Determination of matrilysin activity in gastrointestinal neoplasia

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Abstract
Up-regulation of matrilysin (matrix metalloproteinase-7), a target gene of the APC-Wnt pathway, has been found in epithelial cells of colonic tumours, where it is involved in the degradation of extra-cellular matrix and the activation/shedding of cytokines, growth factors, and adhesion molecules. Because the proteolytic effect of matrilysin depends on the co-expression of activators, measurement of matrilysin activity should in principle be more relevant for clinical purposes than the detection of mRNA or protein. We developed an immunocapture bioactivity assay (BIA) that simultaneously detects the total amount as well as the activity of matrilysin in biological samples, including tissue homogenates. The BIA was validated for gastric cancer homogenates using an established commercial ELISA and immunoblotting. The matrilysin BIA was used for a pilot study on homogenates from human colonic adenomas and carcinomas. Both active as well as total matrilysin levels were enhanced in neoplastic tissues compared to normal mucosa. However, samples from adenomatous origin showed more pronounced differences between active and total matrilysin levels than carcinoma samples. Low matrilysin activity accompanied by an enhanced total level indicates an incomplete activation mechanism, which reflects the multi step nature of colon carcinogenesis. Therefore, the simultaneous detection of total and active matrilysin levels could be of clinical relevance.
Introduction

Matrilysin (matrix metalloproteinase-7, MMP-7) is the smallest member of the MMP family. It is secreted as a 28 kDa inactive pro-enzyme and activated by cleavage to a 19 kDa form\(^1\). In contrast to other secreted MMPs, production of matrilysin has been shown mainly in epithelial cells. Targets of matrilysin include collagen, osteopontin, pro-TNF-\(\alpha\), \(\beta\)-integrin, \(\alpha\)-defensins, E-cadherin, and Fas-Ligand\(^2\). The nature of its substrates, combined with the enhanced expression by malignant epithelial cells suggests an important role for matrilysin during carcinogenesis. Knock-out studies confirm that intestinal tumourigenesis is suppressed in mice lacking matrilysin\(^3\). Matrilysin is a target of the APC-Wnt pathway, often affected in early stages of colorectal carcinogenesis. Immunohistochemical studies have indeed shown the enhanced presence of matrilysin in early stage colorectal neoplasia\(^4\), but also a correlation of matrilysin with Dukes classification and metastasis\(^5\). The clinical potential of matrilysin has recently been emphasized by different studies reporting the predictive value of enhanced matrilysin mRNA levels for the presence of metastasis and survival of patients with colonic or rectal cancer\(^6,7\). If matrilysin mRNA levels are already clinically relevant, than protein levels, especially of the active form, should be even more informative. The primary aim of our study was to measure matrilysin protein levels in homogenates derived from human gastrointestinal cancer tissues. For this purpose we developed a simple 96-well immunocapture bioactivity assay (BIA) for determining the activity of matrilysin in tissue homogenates. The BIA is based on a well established substrate consisting of modified pro-urokinase, in which the activation sequence was replaced by an amino acid sequence which is specifically recognized by MMPs\(^8\). The BIA uses specific antibodies to capture matrilysin and a chromogenic peptide substrate for urokinase for detection. In principle the BIA detects the amount of matrilysin that is present in the activated form. Activation of pro-matrilysin using APMA (4-aminophenylmercuric acetate) in parallel incubations should express the total amount of matrilysin in the sample.
Patients, materials and methods

Fresh tissue specimens from patients who underwent resection for gastric or colonic neoplasia at the department of Oncologic Surgery of the Leiden University Medical Centre were collected. Normal colonic tissue samples were taken at least 5 cm from the carcinomas (n=15). Representative parts of the whole carcinomas and adenomatous polyps (n=15) were selected. Samples were snap-frozen and stored at -70°C until extraction. Homogenization of tissue specimens and determination of protein concentrations were performed as described previously. Plasma from citrated blood and early morning urine were collected from patients with colorectal carcinoma before surgical therapy (6♂/2♀, age 40-70 years). The study was performed according to the instructions and guidelines of the LUMC medical ethics committee. Anti-matriplysin antibodies were mouse monoclonal and goat polyclonal (both from R&D Systems Europe, Abingdon, UK) and rabbit polyclonal (Abgent, San Diego, USA). The commercially available ELISA for total human matriplysin (Quantikine, R&D Systems Europe) was carried out according to the manufacturer's instructions using 10 μl of tissue homogenate. The matriplysin specific immunocapture activity assay was adapted from a MMP-9 activity assay as described previously. In short, 96-well plates (Maxisorb, Nunc) were coated with 100 μl matriplysin specific antibody for 2 hours at 37°C, and blocked for 2 hours with StabilCoat (Diarect AG, Freiburg, Germany). After washing (4x PBS containing 0.05% (v/v) Tween-20), matriplysin from standard (human recombinant 0-32 ng/ml, R&D) or sample was allowed to bind for 16 hours at 4°C in assay buffer (50 mM Tris-HCl pH7.6, 1.5 mM NaCl, 0.5 mM CaCl₂, 1 μM ZnCl₂ and 0.01% (v/v) Brij-35). After activation of parallel incubations in assay buffer containing 1 mM APMA (Sigma-Aldrich), the plates were washed (4x) and incubated with assay buffer to which 7.5 μg/ml modified pro-urokinase and 0.96 mM chromogenic substrate S-2444 (Chromogenix, Mölndal, Sweden) were added. Color development was recorded by measurement of OD₄₅₀ using a Molecular Devices Microplate Reader during 7 hours.
Results and discussion

Figure 1a shows standard curves of APMA-activated recombinant matrilysin detected after coating with various dilutions of 3 different catching antibodies. The mouse monoclonal antibody which performed well in immunohistochemistry and western blotting, did not capture detectable levels of matrilysin in the BIA, not even in an coating-antibody concentration of 10 μg/ml. The rabbit antibody worked better, but the highest levels of matrilysin were captured by the goat polyclonal antibody. The low cross-reactivity (<5%) of this particular antibody with rhMMP-1, -2, -3, -8, -9, -10, -12, and -13, as indicated by the manufacturer, was confirmed in the BIA by spiking with 32 ng/ml active MMP-2, -3, -9, -14 (0-8% cross-reactivity).

To validate the BIA we first measured the total matrilysin content of 11 tissue homogenates derived from gastric cancer patients using a commercial ELISA detecting total matrilysin (active and pro-form). The homogenates were selected for increasing matrilysin content (numbers 1-11 in Figure 1b) consisting of 3 normal tissue and 8 carcinoma samples. These levels were compared with the matrilysin data as found with the BIA in parallel determinations, i.e. with or without APMA activation. The total amount of matrilysin according to the BIA correlated significantly with the data obtained by ELISA (Pearson’s r = 0.910; P<0.0001, n=11), but active matrilysin did not (Figure 1b, open versus closed circles respectively). Apparently, some gastric carcinoma samples contained relatively little active matrilysin compared to the total amount, whereas other samples contained almost exclusively active matrilysin. This phenomenon was confirmed using western blotting. The two most pronounced examples from the BIA (majority pro- versus majority active matrilysin) are depicted in the insert in Figure 1b, showing the corresponding prominent bands in the respective homogenates. To validate the BIA further, we spiked all these samples with a fixed amount of active recombinant matrilysin, leading to a steady increase in all homogenates, except for the 2 highest samples (Figure 1b). The latter was due to the high endogenous matrilysin level within these samples. Repeated (3- to 5-fold) measurements of the same samples within and between different assays resulted in an intra-assay coefficient of variation of 3.2% and an inter-assay variation of 8.4%. Gastric cancer tissues showed markedly enhanced levels of active as well as total matrilysin compared to the normal mucosa (Figure 1b), confirming our previous ELISA data on matrilysin in gastric cancer11,12. The matrilysin BIA was then used for homogenates from colonic tissue samples. Figure 1c shows the active and total fraction of matrilysin in respectively colonic mucosa, adenomas and carcinomas.
High urinary levels of MMP-2 and MMP-9 have previously been shown to be associated with cancer patients (n=8) contained 31-46 ng/ml active and 40-75 ng/ml total matrilysin. In the applicability of the assay was also determined for plasma and urine. Plasma from colorectal
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In general, matrilysin levels were strongly enhanced in neoplastic tissues of the colon. 
Especially in case of the pre-malignant adenomas, specific samples showed a dramatic
difference between the total amount of matrilysin and the active form, indicating a large pool
of pro-matriylisin and suggesting a (still) ineffective activation mechanism. Finally, the
applicability of the assay was also determined for plasma and urine. Plasma from colorectal
cancer patients (n=8) contained 31-46 ng/ml active and 40-75 ng/ml total matrilysin. In the
urine from the same patients all matrilysin was present in the active form (27-208 ng/ml).
High urinary levels of MMP-2 and MMP-9 have previously been shown to be associated with
tumour stage and grade in urothelial carcinomas\textsuperscript{13,14}. The fact that matrilysin is most likely derived from malignant epithelial cells, in contrast to MMP-2 and MMP-9, suggests a potential application for urinary matrilysin as tumour marker, which is presently under study. In conclusion, this study introduces an assay to determine total as well as active matrilysin and the applicability was validated for biological samples from patients with gastrointestinal (pre-)malignancies. The assay was constructed in the light of the recent publications suggesting a predictive value of matrilysin mRNA in colorectal cancer tissue for the presence of metastasis and the survival of the patients\textsuperscript{6,7}. Measurement of matrilysin protein levels, especially of the active form, is expected to be at least as clinically relevant, because the oncogenic function of the proteinase should be directly correlated to the enzyme activity and only indirectly to the potential pool of enzyme (i.e. up-regulated mRNA and/or protein). Our preliminary results indicate indeed a discrepancy between total and active levels of matrilysin in particular neoplastic samples, illustrating regulatory processes in the effectiveness of the matrilysin activation mechanism. The clinical relevance of this phenomenon should still be verified however, using larger groups of patients.

\textbf{References}


