Tumor development of colon cancer in rat liver
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Citation for published version (APA):

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Chapter 1

General introduction
Colorectal cancer and liver metastasis

Colorectal cancer is the second most common cancer in the Western world. At least 8600 new cases are diagnosed in the Netherlands each year and each year approximately 4400 patients die due to colorectal cancer (www.kankerbestrijding.nl/nieuws/ cijfersoverkanker/2002). The most successful treatment of colorectal cancer is still surgical resection. The liver is the main organ of colorectal cancer metastasis. At the time of diagnosis, approximately 25% of patients have liver metastases whereas another 25% of patients develop liver metastases after resection of the primary tumor (1). The overall 5-year survival of colorectal cancer patients is 56%. The strongest prognostic factor of survival is stage of disease. The stage of colorectal cancer is determined by the depth of penetration of the colorectal wall (T-stage), the presence and number of lymph node metastases (N-stage) and existence of distant metastases (M-stage). Table 1 shows that the presence of either lymph node metastases or distant metastases is a poor prognostic factor. When distant metastases are present, the 5-year survival is less than 5%.

<table>
<thead>
<tr>
<th>TNM stage</th>
<th>TNM characteristics</th>
<th>Dukes' equivalent</th>
<th>Astler-Coller equivalent</th>
<th>5-Year survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>T1N0M0</td>
<td>A</td>
<td>A</td>
<td>90-100%</td>
</tr>
<tr>
<td>II</td>
<td>T2N0M0</td>
<td>A</td>
<td>B1</td>
<td>60-90%</td>
</tr>
<tr>
<td>III</td>
<td>T3, N0, M0 or T4, N0, M0</td>
<td>B</td>
<td>B2 and B3</td>
<td>60-90%</td>
</tr>
<tr>
<td>IV</td>
<td>Any T, N1, M0 or any T, N2, M0</td>
<td>C</td>
<td>C1-3</td>
<td>25-60%</td>
</tr>
<tr>
<td></td>
<td>Any T, any N, M1</td>
<td>D</td>
<td>D</td>
<td>&lt; 5%</td>
</tr>
</tbody>
</table>

Table 1: Comparison of classification systems of colorectal cancer, TNM, Dukes’ and Astler-Coller. T1: the tumor has invaded the submucosa. T2: the tumor has invaded the muscularis propria. T3: the tumor has invaded through the muscularis propria into the serosa, and/or nonperitonealized pericolic or perirectal tissues. T4: the tumor has invaded other organs or structures, and/or has penetrated the peritoneum. N0: invasion of regional lymph nodes (metastasis) is not apparent. N1: one to three regional lymph nodes are invaded. N2: four or more regional lymph nodes are invaded. M0: distant metastasis is not apparent. M1: distant metastasis is apparent. See: http://www.oreilly.com/medical/colon/news/equivalents.html

Treatment of colorectal cancer

Standard treatment of stage I colorectal cancer consists of surgical resection of the tumor without any further treatment. Stage II colorectal cancer is treated with surgical resection whereas adjuvant therapy is not indicated for most patients. Some patients may be included in
a clinical trial. Stage III colorectal cancer is treated with surgical resection and postoperative chemotherapy with 5-FU/levamisole, resulting in a reduction in overall death when compared with 5-FU alone (2). More recent studies have shown that treatment with 5-FU/leucovorin (a biochemical modulator of 5-FU) has a 5% survival advantage over 5-FU/levamisole (3). Nowadays, adjuvant chemotherapy with 5-FU/leucovorin is standard treatment for patients diagnosed with stage III colorectal cancer. Eligible patients should be considered for entry into carefully-controlled clinical trials to compare postoperative chemotherapy regimens, postoperative radiation therapy and/or biological therapy to stimulate the immune system, alone or in combination. When colon cancer has metastasized to the liver (stage IV), ablation of metastases by surgical resection, cryotherapy or radiofrequency therapy is the only curative treatment possible (4). However, the majority of these patients cannot be treated by surgical resection due to excessive numbers of metastases, the location of the metastases or a general bad condition of the patient. For these patients, improvement of quality of life and survival are studied at the moment with adjuvant treatment with 5-FU, leucovorin, oxaliplatin and isolated liver perfusion with melphalan (5, 6).

Mechanisms of metastasis

Metastatic capacity of cancer cells is determined by several factors, including enhanced cell motility, altered adhesion and enhanced protease expression. The preference of colon cancer to metastasize to the liver has been explained by two mechanisms. First, the "seed and soil" theory of Paget (1889) postulates that a "seed" (cancer cell) requires a suitable "soil" (target organ) for successful growth at a secondary site. Cross-talk between cancer cells and the microenvironment of the target organ seems to be responsible for organ-specific metastasis. Second, Ewing (1928) postulated the "hemodynamic theory" which challenged the theory of Paget. According to Ewing, formation of metastases is determined by the anatomical position of the target organ in the vascular system indicating that cancer cells lodge in the capillary bed that they encounter. The liver is the first capillary bed for colorectal cancer cells when they are disseminated in the blood stream. When the cancer cells are disseminated in the lymph vessels, they can enter the circulation via the thoracic duct and do not encounter the liver as first capillary bed. Of course, a combination of both is also a possibility. This means that cancer cells can become arrested in the first capillary bed they encounter (Ewing) but only cancer cells that are arrested in the "appropriate" target organ have the capacity to form metastases (Paget).
Early events in metastasis

The mechanisms involved in the first event in metastasis, arrest in the target organ, is still a matter of debate. The mechanism of cancer cell arrest has been addressed in several experimental studies using different experimental approaches. For example, expression of specific adhesion molecules on the cell surface of cancer cells in combination with the presence of their ligands in the target organ has indicated a functional role of integrin-ligand interactions in specific homing of cancer cells (7). On the other hand, hepatic metastasis has been shown to be dependent on the first-pass trapping of cancer cells (8). Moreover, intravital microscopy has shown that cancer cells arrest in rodent liver due to size restriction (9). Cell arrest was shown to be independent of the metastatic capacity of cancer cells. Therefore, other factors besides cell arrest seem to be important for metastasis to occur.

The next step after cell arrest is assumed to be extravasation of cancer cells out of the capillary into surrounding tissue. In this step, cell motility, cell adhesion and secretion of proteases are considered to be essential for the process of extravasation. Cell motility and cell adhesion have been studied in vitro and in vivo. Adhesion of cancer cells to specific substrates in vitro can provide clues which adhesion molecules play a role in metastasis. In vivo experiments have shown that interfering with adhesion by the administration of RGD peptides (peptide sequence recognized by integrins) to the circulation reduces liver metastasis of colorectal cancer (10) but information on the dynamics was not obtained. Intravital microscopy has shown that cancer cells with different metastatic capacities arrest and extravasate equally well in the liver. Furthermore, overexpression of tissue inhibitor of matrix metalloproteinases (TIMP-1), a natural inhibitor of matrix metalloproteinases (MMPs) which are considered to be important in metastasis, does not affect the capacity to extravasate. This implies that post-extravasation events are more important for metastasis than extravasation itself and that MMPs are not involved in the process of extravasation. On the other hand, it was recently shown that extravasation is not a requirement for metastasis because lung colonization was preferentially initiated by intravascularly-growing cancer cells rather than by extravasated cancer cells (11).

Animal models of colorectal cancer

Metastasis formation in animal models is usually studied with end-point assays where tumors are induced, a treatment is given and the effect is scored at a given time. However, the process
of metastasis is a dynamic process including several steps. These steps occur in different stages of tumor development both spatially and temporally. Therefore, treatment may affect tumor development in some steps but not in others and visualization of tumor development in time may elucidate these time-related mechanisms in tumor development. Transfection of cancer cells with marker molecules enables visualization of cancer cells in living animals (12-14). This allows the analysis of development of metastases in time at the single cell level. However, these techniques are invasive and therefore not ideal to follow tumor formation. Noninvasive imaging can in principle be performed with magnetic resonance (MR) imaging, but spatial resolution at the single cell level is still an illusion at present.

Matrix metalloproteinases and colorectal cancer metastasis

The involvement of proteases, and in particular MMPs, in the progression of colorectal cancer and metastasis has been the subject of many studies. In humans, correlations between elevated MMP expression and colorectal cancer progression have been found (15, 16). As a consequence, low-molecular-weight synthetic MMP inhibitors have been developed for the treatment of cancer patients. Animal studies have shown that, in general, MMP inhibitors inhibit cancer progression and metastasis (17-19). On the other hand, induction of metastases by treatment with MMP inhibitors has also been reported in animal models (20). Clinical trials using MMP inhibitors for the treatment of various types of cancer have shown little benefit so far or even did worse than placebo treatment (21). These data indicate that inhibition of MMPs can either inhibit or promote tumor growth. Underlying mechanisms of tumor promotion are yet largely unknown.

Detection of matrix metalloproteinases involved in cancer and metastasis

MMPs are produced and secreted as inactive enzymes and become activated extracellularly, usually by other proteases. Activity of proteases is tightly regulated by pro-enzyme activation and inhibition of active proteases by their endogenous inhibitors, such as TIMPs. There are several ways to study the involvement of MMPs in cancer and metastasis. However, most techniques allow either only localization of the protein and thus do not provide information on their activity or determine activity in tissue homogenates and thus do not provide information whether the activity is localized in tissue regions that are relevant for tumor growth. For a better understanding of the role of MMPs in cancer progression and metastasis, the combination of both, localization of activity, may provide more relevant information. One
of the few techniques available for the localization of proteolytic activity is in situ zymography. The first report on in situ zymography for gelatinolytic enzymes was presented by Galis et al. (22). They used FITC-labelled gelatin that was placed on top of unfixed cryostat sections. Decreased fluorescence was used as a parameter of gelatinolytic activity in situ. However, this often resulted in a poor spatial resolution and low sensitivity. Recently, novel substrates for proteases including MMPs have been introduced that fluoresce after proteolytic cleavage and can be applied to visualize proteolytic activity in situ (this thesis). Application of these substrates has increased sensitivity and spatial resolution because disappearance of fluorescence is less sensitive than formation of fluorescence. Localization of gelatinolytic activity as precisely as possible may contribute to our understanding of its role in tumor progression and metastasis.

**Studies with inhibitors of MMPs**

The most direct way to study the involvement of MMPs in colorectal cancer is by intervention studies in animals. Usually, an animal model is selected that represents the human situation as closely as possible. In such a model, tumors are induced and the involvement of proteolytic enzymes in the development of tumors is studied by administration of MMP inhibitors to the animals (18, 20).

**Aim of the study**

The main issue addressed in this thesis is what processes are involved in tumor development of colon cancer in the liver and in particular whether MMPs are functionally involved in the formation of liver tumors of colorectal cancer. In chapter 2, an experiment of visualization of tumor development with MR imaging in live rats is presented. This study shows that kinetics of tumor development within individual rats can be obtained by imaging rats multiple times. The major advantage of MR imaging is that rats can be imaged in a noninvasive way in time without restriction, and thus tumor growth is affected as little as possible. Furthermore, it provides information additional to end point measurements. To study early events in invasion and metastasis like cancer cell arrest, extravasation and migration, rat colon cancer cells were labelled with GFP and used to visualize these processes in livers of live rats (chapter 3). It is shown that cancer cells are arrested in livers of rats due to size restriction and that these cancer cells do not extravasate but proliferate inside sinusoids, the capillaries of the liver. This implies that MMPs are not necessary in these early stages. Interestingly, early interactions
between cancer cells and hepatocytes were observed instead of interactions between cancer cells and the lining sinusoidal endothelial cells. To identify the interactions between cancer cells and hepatocytes, adhesion molecules were studied (chapter 4). Candidates of the integrin family were identified that may play a role in adhesion between cancer cells and hepatocytes. In chapter 5, methods to detect and localize in situ proteinase activity in general and gelatinolytic activity in particular are reviewed. Detection of gelatinolytic activity in situ was introduced in 1995 (22), but the introduction of quenched fluorescent substrates for gelatinases greatly improved precision of localization and sensitivity of the assay. Localization of gelatinolytic activity in metastases of colon cancer in rat liver was demonstrated with dye-quenched (DQ)-gelatin as substrate (chapter 6). Gelatinolytic activity was localized in these metastases and suggests a role in remodelling of tissue related with tumor development. However, definite conclusions could not be drawn. Previous studies have shown that MMPs in general and gelatinases (MMP-2 and MMP-9) in particular are involved in colon cancer progression and metastasis. However, gelatinases are multifunctional proteins that can result in both tumor promoton and tumor inhibition (chapter 7). Therefore, a selective MMP inhibitor was administrated to rats to investigate the biological role of MMPs in tumor progression (chapter 8). It was found that daily administration of MMP inhibitor reduced the diameters of tumors that had developed at three weeks after their induction. These findings are in line with the findings presented in chapter 6, that suggest a role of gelatinolytic activity in tissue remodelling and thus inhibition of remodelling should slow down tumor growth. However, daily treatment of the rats with the MMP inhibitor did increase the number of metastases that had developed at three weeks after induction by two-fold. It is suggested that MMP inhibition also affects the immune response in early stages of metastasis and therefore, may be responsible for the increased numbers of metastases observed at three weeks after induction. The general discussion (chapter 9) puts the findings described in this thesis into perspective of the present views on processes involved in invasion and metastasis of colon cancer in the liver.

References


