that the expression levels of MMP-2 and MMP-9 correlate with tumour progression, but the attempts to develop MMP inhibitors have not been successful so far. However, this therapeutic concept remains a topic of interest because of the enormous role of MMP-2 and -9 in the pathogenesis of CC that has been demonstrated (Kitamura and Taketo, 2007).

Conclusion

We observed significantly increased activities of MMP-2 and -9 in the sera of CC patients compared to the healthy subjects. The increase in the circulating levels of MMPs was TNM stage-specific. The existing redox imbalance was followed by an increase of lipid peroxidation and membrane damage markers (LDH and MDH). In this study it was shown for the first time that the gelatinolytic activity in CC is associated with redox imbalance. Also, the serum MDH activity was evaluated for the first time in colorectal tumour and shown to rise in conjunction with the CC progression.

Discloser of conflict of interest

No conflict of interest was declared by the authors.

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