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Chapter 8

The biphasic effects of a selective MMP inhibitor on tumor development of colon cancer in rat liver

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submitted
Abstract
Colon cancer metastasis is the major cause of death in colorectal cancer patients. Enhanced expression of matrix metalloproteinases (MMPs) and in particular MMP-2 and MMP-9 has been correlated with cancer progression and metastasis. Broad-range and MMP inhibition inhibits cancer growth, however, clinical trials with MMP inhibitors have been a disappointment so far.

An animal model of tumor development of colon cancer in the liver has been used to study the role of tumor progression in the liver. We have previously shown that gelatinolytic activity is present in these tumors but its pathological function was not established. Therefore, in the present study, we evaluated the effect of a selective MMP inhibitor on the in vivo development of liver tumors.

Daily treatment with a low-dose and a high-dose selective MMP inhibitor resulted in a marginal inhibition of tumor volume only in the high-dose inhibitor group. However, the number of tumors was increased 2-fold due to selective MMP inhibition. The effect of selective MMP inhibition on the involvement of the host immune response was therefore investigated. Treatment of rats with the selective MMP inhibitor had no effect on the number of circulating white blood cells in unchallenged rats, however, when rats were challenged with cancer cells, it seems that recruitment of neutrophils was affected.

It is concluded that treatment of cancer patients with MMP inhibitors should be considered because induction of adverse effect is a possibility.

Introduction
Colorectal cancer is one of the most frequent cancers in the western world. The 5-year survival in 1995 in Europe was 40% (1). Nowadays, the 5-year survival in The Netherlands is 50-60% (www.kankerbestrijding.nl/nieuws/cijfersoverkanker/2002). Poor prognosis is mainly related with metastasis. Tumor invasion and metastasis require several crucial steps among which degradation of extracellular matrix (ECM) and basement membranes (BM) is considered to be essential. Various types of proteinases participate in matrix turnover, but matrix metalloproteinases (MMPs) are the principal matrix-degrading enzymes. MMPs can be expressed by cancer cells as well as stromal cells including endothelial cells, macrophages, T-cells, neutrophils and fibroblasts (2). It has become evident that MMPs also play a role in
other steps in the metastatic cascade i.e. angiogenesis, immune surveillance, proliferation and apoptosis (3). Therefore, MMPs are considered important regulators of tumor growth, both at the primary site and in distant metastases (4). MMPs are a family of at least 25 matrix-degrading proteinases, which in concert can degrade all ECM components. One particular group, the gelatinases, also known as 72 kD and 92 kD type IV collagenase, or gelatinase A and gelatinase B, or MMP-2 and MMP-9, respectively, are of particular interest with respect to the development and progression of colorectal cancer. MMP-2 and MMP-9 are elevated in colorectal cancer tissues at the mRNA level (5, 6), protein level (7, 8) and activity level (9). Furthermore, it has been reported that metastases have higher levels of MMP-2 and MMP-9 as compared with the primary tumor (10).

These aspects have provided the rationale to block MMP activity as a therapeutic strategy in the treatment of cancer. In animal models, both broad range and more selective MMP inhibitors have shown growth inhibition of primary tumors and decreased distant metastasis in colon (11), prostate (12), mammary (13) and lung cancer (14). On the other hand, the broad range MMP inhibitor, batimastat, introduced liver metastases in a breast cancer model whereas liver metastases were not observed in control animals (15). Similarly, liver metastases were increased in number in T-cell lymphoma after treatment with batimastat. Furthermore, daily administration of batimastat to healthy animals induced MMP-9 expression and increased expression of MMP-2 in liver tissue (15).

MMP inhibitors have shown disappointing results in clinical trials to prevent cancer progression of various types of cancers (16). MMP inhibitors of the hydroxamate-type (like batimastat) never have shown any significant advantage over placebo so far. Prinomastat (AG3340) phase III studies were terminated preliminarily because beneficial effects over chemotherapy were not observed (www.agouron.com/Pages/press_releases/pr080400.html). The application of BAY 12-9566 in a phase II clinical trial showed stabilization of the disease in some cases but a phase III trial was terminated preliminarily as treatment did worse than the placebo group (www.bayerusa.com/news/co0221.asp). These disappointing results may be explained by the multifunctionality of MMPs which are involved in more than matrix degradation alone. For example, MMPs can be involved in cell proliferation, (cancer) cell survival, angiogenesis, invasion and immune surveillance (reviewed in (3)).

An animal model of tumor development of colon cancer in liver has been developed in rat by administration of CC531(s) cancer cells to the portal vein (17-19). With respect to the role of
proteases, it was found that inhibitors of extracellular cathepsin B rather delayed than inhibited colon cancer metastasis in rat liver. It was concluded that active extracellular cathepsin B was involved in the development of these tumors but that its function was not an essential requirement (20). Later, it was shown that gelatinolytic activity was present in these tumors (21) but whether it plays a decisive role in tumor development is not yet established. Therefore, we investigated in the present study the functional role of gelatinolytic activity in the development of tumors of colon cancer in liver by daily oral administration of a selective inhibitor of MMP-2, MMP-9 and MT1-MMP.

Material and Methods

Animals

Male WagRij rats (Broekman, Someren, The Netherlands) with a body weight of 200-250 g were maintained for 2 weeks under constant environmental conditions with free access to food and water. All animal experiments were performed in agreement with the Animal Ethics Committee of the Academic Medical Center, University of Amsterdam.

Cancer cells

An established colon carcinoma cell line, CC531s, was developed by Marquet et al. (22) and stably transfected with eGFP by us (23). The cells were cultured at 37°C as monolayers in RPMI-1640 Dutch Modification without L-glutamine supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 IU penicillin/ml and 100 mg streptomycin/ml (all obtained from GIBCO/BRL, Grand Island, NY, USA). Cells were washed with phosphate-buffered saline (PBS) and after detachment by treatment with trypsin (0.05% w/v; GIBCO) and ethylenediaminetetraacetic acid (EDTA; 0.02% w/v; Sigma, Zwijndrecht, The Netherlands) in PBS and centrifugation (250 g, room temp, 10 min), single cell suspensions were obtained with a viability of at least 95% (19).

Surgery

A small midline incision was made in the abdominal wall of rats under anaesthesia with FFM mix (1ml Hypnorm, 1ml Midazolam and 2 ml water, 0.27 ml/100g body weight, intraperitoneally). A suspension containing 2.5x10^6 cancer cells in 500 µl PBS was injected into the portal vein with a 27-gauge needle.
Drug treatment end-point studies

The synthetic MMP inhibitor RO 28-2653 (5-biphenyl-4-yl-5-[4-(nitro-phenyl)-piperazin-1-yl]-pyrimidine-2,4,6-trione) was kindly provided by Dr. H-W. Krell (Roche Diagnostics GmbH, Penzberg, Germany). Animals were treated daily by oral administration of 45 mg/kg/day (n = 9) or 100 mg/kg/day (n = 9) RO 28-2653 dissolved in vehicle, starting at the day of cancer cell administration until sacrifice. Control animals (n = 17) received vehicle (0.2% carboxymethylcellulose) only. Three Weeks after administration of the cancer cells, animals were sacrificed and livers were removed. Tumors that were visible by the naked eye, were quantified by counting the numbers and measuring diameters with a callipers as described previously (24).

Immune response studies

To evaluate the effects of RO 28-2653 on the immune response of the animals to the administration of cancer cells, rats were treated with vehicle (n = 4) or 100 mg/kg/day RO 28-2653 (n = 4) respectively. Administration of vehicle and inhibitor started at 2 days before administration of the cancer cells, until the day of sacrifice at 4 days after administration of the cancer cells. Prior to both administration of the cancer cells and sacrifice of the animals, EDTA blood was collected from the lateral tail vein. The number of circulating lymphocytes, monocytes, neutrophils, eosinophils and basophils was determined with a CellDyn 4000 autodifferential cell sorter (Abbott Diagnostics, Chicago, MI, USA). Liver tissues were collected and the immune response in the liver was analyzed quantitatively.

Histology

Liver tissue containing tumors of control animals and inhibitor-treated animals were frozen in liquid nitrogen and stored at -80°C until used. Serial sections (8 μm thick) were cut at a cabinet temperature of -25°C using a motor-driven cryostat (Bright, Huntingdon, UK). Sections were air-dried for one hour and fixed at room temperature in either 4% formaldehyde in PBS (to detect MMP-9) or acetone (to detect ED1, OX8 and RP1). MMP-9 was demonstrated with a mouse monoclonal antibody against human active and inactive MMP-9 (diluted 1:400; Neomarkers, Fremont, CA, USA). Newly recruited monocytes, macrophages and Kupffer cells were demonstrated with ED1 (diluted 1:500 in PBS in the presence of 0.2% (w/v) BSA and 1% (v/v) normal rat serum), suppressor T lymphocytes and natural killer cells were demonstrated with OX8 (diluted 1:100 in PBS in the presence of 0.2% (w/v) BSA and
1% (v/v) normal rat serum) both obtained from Serotech (Oxford, UK) and neutrophils were detected with RPI (diluted 1:2000 in PBS in the presence of 0.2% (w/v) BSA and 1% (v/v) normal rat serum) obtained from BD PharMingen, San Diego, CA, USA. Secondary antibodies were labelled with Alexa 488 (Molecular Probes, Eugene, OR, USA). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI; 1.0 μg/ml; Molecular Probes). Fluorescence of Alexa 488 was detected with excitation at 460-500 nm and emission at 512-542 nm. DAPI was detected with excitation at 340-380 nm and emission at 425-492 nm.

To determine the number of cells positive for these antigens, 3 different parts of the liver were sectioned and stained with each antibody. From each section, five random fields of view were recorded and scored for the number of positive cells present in the liver parenchyma at 4 days and 3 weeks after induction of metastases.

Data analysis

Statistical analysis was performed with GraphPad Prism software (GraphPad, San Diego, CA). The one-way analysis of variance (ANOVA) with the Turkey-Kramer multiple comparison test and the unpaired Student t test were used. Differences of p<0.05 were considered significant. Data are presented as mean ± SEM.

Results

Effects on tumor development after 3 weeks of selective MMP inhibition

Animals were either treated daily with vehicle (n = 17; control group), 45 mg/kg/day RO 28-2653 (n = 9; low-dose group) or 100 mg/kg/day (n = 9; high-dose group) starting at the day of administration of cancer cells until sacrifice at 3 weeks after the administration of cancer cells. All tumors appeared on the surface of the liver lobes as globular structures. The average diameter of the tumors was 1.86 ± 0.06 mm in the control group, 1.67 ± 0.10 mm in the low-dose group and 1.53 ± 0.08 in the high-dose group (Fig. 1A). The difference between the control group and the low-dose group was not statistically significant whereas the difference between the control and the high-dose group was significant (p<0.05). The number of tumors found macroscopically at the surface of the liver at 3 weeks after administration of the cancer cells was 89 ± 13 in the control group, 169 ± 15 in the low-dose group and 171 ± 33 in the
high-dose group (Fig. 1B). Differences between the control and both treatment groups were statistically significant (\(p<0.01\)).

![Figure 1. Effects of MMP inhibition on mean size (A) and mean number (B) of tumors in rat liver developed at 3 weeks after intraportal administration of colon cancer cells. The mean diameter of the tumors in the control group (C) and the low-dose group were not significantly different, but the difference between the control group and the high-dose group was statistically significant (\(p<0.05\)). The mean number of tumors in the treated group were both significantly different from those in the control group (C) (\(p<0.01\)).](image-url)

**Analysis of white blood cells**

To evaluate the effects of RO 28-2653-treatment and administration of cancer cells on the host defence, blood was collected from control animals \(n=4\) and animals treated with 100 mg/kg/day MMP inhibitor \(n=4\). Blood was collected prior to induction of metastases and at 4 days after induction of metastases and was analyzed for the number of circulating neutrophils, eosinophils, basophils, lymphocytes and monocytes. Prior to administration of cancer cells but 2 days after the start of treatment with the inhibitor, differences in the numbers of white blood cells were not found (Fig. 2A). At 4 days after induction of tumors, differences in the number of circulating eosinophils, basophils, lymphocytes and monocytes between untreated animals and animals treated with inhibitor were not observed but the number of circulating neutrophils was significantly lower in the RO 28-2653-treated rats as compared with control animals \((2.0 \pm 0.4 \times 10^9/l \text{ versus } 3.6 \pm 0.5 \times 10^9/l, \text{ respectively; } p<0.05; \text{ Fig. 2B})\). Because numbers of circulating neutrophils prior to induction of tumors were similar \((1.7 \pm 0.1 \times 10^9/l \text{ versus } 1.5 \pm 0.3 \times 10^9/l, \text{ respectively})\), it is likely that recruitment of neutrophils was impaired in RO 28-2653-treated animals.
Figure 2. Number of circulating white blood cells prior to induction of tumors (A) and at 4 days after induction of tumors of colon cancer in livers of rats (B). Prior to induction of tumors, there were no differences between control animals (C) and animals treated with the inhibitor (RO) in the circulating numbers of neutrophils (Neu), lymphocytes (Lym) (left Y-axis), eosinophils (Eos), basophils (Baso) and monocytes (Mono) (right Y-axis). At 4 days after induction of tumors, all types of white blood cells were similar in the control group and the treated group except for the number of neutrophils. In the control group, the number of circulating neutrophils had more than doubled due to the administration of cancer cells whereas treatment with the inhibitor prevented that significantly (p<0.05).

Analysis of the local immune response at 4 days and 3 weeks after induction of tumors

3 Weeks after induction of tumors, numbers of MMP-9-positive cells, monocytes/macrophages (ED1-positive cells), natural killer cells (NK)/cytotoxic T-lymphocytes (CTL) (OX8-positive cells)) and neutrophils (RP-1-positive cells) were not significantly different in liver parenchyma of control animals and RO 28-2653-treated animals (Fig. 3A). To investigate whether differences between control animals and RO 28-2653-treated animals with respect to circulating white blood cells at 4 days after induction of the tumors were also reflected in liver parenchyma, MMP-9-positive cells, monocytes/macrophages (ED1), natural killer cells (NK)/cytotoxic T-lymphocytes (CTL) (OX8-positive)) and neutrophils (RP-1) were also scored in liver parenchyma of these animals. At this stage, numbers of MMP-9-positive, ED1-positive, OX8-positive cells and RP1-positive cells were not different in control as compared with RO 28-2653-treated animals (Fig. 3B). When comparing Fig. 3A and Fig. 3B, it becomes clear that numbers of ED1-positive cells, MMP-9-positive cells and RP1-positive cells are larger in liver parenchyma at 4 days after administration of cancer cells as compared with 3 weeks after administration of cancer cells, whereas there is no difference in the number of OX8-positive cells between these 2 stages.
Figure 3. Number of cells positive for ED1 (monocytes and macrophages) (left Y-axis), MMP-9, OX8 (cytotoxic T lymphocytes and NK cells) and RP1 (neutrophils) (right Y-axis) per 0.1 mm² in liver parenchyma at 3 weeks (A) and 4 days (B) after induction of tumors in control animals (C) and inhibitor-treated animals (RO). Significant differences between animal groups were not observed.

Discussion

General and more selective MMP inhibitors have shown to inhibit cancer progression and metastases in animal models (11, 14, 25). We have previously shown in situ gelatinolytic activity in tumors of colon cancer in rat liver, the same animal model as was used in the present study. It was found that gelatinolytic activity was localized in the extracellular matrix of tumors but conclusions on the biological function could not be drawn (21). Therefore, in vivo inhibition of gelatinase activity was applied to define the relevance of gelatinolytic activity in this tumor model. For that purpose, tumors were induced in rat liver by administration of colon cancer cells into the portal vein. Daily administration of a low dose and high dose of a selective MMP inhibitor (RO 28-2653; selective for MMP-2, MMP-9 and MT1-MMP) was applied to test the involvement of those MMPs in the development of liver tumors of colon cancer cells. Blood plasma levels of this selective inhibitor have been determined previously (12). It appeared that plasma levels between 30 µg/l and 50 µg/l were reached in rats treated with 30 mg/kg/day whereas plasma levels ranged between 80 µg/l and 120 µg/l in rats treated with 100 mg/kg/day. Therefore, we selected treatment with a daily low dose of 45 mg/kg/day and a high dose of 100 mg/kg/day.

Daily oral treatment with RO 28-2653 starting at the day of administration of cancer cells until the animals were sacrificed resulted in a limited decrease in size of the tumors (approximately 30% after treatment with the high dose). Histopathology of the tumors was
not clearly affected by treatment with the inhibitor. The relative portion of cancer cells, stroma and immune cells was similar in all 3 animal groups. The reduced size of the tumors may be in line with our previous findings that at 3 weeks after administration of the cancer cells, gelatinolytic activity is present in the extracellular matrix in tumor stroma and may be involved in the growth of the tumors. This activity may well be involved in matrix remodelling necessary for tumor expansion and consequently inhibition of gelatinolytic activity results in reduced tumor growth. Inhibition of angiogenesis may also have had an effect but angiogenesis was not investigated in the present study. The marginal reduction of 30% in the high-dose group and the lack of reduction in tumor size in the low-dose group indicates in our opinion that the inhibitor is not significant for the treatment of cancer.

Moreover, numbers of tumors were increased by 2-fold in both treatment groups as compared with the control group. Because the total tumor load in livers of rats treated with the inhibitor is in fact higher than that in livers of control rats and histopathological features of the tumors were not different, it can be concluded that treatment did not reduce the cancer cell mass in the animals at all. In agreement with these findings, it has been reported that metastases of T-cell lymphoma and breast carcinoma are induced in liver by batimastat treatment (15). It was shown that treatment with batimastat lead to increased expression of MMP-2 and induced expression of MMP-9 in healthy livers and upregulation of angiogenesis-promoting factors (15). It was concluded that these effects may even promote cancer metastasis.

Increased numbers and reduced sizes of tumors suggest, unlike the study of Kruger (15), a biphasic effect of the treatment with the inhibitor. Reduced tumor size as discussed above is opposed to increased numbers of tumors. A possible explanation is that there is a survival advantage for cancer cells in the early steps of tumor formation due to treatment with the inhibitor. The immune response is held responsible for clearance of cancer cells in a target organ (26-28). Therefore, an impaired immune response to cancer cells could result in a survival advantage of cancer cells and subsequently result in more tumors. Gelatinases are also expressed by immune cells including macrophages, lymphocytes and neutrophils (reviewed in (29)). Macrophages are involved in inhibition of tumor development (30) and macrophage-derived MMP-9 (and uPAR) inhibits hematogenous metastasis (27). Furthermore, depletion of macrophages before tumor cell administration induces the number of macroscopic tumors dramatically (31). T-lymphocytes isolated from patients with myelopathy have been shown to use gelatinases for migration across artificial basement
membranes (32) and it has been postulated that gelatinases expressed by NK cells play a crucial role in their migration towards and into tumors (33). Furthermore, neutrophils have been shown to be cytotoxic to cancer cells in vitro (34). In vivo, neutrophils have been implicated in tumor cytotoxicity (35). Therefore, it can be concluded that because gelatinases are expressed by a variety of immune cells and are involved in their migration and because immune cells are involved in tumor growth inhibition, inhibition of the immune response in early stages of tumor development due to the administration of RO 28-2653 may be responsible for increased numbers of tumors at 3 weeks after their induction.

The amounts of cells positive for MMP-9, ED1, OX8 and RP-1 were similar in liver parenchyma containing tumors of control animals and RO 28-2653-treated animals at 3 weeks after induction (Fig. 3A). However, tumors are established after 3 weeks of growth and are unlikely to be very much affected by the immune system. Therefore, we also studied effects of RO 28-2653 treatment on the immune response in early stages of tumor development. It was found that treatment with RO 28-2653 for 2 days prior to the administration of cancer cells had no influence on the amount of circulating white blood cells. However, when rats were challenged with cancer cells, blood neutrophil counts were increased more than 2-fold in rats not treated with the inhibitor whereas in RO 28-2653-treated animals this increase was not found. This indicates that recruitment of neutrophils was altered in RO 28-2653-treated animals. Impaired recruitment of neutrophils was shown in MMP-9-knock-out mice as well (36). The importance of neutrophils in cytotoxicity towards cancer cells has been shown in several studies (28, 37-39). Therefore, decreased neutrophil recruitment in the blood due to RO 28-2653 treatment may well be responsible for decreased cancer cell clearance in early stages of tumor development and therefore result in increased numbers of tumors at 3 weeks. However, the number of neutrophils in livers of rats as detected with the RP1 antibody was not different in inhibitor-treated animals and control animals. One possible explanation is that the RP-1 antibody reacts only with neutrophils at a certain stage of development whereas circulating neutrophils are detected in every stage of development. As a consequence, neutrophil values in livers do not necessarily reflect blood values. Another explanation could be that neutrophil counts in the liver lag neutrophil counts in the blood and therefore, differences in the liver could appear later.

In conclusion, our study in a rat model of tumor development of colon cancer in the liver suggests a dual effect of MMP inhibition on the formation tumors. First, MMP inhibition only
marginally reduced the size of tumors and this small reduction has little clinical significance. Second, MMP inhibition induced increased numbers of tumors at 3 weeks after administration of the cancer cells, likely because MMP inhibition affects recruitment of neutrophils and impaired recruitment of neutrophils may have resulted in the development of a higher number of tumors.

In the past, MMP inhibitors have been tested in the treatment of advanced stages of cancer in clinical trials. However, clinical trials have shown poor efficacy of MMP inhibition and treated patients have even been reported to do worse than the placebo group (40). It was speculated that MMP inhibitors could be more effective when given in early stages of the disease or to prevent development of undetected micrometastases (3). However, our data suggest that the use of MMP inhibitors in early stage of the disease or as preventative drug may well induce adverse effects i.e. promotion instead of inhibition of tumor development because in our model the host’s immune system seems to be impaired by MMP inhibitors.

References


