Human Colorectal Cancer:
Experimental Staging and Therapeutics

Kjell Dahl
HUMAN COLORECTAL CANCER: EXPERIMENTAL STAGING AND THERAPEUTICS

Kjell Dahl

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ABSTRACT

Colorectal cancer is the third most common cause of death in cancer worldwide and approximately one million individuals are diagnosed yearly. Surgery is the primary cure, and if that’s not feasible, only palliative therapy remains.

Colorectal cancer disseminates into peritumoral lymphatic vessels and further into the regional lymph nodes. In the lymph nodes the tumor cells may arrest and establish a metastasis, or bypass into the systemic circulation through lymphovenous shunts. A tumor may shed millions of cells daily, but fewer than 0.1% reside in organs. Metastases to regional lymph nodes or more distant organs are of crucial prognostic importance in colorectal cancer and constitute the largest clinical challenge.

The sentinel node is the first draining lymph node to a tumor and may therefore reflect the tumor status of the entire lymphatic field. We have demonstrated the feasibility of identifying the sentinel node in colon cancer with an accuracy of 100% in 30 patients, and in 93.8% the sentinel node was diagnostic for lymph node metastases. The false negative rate in our first study was 16.7%. At follow up after at least 30 months, only patients with lymph node metastases at surgery had died from colon cancer. We believe that the sentinel node technique may improve staging in colorectal cancer, leading to more patients receiving correct prognosis and follow-up.

When tumor cells are arrested in regional lymph nodes they are exposed for the immune system, which has the capacity of eliminating tumor cells. The first place of encounter is the sentinel node. We have studied the immunological function of sentinel node derived lymphocytes in vitro. The lymphocytes from non-metastasized sentinel nodes proliferated dose-dependently upon stimulation with tumor cells, interleukin-2 and antigen presenting cells. Lymphocytes from metastasized sentinel nodes and tumor infiltrating lymphocytes were normally unresponsive to stimuli. We have demonstrated the presence of tumor-reactive lymphocytes with a capacity of killing tumor cells in sentinel nodes.

Despite efficient surgical interventions and the use of modern adjuvant therapy, about half of the colorectal cancer patients will die of disseminated disease within five years of diagnosis. Clearly, there is a need for improved ways of treatment. In our 3rd paper we used in vitro expanded sentinel node derived lymphocytes as adoptive immunotherapy. Following the principles outlined in the 2nd paper, we multiplied tumor-reactive lymphocytes, mainly of T-helper type 1, to high numbers, before giving them back to the patients as an autologous blood transfusion. In all studied sixteen patients we managed to perform immunotherapy after on average 36 days of expansion in vitro. The therapy had no side-effects. All patients responded to the therapy. Out of the nine patients in the study having Dukes’ D disease, four patients achieved complete response with regress of metastases, and two patients had partial responses. The remaining ten patients displayed stable disease. None of the patients in the study received any regular chemotherapy regimens after the immunotherapy. To our knowledge, this is the first study showing therapeutic effects related to sentinel nodes.

The sentinel node technique is established in staging for malignant melanoma and breast cancer and has been evaluated in several other malignancies. Probably, most solid tumors disseminate through the lymphatics. Since it is proven that metastases have the capacity to metastasize and to induce tumoral lymphangiogenesis, we wanted to evaluate the lymphatic drainage from metastases. We identified metastases-draining lymph nodes in all nineteen investigated patients and we named these nodes “metinel nodes”. Analyses demonstrated that the metinel nodes contained tumor-reactive lymphocytes. The metinel node derived lymphocytes proliferated in vitro upon stimulation with tumor homogenate and in nine patients we proceeded and performed adoptive immunotherapy.
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<tbody>
<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytotoxicity</td>
</tr>
<tr>
<td>AJCC</td>
<td>American Joint Committee on Cancer</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>BEC</td>
<td>Blood vessel endothelial cells</td>
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<tr>
<td>CD</td>
<td>Clonal differentiation</td>
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<tr>
<td>CEA</td>
<td>Carcinoembryonic antigen</td>
</tr>
<tr>
<td>Con A</td>
<td>Concanavalin A</td>
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<tr>
<td>CRC</td>
<td>Colorectal cancer</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
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<tr>
<td>FAP</td>
<td>Familial adenomatous polyposis</td>
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<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
</tr>
<tr>
<td>FOLFIRI</td>
<td>Combination chemotherapy including 5-Fluorouracil, leucovorin and irinotecan</td>
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<tr>
<td>FOLFOX</td>
<td>Combination chemotherapy including 5-Fluorouracil, leucovorin and oxaliplatin</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>HER-2</td>
<td>Herceptin-receptor 2</td>
</tr>
<tr>
<td>HNPCC</td>
<td>Hereditary non-polyposis colon cancer</td>
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<tr>
<td>HPV</td>
<td>Human papilloma virus</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat shock protein</td>
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<tr>
<td>IFP</td>
<td>Interstitial fluid pressure</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>LNR</td>
<td>Lymph node ratio</td>
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<tr>
<td>LMVD</td>
<td>Lymphatic micro vessel density</td>
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<tr>
<td>LS</td>
<td>Lymphoscintigraphy</td>
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<tr>
<td>LV</td>
<td>Leucovorin</td>
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<tr>
<td>LYVE-1</td>
<td>Lymphatic vessel endothelial HA receptor</td>
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<tr>
<td>MAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MN</td>
<td>Metinel node</td>
</tr>
<tr>
<td>MOSAIC</td>
<td>Multicenter international study of oxaliplatin/5-fluorouracil/leucovorin in the adjuvant treatment of colon cancer</td>
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<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NK</td>
<td>Natural killer cell</td>
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<tr>
<td>NK T</td>
<td>Natural killer T cell</td>
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<tr>
<td>NSABP</td>
<td>National Surgical Adjuvant Breast and Bowel Project</td>
</tr>
<tr>
<td>PB</td>
<td>Patent blue dye</td>
</tr>
<tr>
<td>PBL</td>
<td>Peripheral blood lymphocytes</td>
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<tr>
<td>PROX-1</td>
<td>Homeobox prospero-like protein</td>
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<tr>
<td>RT</td>
<td>Radioactive tracer</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
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<tr>
<td>SN</td>
<td>Sentinel node</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TAA</td>
<td>Tumor associated antigen</td>
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<tr>
<td>TCR</td>
<td>T-cell receptor</td>
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<tr>
<td>Th</td>
<td>T helper cell</td>
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<tr>
<td>TIL</td>
<td>Tumor infiltrating lymphocytes</td>
</tr>
<tr>
<td>TME</td>
<td>Total mesorectal excision</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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<tr>
<td>TNM</td>
<td>Tumor nodes metastasis</td>
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<tr>
<td>Treg</td>
<td>T regulatory cells</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>X-ACT</td>
<td>Xeloda in Adjuvant Colon Cancer Therapy</td>
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INTRODUCTION

INCIDENCE, PREVALENCE AND SURVIVAL

Colorectal cancer (CRC) accounted for about one million new cases in 2002, representing 9.4% of the world total cancer and ranked fourth in worldwide incidence for men and third for women. The incidence is at least 25-fold higher in the western countries compared to developing countries, with the highest rates among African American men residing in the USA, 72.2/100,000. In general frequency ratio of colon to rectal cancer is about 2:1, slightly higher among women. The overall 5-year survival (for men) with CRC is 65% in North America, 54% in Western Europe, but only 30% in developing countries (India). The overall mortality is about 50%, thus 2.8 million people are alive with CRC, within five years of diagnosis, making the worldwide prevalence only second to breast cancer.

In Sweden the average incidence per year for CRC in 1993-97 was 30.3/100,000 for men, 23.2/100,000 for women, and almost 5500 new cases of CRC are diagnosed yearly. The incidence is slightly increasing, especially for men, but the incidence of rectal cancer is decreasing slowly. The 5-year relative survival in Sweden during 1995-1999 for CRC was about 57% among both genders and it has increased during the last decades.

In Sweden colon cancer is the fourth most common cancer in men and second most common cancer among women. Rectal cancer is ranked as number six and number eight, respectively.

DEFINITION AND ETIOLOGY

Colon cancer is most often an adenocarcinoid neoplasm with invasive growth and metastatic capacity located in the colon whereas rectal cancer usually has similar histopathology but starts in the rectum within 15 cm from the anal verge. The distance should be measured with a regular rectoscope. Although adenocarcinomas in more than 95% of all cases, CRC may be of mucinous adenocarcinoid origin or signet-ring cell cancer and in rare cases other carcinomas, lymphomas or squamous cellular cancer. CRC is often mentioned as one entity, but usually regarded as two separate types of cancers due to differences in treatment and prognosis.

The etiology of CRC is multifactorial, but consists mainly of environmental and hereditary factors. FAP (Familiar adenomatous polyposis), caused by a mutation in the APC-gene, leads to the development of multiple colonic polyps early in life. Patients have a 100% risk of developing CRC and prophylactic surgery indicated with proctocolectomy and pelvic pouch.

HNPCC (Hereditary non-polyposis colon cancer) is caused by mutations within DNA-mismatch-repair systems and carriers have a 70% life-time risk of developing cancer. The number of polyps is less than in FAP and regular colonoscopies with polypectomies often suffice as screening procedure.
Common hereditary CRC (HCRC) is as frequent as FAP and HNPCC, but the genetic causes are so far unknown. The life-time risk of developing CRC is similar to HNPCC, but the disease often presents itself at a higher age. There are large geographical differences in incidence of CRC and migrants moving from low-risk to high-risk areas show rapid increase in incidence. Western life-style factors, supported by strong epidemiological evidence are regarded as the main explanations. There are correlations between development of CRC and high consumption of meat, fat, carbohydrates, but low fiber-intake. Physical inactivity, excess body weight and an abdominal deposition of fat also increase the risk.

SURGERY

Anatomy

Surgery cures most CRC, and when surgery is not feasible only palliative therapy remains. In addition, adjuvant chemotherapy in colon cancer increases the 5-year survival in general by 10% when given to Dukes’ C patients. Preoperative irradiation therapy in rectal cancer patients increases 5-year survival by about 15%. Within CRC surgery there are some standard operations in patients with colon cancer and also regarding patients with rectal cancer. The operations are performed in the same accustomed way due to anatomical and tumor-biological reasons. In short, curative CRC surgery is performed within embryological well-defined planes surrounding colon or rectum, facilitating the mobilization of the bowel and mesentery. Within the mesocolon and mesorectum the draining lymph vessels usually follow the vascular pedicles proximally, and by performing these standardized resections, most regional draining lymph nodes should be removed. This optimized surgery may be curative in about 60% of the patients.

The importance of these principles may be illustrated by the surgery for rectal cancer. Thirty years ago, rectal cancer operations did not follow the embryologic planes and local recurrences occurred in up to 55%, leading to a high mortality. In the 1980s the operation techniques changed dramatically as the total mesorectal excision (TME) technique, meaning a careful dissection in the avascular border of the mesorectum (the holy plane), was found to be superior to the previously used technique. Currently, local recurrences are few, about 6% (also lowered by preoperative irradiation therapy) and mortality has decreased considerably to about 9%. Current discussions concern the possibilities of emerging similar improvements in operation techniques within colon cancer surgery.

Lymph nodes in colorectal cancer

The local lymph node status in CRC is an established prognostic factor and governs the treatment. At diagnosis roughly one-third of all patients with CRC has node-positive disease, one-third has node-negative disease and one-third has distant metastases. The classification of Dukes’ from 1932 has been the most used staging system for CRC and classifies into Dukes’ A to D. Dukes’ A and B defines resectable tumor growth restricted to the bowel wall or adjacent organs, C defines the presence of lymph node metastases and D means metastases to distant organs. The Dukes’ classification has been criticized for being too rough and for not considering the number of lymph nodes in specimens. Thus, the TNM-system was introduced by the
American Joint Committee on Cancer (AJCC), constituting a shorthand notation for the anatomic extent of malignant neoplasm. T stands for tumor growth, representing the size or extent of the primary tumor; N is the presence and extent of regional lymph node metastases, and M is the presence of distant metastasis. Once T, N and M have been classified, the information is used to determine the stage of the tumor. AJCC and Union Internationale Contre le Cancer (UICC) have together introduced a common staging system (based on the TNM system), which describes the extent of a cancer's spread in Roman numerals from 0 through IV.

Relative five-year survival in Dukes’ A (T1-2 N0 M0/stage I) is 90-95%, Dukes’ B (T3-4 N0 M0/stage II) 60-80%, Dukes’ C (T1-4 N1-2 M0/stage III) 40-60% and Dukes’ D (T1-4 N1-2 M1/stage IV) <5%. The most common sites of distant metastases are liver, 33-60%, and lungs, 20-25%. Patients classified as Dukes’ D often have unresectable metastases and an expected average survival of 6-9 months from diagnosis, but in case of resectable metastases, the 5-year survival following liver or thoracic surgery increases to 20-30%. Studies have also shown that surgery for remetastases in selected patients is safe and results in excellent long-term prognosis.

Many studies confirm a positive relationship between the number of examined lymph nodes in a colorectal cancer specimen and prognosis. The number of lymph nodes that must be examined for correct staging of node negativity varies between 10-17. When fewer than eight nodes are examined, the possibility of correct staging, if at all possible, declines significantly.

Another factor restricting staging may be the quality of the pathology. The search for lymph nodes in a specimen may be time-consuming, laborious and expensive work and some patients will suffer from understaging. The prognostic significance and importance of careful and scrupulous histopathological examinations of the specimens are unmistakable and local tradition may be a confounder. Thus, it is important that the surgeon resect a sufficient number of lymph nodes and also that the pathologist retrieve most if not all of them.

In a Swedish study on 3735 patients who had undergone resection of colon cancer, only 19% of the pathology reports fulfilled the recommendations of the Swedish Society of Pathology. However, recent results indicate an increased awareness by surgeons and pathologists, and the numbers of investigated lymph nodes in specimens are consistently rising. Consequently, it is of utmost importance for correct staging that pathologists analyse a sufficient amount of lymph nodes.

**Micrometastases and lymph node ratio**

Despite the efficient surgical and oncological interventions of today, about half of the patients with CRC will die from their disease within five years of diagnosis. Even when radical surgery was performed primarily and no histopathological signs of lymph node metastases or radiological distant metastases existed, the 5-year survival may be only 60% (Dukes’ B). This contradiction may be due to insufficient surgery or pathology, but it may also be due to the presence of micrometastases. A tumor sheds malignant cells into the lymphatic and systemic circulation, which may arrest in regional lymph nodes or in the small capillaries of a distant organ, such as the liver or the lungs, establishing a metastasis. Histopathologically negative lymph nodes draining CRC, and other tumors, may contain a minute amount of tumor cells. Since the
micrometastases consist of only one single cell or minor clusters of malignant cells, they cannot be identified by routine histopathological examinations with hematoxylin and eosin.

In the search for micrometastases in regional lymph nodes, immunohistochemical techniques and reverse transcription-polymerase chain reaction (RT-PCR) have been used and the presence of micrometastases has been demonstrated. The detection of micrometastases may lead to improved staging and better adjuvant treatment, follow-up and prognosis. Liefers et al identified micrometastases by carcinoembryonic-antigen (CEA) RT-PCR in 36 out of 192 (19%) investigated lymph nodes in 14 of 26 patients classified as node-negative. The relative 5-year survival was only 50% in the group of patients having micrometastases versus 91% in the group without micrometastases. Another study, using cytokeratin 20 RT-PCR, showed that 48 out of 141 (34%) histologically node-negative patients had evidence of occult metastases in regional lymph nodes. After a median follow-up of 42 months, the upstaged patients had a 9% significant reduction in overall survival (p< 0.0001). Other studies do not support the prognostic importance of micrometastases.

Contradictory results may be explained by too small and heterogeneous studies. Along with the total number of investigated lymph nodes and the search for micrometastases in a specimen, another related measure is the lymph node ratio (LNR). By definition, LNR is the number of positive metastatic regional lymph nodes divided by the number of examined lymph nodes in the specimen. LNR has been shown to be a prognostic tool in CRC as well as in other malignancies such as gastric cancer or pancreatic cancer. A retrospective study on 20,702 eligible patients with stage III colon cancer subclassified the patients into stage III A, B and C (subclassification of stage III according to the AJCC/UICC system), and the LNR proved to be an independent positive prognostic factor for disease-free survival and mortality in stage III B and C. For patients in stage III B the 5-year mortality was 27% when >13 nodes were negative in the specimen, while the 5-year mortality was 45% when <3 negative nodes were examined. In stage III C the same registrations were 42% and 65%, respectively, but in stage III A no association could be found. Another study demonstrated the associations between LNR and overall survival, relative survival and disease-free survival. After curative surgery with a sufficient amount of resected lymph nodes (10), an as low as possible LNR was shown to be a positive prognostic factor for overall and relative (cancer-specific) survival. The cancer-specific survival when having a LNR >0.4 was 67% for a N1 (1-3 resected metastatic lymph nodes according to the TNM-system) patient versus 79% when the LNR <0.2.

This type of differences is not currently considered in the AJCC staging system, the TNM-system or any other accepted staging system for colorectal cancer.

ADJUVANT CHEMOTHERAPY

Chemotherapy

By the term adjuvant chemotherapy it is understood that some patients despite the absence of remaining visible tumor burdens postoperatively, still have a risk of developing recurrences later on. These observations are likely due to residual cancer existing in occult or microscopic stage and that is the reason for giving patients with
the highest risk of recurrent colon cancer postoperative adjuvant chemotherapy. Since chemotherapy kills dividing cells, it has the capacity of killing circulating tumor cells or minor clusters of tumor cells, or in some occasions also large volume tumors (hypo-sensitive for the drug).

**Dukes’ A**

No studies have been performed to evaluate the role of adjuvant chemotherapy in Dukes’ A colon cancer. Since the 5-year relative survival is 90-95%, such studies must be of huge dimensions to prove statistical significances in survival. In addition, adjuvant chemotherapy is expensive and may have severe side-effects.

**Dukes’ B**

Adjuvant therapy in patients having lymph node negative disease (Dukes’ B) at surgery has been debated since long and is still a controversial issue. There is no convincing evidence for improvement in survival or reduction in recurrence rates and recommends mainly only in high risk patients. This may be due to the fact that the tumor biology in Dukes’ B and C is inherently different or that the studies conducted to prove the benefits of adjuvant chemotherapy in Dukes’ B have been underpowered. To demonstrate a 2.5% absolute improvement in overall survival in Dukes’ B colon cancer with 90% power, more than 10,000 patients must be studied. The first study reporting a significant increase in disease-free survival and overall survival by adjuvant chemotherapy in stage II and III was the NSABP Protocol C-01, although both stages were regarded as one entity.

The International Multi-center Pooled Analysis of B2 Colon Cancer Trials (IMPACT) compared 6 months of 5-Fluorouracil and leucovorin (5-FU/LV) chemotherapy with surgery alone in 1,016 stage II colon cancer patients from five different trials. The absolute reduction in risk of death in patients receiving chemotherapy was only 2%, which was not statistically significant. On the basis of these results, routine use of adjuvant chemotherapy is not recommended for Dukes’ B colon cancer. More recently, Gill et al. presented a pooled data set of 3,302 patients with stage II colon cancer from seven randomized trials comparing 5-FU/LV or 5-FU/levamisole versus surgery alone. A disease-free survival benefit was seen for patients receiving chemotherapy, but no significant overall survival benefit.

The National Surgical Adjuvant Breast and Bowel Project group (NSABP) analysed outcome data from four of their trials (C-01 to C-04) comprising more than 1,500 stage II patients in total. Adjuvant therapy reduced mortality by 30%. Subgroup analysis revealed best benefit of adjuvant therapy in the poor prognosis groups of T4 tumor stage and patients with obstruction or perforation. Tumors in Dukes’ B disease presenting with perforations, obstructions or with poor differentiation are defined as high-risk tumors, with a higher risk of recurrences and are associated with worse prognosis. Several studies show clinical benefit of adjuvant chemotherapy in those patients.

A recent meta-analysis on adjuvant therapy for stage II colon cancer reported data for 4,187 patients. The reduction in mortality for treated patients was approximately 13% but the result just failed to reach significance.
The use of adjuvant chemotherapy for Dukes’ B patients varies according to differences in routines between countries and physicians, but are not in practical use, with the exception of a wider use in high-risk tumors in node-negative patients. In 2006 the Swedish National Board of Health and Welfare presented a ‘state of the art’ document on CRC and concluded that regimens based on 5-FU decrease the risk of recurrences and death with 3-4% by adjuvant chemotherapy in Dukes’ B patients.57

Dukes´ C

Before Moertel et al presented their trailblazing study on chemotherapy in colon cancer in 1990,58 the general consensus was that the best standard treatment was surgery alone. Many studies had been performed evaluating various forms of immunotherapy and chemotherapy, mainly based on 5-FU either alone or in combination with other agents, but none had showed any convincing evidence of clinical benefits.59 Moertel et al demonstrated that the combination of 5-FU and levamisole given in an adjuvant setting to patients surgically treated for Dukes’ C disease, had a reduced risk of recurrences and an improved overall death rate. The year before, they also proved that the combination of 5-FU and Levamisole improved survival and reduced tumor recurrence in patients having colon cancer Dukes’ C.60

The study had a great impact and the American National Institute of Health (NIH) issued a consensus that all patients staged Dukes’ C should receive adjuvant chemotherapy unless medical or psychosocial contra-indications existed.61 Following long term results, with a median of 6.5 years, the reduction in recurrences by 40% and overall death rate by 33% was maintained.52

The 5-FU based chemotherapy has thereafter been evaluated in combination with levamisole or LV (folinic acid) in several studies,63-65 demonstrating a relative reduction in mortality of about 20-30%. The absolute reduction in mortality is about 10%, leading to it that the roughly expected 5-year survival in Dukes’ C disease has improved from 50% to 60%.

In the Quasar study almost 5000 patients with Dukes’ B and C colorectal cancer were randomized to 5-FU/ high-dose LV, 5-FU/low-dose LV or 5-FU/placebo or levamisole regimens. All three groups demonstrated a similar three-year survival of 70%. Patients treated with levamisole had worse survival than placebo.66 The authors recommended future trials of chemotherapy versus no chemotherapy to show if both treatments were equally effective or ineffective. Randomized studies demonstrate that LV in combination with 5-FU during six months is as efficient as the combination with levamisole during 12 months67 and that both groups have similar toxicity. Thus, the current standard is the combination of low-dose LV and 5-FU in a weekly schedule or on five consecutive days every 4 weeks, during six months.

Two large randomized studies, X-ACT68 and NSABP-CO6,69 have demonstrated that capecitabin (per oral 5-FU) is equal in efficiency to intravenous 5-FU and has lower toxicity.

In the MOSAIC study, oxaliplatin was added to the combination of 5-FU and LV (FOLFOX).70 The rate of disease-free survival at three years was 78.2 % in the group given oxaliplatin versus 72.9% in the group given only FL (p=0.002).
The combination of the topoisomeras 1 inhibitor irinotecan and 5-FU/LV (FOLFIRI) has not achieved the same use as FOLFOX due to lack of convincing clinical evidence. The studies performed regarding the use of adjuvant chemotherapy within rectal cancer have in general been small and often in combination with irradiation therapy. Some of the studied drugs are no longer in use, and above all, operation technique has improved dramatically. The studies performed were based on 5-FU regimens and there are no current studies concerning the use of the new drugs irinotecan or oxaliplatin.

**Dukes’ D**

For patients with disseminated disease (Dukes’ D) only palliative treatment remains, unless curative surgery can be performed, and the expected median survival is 6-9 months from diagnosis. Today, no facts support that chemotherapy alone cures Dukes’ D patients. Since the 1950s the dominating chemotherapy regimen has been 5-FU based and after the addition of LV, optimal scheduling has rendered an average of 12 months survival, equal to the best supportive care of the time.

During recent years, the introduction of oxaliplatin and the topoisomeras 1 inhibitor irinotecan have lead to new combinations of drugs. Infusional irinotecan in combination with 5-FU/LV (FOLFIRI) has been proved to be superior to the previous 5-FU/LV regimen, improving overall survival by a further 2-3 months. The combination of infusional 5-FU/LV and oxaliplatin (FOLFOX) has the same effect on recurrent disease, time to progression and median survival as FOLFIRI. The combinations have similar grades of toxicity, but slightly different symptoms, and prolong the median survival to 14-16 months. They are both approved as first-line therapy in otherwise healthy patients. In case of failure in one therapy, a switch to the other combination of chemotherapy, no matter what sequences, may further prolong the median survival to slightly more than 20 months in total.

Peritumorally there is an angiogenesis, stimulated by preferentially vascular endothelial growth factor A (VEGF-A) produced by the tumor, and 80% of all tumors also express the epidermal growth factor receptor (EGFR). New ways of targeting therapies have contributed to the creation of monoclonal antibodies (MAbs) towards VEGF-A, bevacizumab, and EGFR, cetuximab. The use of bevacizumab in addition to a combination of irinotecan and 5-FU/LV (IFL) prolongs the median survival significantly from 15 months to more than 20 months, compared to IFL alone. The combination of bevacizumab and FOLFOX has been reported to improve median survival from 10.7 months to 12.5 months.

The combination of Cetuximab and irinotecan given to irinotecan refractory patients, has shown prolonged disease-free interval of nearly three months.

It must be kept in mind that the more extensive combinations of chemotherapeutical drugs are given to patients, the more side-effects may ensue. Chemotherapy may also be used as an adjuvant for down-staging of e.g. metastases to the liver or lung, making primarily unresectable Dukes’ D patients curable. Interestingly, prognosis in patients undergoing surgery after tumor shrinkage by neoadjuvant therapy is similar to those with primarily resected metastases.
LYMPHANGIOGENESIS

Anatomy and physiology

The clinical observation that metastases to regional lymph nodes or distant organs are of crucial prognostic importance in cancer disease was made as early as during the 19th century.²⁰

As a tumor grows in size, it requires an increased peritumoral angiogenesis and lymphangiogenesis. Tumor cells may theoretically spread by lymphogenous or hematogenous routes into the systemic circulation and disseminate the disease.⁸⁵, ⁸⁶

During many years the lymphatic system and its role in tumor development was relatively unknown, much due to the difficulties in discriminating between blood vessels and lymphatic vessels. It is only in the past decade that molecular markers have been detected that help us differentiate between lymphatic endothelial cells (LEC) and blood vessel endothelial cells (BEC). The markers Podoplanin,⁸⁷ LYVE-1⁸⁸ and Prox-1⁸⁹ are identified almost exclusively on LEC.

When a tumor increases in size, the intratumoral pressure rises and potentiates the opportunities of the cancer cells to migrate out of the tumor. The interstitial fluid pressure (IFP) is lower than the pressure inside the tumor and therefore the hydrostatic pressure may transport malignant cells into the extracellular matrix (ECM). The intratumoral lymphangiogenesis facilitates the passive entry of tumor cells into the lymphatics, not dependent on osmotic pressure.⁹⁰

Cytokines secreted by tumor cells or by host cells and stroma, initiate the lymphangiogenesis.⁹⁰ The secreted cytokines are glycoproteins of the vascular endothelial growth factor (VEGF) family and an established signalling pathway is via VEGF-C and VEGF-D and the VEGF receptor 3 (VEGFR-3).

Lymphatic capillaries consist of low-pressure, highly permeable vessels with thin walls, and they are like cul-de-sacs. The LEC differ from the BEC in that, among other things, they lack a basement membrane. Their inter-cellular junctions are less tight than in BEC, due to their connection to the surrounding ECM by specialised fibrillin-containing anchoring filaments. At increased IFP, the filaments exert tension on the LEC, thereby widening the capillary lumen and opening the intra-cellular junctions. This process allows for the passage of fluids, molecules and cells into the lymphatics. The lymphatic flow is directed into collecting lymphatic vessels and further into the afferent vessels of the lymph nodes.

In the lymph nodes the tumor cells are exposed to antigen presenting cells and the cells of the humoral and cellular system (the consequences of this will be discussed in the following text). The cells may arrest and invade the cortex of the node to form a regional lymph node metastasis or bypass the node into the systemic circulation. A lymph node has lymphovenous shunts into the systemic blood circulation and about half of the lymph flow entering a node continues into the blood circulation.⁹¹ The other half of the lymph flow leaves the node in efferent lymphatic vessels to the following lymph nodes and vessels. Finally, the minor lymphatic trunks lead into major lymphatic trunks (thoracic duct and right lymphatic duct) which converge in the left or right subclavian vein, respectively.

Clinical relevance of the lymphangiogenesis

Several studies support the importance and clinical relevance of the lymphatic system in cancer disease and the existence of peritumoral lymphangiogenesis have been demonstrated, initiated by a primary tumor or a metastasis.⁹², ⁹⁶
Kawakami et al demonstrated, in a study on 24 human CRC specimens, a significant relationship between the levels of VEGF-C mRNA in the primary tumors and lymphatic invasion.93 Two-thirds of the specimens showed an overexpression of VEGF-C mRNA in the tumors and of VEGFR-3 in the stromal tissues surrounding the cancer cells and the higher their levels, the more signs of lymphatic invasion and lymph node metastases. Similar results were achieved by Akagi et al who in 99 primary tumors and 18 metastatic lymph nodes from CRC patients examined VEGF-C mRNA expression. The expression of VEGF-C correlated with lymphatic involvement, lymph nodes metastasis and depth of invasion. Survival time was shorter for VEGF-C positive groups than for VEGF-C negative ones, but with no statistically significant difference.94

White concluded that patients operated on for colon cancer had an overexpression of VEGF-D, but not its receptor VEGFR-3, and it correlated with a decrease in disease-free interval and survival.95

Recently, a Chinese study investigated the correlations of lymphatic microvessel density (LMVD) and expression of VEGF-C in relation to the development and prognosis of colon cancer in 44 patients.96 VEGF-C was overexpressed in 43.2% (19/44) tumor specimens, but its expression correlated significantly to lymph node metastases and Dukes’ classification. Increased LMVD correlated to an increase in lymph node metastases and to a more advanced Dukes’ stage. Patients whose tumors overexpressed VEGF-C or had increased LMVD, had a significant (p= 0.0225 and p=0.0036) decreased survival.

THE SENTINEL NODE CONCEPT

Background to the sentinel node technique

The sentinel node (SN) concept implies that the lymphatic drainage from a primary tumor first drains to a specific regional lymph node and that the tumor status of this unique lymph node (sometimes more than one) reflect the status of the entire regional lymphatic field.

An SN was first described by Gould in 1960,97 without the use of any tracers, in a patient with carcinoma of the parotid gland. The observation was used again, 17 years later, by Cabanas98 in penile cancer, the first to perform lymphangiograms to identify the SN. The main breakthrough for the SN concept was in 1992 when Morton et al99 demonstrated that the SN technique in patients with early stage melanoma with a high degree of accuracy, false-negative rate of less than 1%, identified patients likely to benefit from radical lymphadenectomy. In 1995 Giuliano et al presented a study to evaluate the SN concept within breast cancer.100 They demonstrated that analyses of the SN predicted the axillary nodal status in 109 of 114 cases (95.6%). Thereafter the SN technique became routine in primary surgery for malignant melanoma and breast cancer. Alex and Krag introduced the use of lymphoscintigraphy (LS) and they were the first to inject radioactive tracer (RT) to identify the SN in patients with malignant melanoma.101, 102 This made a non-invasive procedure possible in which the use of a hand-held gamma-probe or preoperative LS identified the SN with equal precision to blue dye.

The SN technique has also been evaluated in gastrointestinal malignancies103 such as CRC104 or gastric cancer,105 but also in urinary bladder cancer,106 gynecological malignancies,107 thyroid cancer,108 anal carcinoma,109 prostate cancer,110 lung cancer111 and in other tumor types.

The SN technique has mainly been used as a diagnostic tool to improve staging and to direct the extent of local lymph node dissections. Presumably, most solid tumors first
spread through the sentinel node(s) and probably enter the systemic circulation via lymphovenous shunts present in these lymph nodes.\textsuperscript{112, 113} Thus, for staging purposes it may be of value to evaluate the sentinel node status in most solid tumors.

\textbf{Sentinel node in colorectal cancer}

The first study of the SN concept in CRC was presented at the Society of Surgical Oncology, San Diego, in 1997.\textsuperscript{114} 44 Patients were included, most of them having colon cancer, and 1 mL lymphazurine was used as tracer. The first 1-3 blue-colored nodes that were detected in the mesentery were defined as SN and identified in 98\% of all cases. Twelve patients had lymph node metastases and two of them had negative SN, giving a false negative rate of 16.7\% (2/12). The authors concluded that the technique was feasible and of potential clinical value.

The following study showed contradictory results. Fifty patients were included, mainly with colon cancer, and 1-2 mL of Patent Blue dye (PB) was injected at four sites close to the tumor. SN were identified in 70\% of the cases. Out of these 35 succeeded cases, 20 patients had lymph node metastases at surgery and in 12 of them the SN was negative for metastases, giving a false-negative rate of 60\%. The authors concluded therefore that the concept of lymphatic mapping and SN identification was not valid for colorectal cancer. However, due to initial technical problems, such as intraluminal injection of PB or to the fat clearance technique (which dissolved the ink), there are several objections against this conclusion.

Since these two first studies were published, about 20 studies have been presented regarding CRC, with use of different techniques and sometimes with inconsistent results. Most of the studies dealt with patients with colon cancer or patients with a mixture of colon and rectal cancer patients.

Few studies have exclusively involved patients with rectal cancer, probably due to the combination of the difficulties in injecting the tracer endoscopically and the fact that the preoperative irradiation diminishes the success rate in SN mapping.\textsuperscript{115} The studies performed have not shown any conclusive results favouring the staging technique in rectal cancer.\textsuperscript{116-118}

\textbf{TUMOR IMMUNOLOGY}

\textbf{Background}

In 1891 an American surgeon, Dr William B Coley, discovered that patients who developed bacterial infections also demonstrated spontaneous tumor regressions. While studying journals, he noted that one patient, having inoperable bone cancer, had suffered from severe erysipelas at his death-bed and two weeks later had walked out of the hospital. He started inoculating patients with streptococci and the first treated patient developed erysipelas and experienced total spontaneous regression of his cancer of the tonsils. Over the years his method was improved and he achieved remarkable results within e.g. giant cell bone tumors and breast cancer where 79\% and 65\% of the inoperable patients were alive after five years. In 1943 it was discovered that the lipopolysaccharide (LPS), which occurs in the cell walls of gram-negative bacteria, was the biologically active substance in Coley's toxins. Since then, a lot of research work has been done on Coley's toxin, but it has not yet been applied in any clinical praxis.
The role of the immune system within cancer disease was rediscovered in the late 1950s when Burnet and Thomas introduced the cancer immunosurveillance theory, claiming that lymphocytes were responsible for eliminating continuously arising, nascent-transformed cells. The theory was debated and argued within the following years, much due to the lack of obvious experimental evidence supporting the idea. Despite many studies, none could demonstrate that immunodeficient mice were more susceptible to tumors than wild type. It has since then been shown that nude, athymic mice are not totally immunoincompetent, because they have a nonthymic-dependent T cell populations and functioning humoral immune system. In addition, the studies performed often had too short a monitoring period to detect spontaneous tumor formation.

In the past years, improved mouse models and observations in humans have provided convincing and strong evidence supporting the importance of the immune system in cancer. Tumors have been provoked in mice by carcinogens, amputated and dead tumor cells thereafter being inoculated into healthy mice. These immunised mice could thereafter reject living tumor cells that were injected (which non-immunised mice could not). Immunodeficient patients have an overrepresentation of virus-induced malignancies, e.g. HIV-patients and Kaposi's sarcoma, but also an increased probability of developing cancer of non-viral etiology. Cardiac transplant patients have been shown to have a 25-fold higher prevalence of lung tumors than in the general population and Birkeland has shown an increased risk of developing cancer after renal transplantation.

Immunoediting

Elimination

Large amounts of facts support that both the cellular as well as the humoral immune system develop immune responses against tumors. The human MAbs used in HER-2 positive breast cancer patients, trastuzumab, is now a standard cure of treatment and exemplifies the importance of the humoral immune system. The fact that tumor infiltrating lymphocytes (TIL) are a positive prognostic factor for survival in patients with colon cancer and ovarian cancer illustrates the importance of the cellular immune system.

In recent years the term of cancer immunoediting has been introduced and should be considered as an elaboration on the immunosurveillance theory. It consists of three parts; tumor elimination (i.e. the former immunosurveillance), equilibrium and escape, thereby also embracing aspects on why immunocompetent individuals still develop cancer. The first line of defence against the development of uncontrolled cellular changes in progression towards a tumor, constitutes of the intracellular intrinsic system and represents an abundance of mechanisms striving to cell repair, senescence and apoptosis. When the intrinsic system fails and a malignant cell is transformed the extrinsic system, consisting of mechanisms regulating the intercellular relationships and the regular immune system, enters. Its entrance is triggered by local tissue disruption and inflammation, leading to an induced production of proinflammatory molecules and cytokines. There is a rapid turnover of cells in a tumor, lack of oxygen and nutrition, causing a hostile environment, stimulating antigen presenting cells (APC) like dendritic cells and macrophages, to migrate to the place of the transformed cells. The APC are attracted by 'danger signals' such as heat-shock proteins (HSP, released as a result of tumor-cell damage or necrosis), extracellular breakdown products or proinflammatory factors (cytokines released by tumor cells or other cells). The APC are able to phagocyte debris from tumor cells to produce cytokines, such as e.g. interleukin-1,
TNF-α or GM-CSF, thereby promoting their own function and attracting other cells, such as the NK cells.

NK cells, NKT cells and $\delta^+\gamma^+$ T cells are also recruited to the “danger site”, recognizing molecules such as the stress ligand for NKG2D (induced on tumor cells by inflammation or transformation). NK cells preferentially kills tumor cells lacking the expression of MHC I on their cell surfaces. NKT cells can be defined as NK cells carrying a T cell receptor (TCR).

$\delta^+\gamma^+$ T cells are CD4$^+$ CD8$^-$ T cells representing about 10% of all T cells, although having a controversial function. They consist of preformed clones towards a few specific tumor associated antigens (TAA), producing perforin and antitumor cytokines and they mediate cytotoxicity when recognizing TAA. They are considered to be of importance in the early activation of the cellular immune system and possess an ability to kill tumor cells.

NKT cells and T cells recognize the tumor development through expression of TAA on MHC molecules or by direct interaction with their TCR, followed by cell destruction of the tumor cells and production of cytokines. These cytokines control tumor growth and amplify the immune response by several mechanisms including interferon-γ (IFN-γ) secretion which intensifies the immune response and recruits more cells.

The APC phagocytose debris from tumor cells and then migrate via the lymph vessels to the first draining lymph nodes, the SN. In the lymph node APC present the ingested antigen on their MHC class-II molecules to CD4$^+$ T cells. An infinite amount of different types of TCR exists, able to recognise different types of antigens, some of them will recognise the antigen presented by the APC, and may be defined as tumor-reactive lymphocytes. CD4$^+$ Th 1 cells promote the activation of CD8$^+$ T killer cells and thereafter both cell types migrate to the place of the tumor to become TIL. The CD8$^+$ cells recognise the tumor and kill them by direct and indirect mechanisms. The CD4$^+$ cells produce IL-2 that maintain the function of the CD8$^+$ cells and in addition they will initiate the humoral immune response.

Equilibrium

A tumor is heterogeneous and may contain thousands of different mutations, leading to a perpetual challenge for the immune system. Equilibrium refers to that sometimes not all tumor cells are being killed during the elimination phase and an equilibrium of tumor cell growth and killing may be formed in the tumor. The continuously newly created mutations entail continual adjustments in the immune defense leading to the creation of mutations that the immune system does not conquer. This phase is probably the longest of the three phases and has been estimated to persist up to 20 years in humans. Finally the development of tumoral changes leads to that the cancer gets the advantage of the immune system and a cancer tumor becomes clinically detectable and thereby the third phase is initiated.

Escape

There are several ways for tumor cells to escape the humoral and cellular immune system. The selection of mutations with capacity of avoiding the immune system may occur due to the intratumoral development in itself, but also secondary due to a state of immunosuppression in the individual. The tumor conquers both the innate and adaptive part of the immune system due to both intrinsic and extrinsic factors.

CRC cell lines have been reported to produce immunosuppressive factors and to express indoleamine 2,3-dioxygenase (IDO), an enzyme which catalyses tryptophan...
degradation, causing T cell proliferative arrest. Other ways for tumors to avoid the immune system may be by physical exclusion of immune cells from tumor sites, reduced expression of MHC I molecules or costimulatory proteins and disruption of natural killer (NK) and natural killer T (NKT) cell recognition. Tumors may secrete cytokines like VEGF and IL-10, that interfere with dendritic cell, antigen presenting cells, activation and differentiation, or by blocking the production of inflammatory molecules by expressing the STAT 3 protein. In cases of initiated immune responses tumors may loose targeting antigens, rendering tumor-reactive lymphocytes anergic, inducing regulatory T cells or specifically deleting responding T lymphocytes. The tumor that finally emerges is poorly immunogenic and immune-resistant, consistent with the escape mechanism of the immunoediting theory.

IMMUNOTHERAPY

The aim of immunotherapy is to stimulate the natural immunity of the patient with his or hers own tumor antigens or with exogenous antitumor agents. The identification of tumor antigens has created new possibilities for tumor immunotherapy and many immunotherapeutic approaches are now being translated into clinical trials. Both the humoral and the cellular immune system may be used in immunotherapy by e.g. passive administration of monoclonal antibodies or by adoptive cell-therapy, but gene-therapy and the single use of cytokines has also been described. The use of interferon-α always lead (100%) to a complete regress of papilloma virus-induced tumors of the vocal cords, and it has a significant positive effect in the treatment of other tumors, such as hairy cell leukemia (80% CR) and some lymphoma (50%).

Humoral immunotherapy

Monoclonal antibodies

The humoral immune system may be stimulated by the passive transfer of antibodies, MAbs, or by enhancing B cell responses in vivo, i.e. by vaccines, promoting antitumor activity. Administration of vaccines activates B-cells to produce antibodies towards antigens which, like the passive administration of MAbs, leads to an increased antibody-dependent cell-mediated cytotoxicity (ADCC), interfering with tumor cell growth and stimulating APC. In addition, vaccines also stimulate APC, leading to the activation of the cellular immune system and the cytotoxic T cells. Human MAbs are established within treatments for hematological malignancies, but are also approved for the use in solid tumors. Within hematological malignancies there are MAbs for different types of leukemia and one of the most widely used MAbs in general, rituximab, is used in Non-Hodgkin’s lymphoma. HER-2 is a glycoprotein and member of the EGFR-family, which is overexpressed in 25-30% of all breast cancer. The use of a recombinant humanized MAb towards HER-2, trastuzumab, has been shown to reduce the risk of death by 20% in phase III trials in metastatic breast cancer. MAbs for use in solid tumors are bevacizumab and cetuximab, respectively for disseminated metastatic CRC.
Vaccines

Vaccines are available for many diseases like polio or the measles, but not for others like hepatitis-C, HIV or cancer. Cancer-vaccines are directed against defined TAA, but despite extensive research within the field, results are disappointing. For achieving a maximal response of the administered vaccine, there is a need for a simultaneously given adjuvant, creating an inflammatory condition stimulating the general immune system to enhance the effect of the vaccine. The adjuvant may consist of aluminum derivates, bacterial toxins or cytokines like IL-2 or GM-CSF.

Recently a vaccine against cervical womb cancer has been introduced, but in fact, it is an antiviral vaccine that protects against human papilloma virus (HPV) infections, when given prophylactically. Cervical cancer is the second most common cancer in women worldwide and is caused by HPV-infections in 70%. The vaccine has been proved to reduce the degree of HPV infections and thereby lower the incidence and mortality in cervical cancer.\(^\text{142}\)

The difficulties in providing a functioning vaccine for cancer are likely to depend on several causes. Cancer patients are often in a bad general condition and immune suppressed. Cancer cells are initially derived from normal cells, which lead to a certain amount of autoimmunity in the reaction towards tumors, which may be manifested as development of vitiligo in some malignant melanoma patients. Tumors may escape from vaccines by effects of T regulatory cells (Treg) or by down regulating of MHC-I molecules on their cell surfaces.

Humoral cellular therapy

Another humoral strategy, not being based on antigen-driven restimulation, is the autologous transfer of lymphokine activated cells (LAK-cells). LAK-cells are NK cells being cultured in vitro by stimulation of IL-2, thereby being transformed into a more potent killer cell and their action are effected by ADCC. The LAK cells are infused back into the patient with or without supplementary high doses of IL-2.

Rosenberg et al demonstrated in 1985 that LAK cells, generated from multiple leukaphereses, resulted in objective regression of cancer in 11/25 patients.\(^\text{143}\) One patient with disseminated malignant melanoma obtained complete tumor regression. Out of 8 treated patients in the study with colon cancer only 2 showed partial regression of metastases.

Two years later Rosenberg started treating the patients with high-dose IL-2 in two cycles until high-dose toxicity was reached. This administration of high doses of IL-2 to expand and activate NK cells entirely in vitro, has yielded marked anti-tumor activity and complete remission in a subset of patients.\(^\text{144}\) Severe fluid retention and transient dysfunctions in multiple organs were apparent and four treatment-related deaths occurred at the start of the series.\(^\text{145}\) Long-term follow-up results were presented\(^\text{146, 147}\) with complete regression in 7-8% of patients with metastatic renal cancer or melanoma.

Cellular immunotherapy

Adoptive therapy

The majority of TAA are intracellular proteins and thereby not available for the treatment with MAbs or vaccines, leading to the necessity of adoptive immunotherapy.
Besides vaccines or antibodies blocking CTLA-4 (a potent negative regulator of T-cell activation), T-cell immunity can be augmented by adoptive transfer of tumor-reactive T-cells, cytokines or genetically modified T-cells. In a study of 20 patients with metastatic melanoma Rosenberg extracted TIL from the tumor itself or metastatic lymph nodes. TIL were cultured in vitro and given back to the patient in combination with high-dose IL-2 and cyclophosphamide. The results were promising and a subsequent study demonstrated that treatment with TIL and IL-2 in combination with cyclophosphamide resulted in objective responses in about half of the patients with metastatic melanoma. This caused lymphodepletion is considered to eliminate Treg cells, create depletion of endogenous cells competing for activating cytokines (cytokine sink) and increase the function and availability of APC, and hereby promoting the immune response.

Constructive adoptive therapy

In the strive for the optimal T-cell response in cancer several different techniques have been applied. TCR derived from TILs have been identified, cloned and transfused in the PBL having some effect, and are currently being clinically evaluated. Other studies have identified and generated high-affinity TCR in mice, produced lentiviral transduced T cells preselected for specific markers or created chimeric receptors with antibody specificity. Ultimately, the TCR gene-therapy approach might hold the key to the wide-spread application of ACT-based therapy to the treatment of cancers of multiple histologies.

IMMUNOTHERAPY IN COLORECTAL CANCER

Monoclonal antibodies

The only clinically approved immunotherapy for CRC is the use of MAbs. The first reported results for the use of MAbS within CRC concerned the murine anti-17-1A MAb antibody, edrecolomab, in Dukes’ C colon cancer. Treatment seemed very promising, with a 5 year reduction of overall death rate by 30 % and a decrease in recurrence rate of 27%, but later analyses in a large randomized study revealed no greater efficacy than standard adjuvant chemotherapy treatment. Today edrecolomab still has not showed any significant clinical benefit in the treatment of CRC. The MABs of cetuximab and bevacizumab are both approved for use in metastatic CRC, for details see paragraph “Adjuvant chemotherapy”.

CEA is expressed by colon cancers and trials have been conducted to create a MAbs against CEA. In a study of 23 patients with advanced CRC 13 generated true anti-CEA responses, yet without clinical responses.

Vaccines

Other attempts to stimulate the innate immunity have been performed by active specific immunotherapy using different types of vaccines. No vaccination regimen can be currently recommended outside clinical trials, but immunological findings and observations within animals and humans have stimulated researchers. A vaccine based on an antibody towards CEA promoted clinical response dramatic tumor regression in 2/12 patients with colon cancer, and three patients demonstrating
minor responses. However, in some studies vaccines based on CEA antibodies in combination with 5-FU or based on CEA proteins, did not show any clinical responses.

Administration of a vaccine consisting of autologous tumor cells and an adjuvant immunomodulating agent, bacillus Calmette-Guérin (BCG), given in combination, several times, to 98 patients with colorectal cancer, did not show any statistically significant differences in survival but a small decrease in recurrence rate in Dukes’ B. Further studies were performed but no statistical clinical benefit in disease-free interval or survival could be recorded, not even when combined with 5-FU/LV. In addition to these vaccine studies, a large amount of studies have been performed, none of them showing any convincing results. In an adjuvant setting one study demonstrated significant clinical benefits of a vaccine in combination with BCG.

Studies concerning combinations of chemotherapy and active specific immunotherapy are increasing and the GOLF-study (gemcitabin, oxaliplatin, LV, 5-FU + GM-CSF + IL-2) demonstrated 70% objective response rate in 15 patients.

Adoptive therapy

Studies of TIL in human CRC has shown that their presence in tumors are a positive prognostic sign and that TIL from CRC, expanded in vitro in IL-2, display cytokine secretion in response to autologous tumor cells. However, TIL from CRC display reduced levels of CD3 zeta chain and T-cell responses to mitogenic stimulation are decreased in lymphocytes from venous blood draining CRC, as compared to arterial blood supplying the tumor. Thus, maybe TIL are not the optimal source of immunotherapy, and other studies have shown that the activation of naïve T-cells occur within secondary lymphoid organs, such as lymph nodes.

Marits et al have demonstrated the presence of SN derived tumor-reactive lymphocytes stimulated by urinary bladder cancer, which proliferated dose-dependently in vitro upon stimulation with tumor homogenate.

Adoptive transfer of in vitro expanded lymph node cells in human CRC was performed in patients having metastatic cancer. By the use of a radiolabeled MAb towards mucin, expressed by the tumor, draining lymph nodes with tumor cells or debris were identified. Following immunotherapy, one patient achieved a partial response, four patients minor responses and an overall increase in survival was observed.
AIMS OF THE THESIS

Despite optimized staging, surgery and modern ways of adjuvant therapies, about half of all patients diagnosed with colorectal cancer will die within five years. Clearly, there is a need for improved staging and therapeutics. We aimed to investigate new ways of staging and treatment by:

- Investigating if it was possible to find sentinel nodes in patients with colon cancer, if these were diagnostic for the entire regional lymphatic field and if they were associated with survival.

- Analysing functional immunology in sentinel nodes in patients with colon cancer, specifically presence of tumor-reactive lymphocytes and expansion of T cells \textit{in vitro}.

- Exploring the possibilities of using \textit{in vitro} cultured sentinel node acquired lymphocytes as treatment for patients with disseminated or high-risk colorectal cancer.

- Examining if it was feasible to find first draining lymph nodes to metastases, from metastatic colorectal cancer and from other solid tumors, to see if these lymph nodes contained tumor-reactive lymphocytes to use in adoptive immunotherapy.
MATERIALS AND METHODS

IDENTIFICATION OF SENTINEL NODES IN PATIENTS WITH COLON CANCER (PAPER 1)

Patients
The first study included 30 patients with colon cancer, 13 women and 17 men. Mean age at diagnosis was 70 years. Three patients were operated due to a primary colonic tumor in caecum, four patients due to a tumor in colon ascendens, three patients to tumors in colon ascendens and three patients had surgery because of tumors in the transverse colon. Three patients had primary tumors in the splenic flexure, four had primary tumors in the descending colon and thirteen patients had sigmoid tumors. Only patients without intraoperative signs of distant metastases or lymph node involvement were included. The study was approved by the local ethical committee and informed consent was given by each patient.

Methods
The part of the colon with the tumor was mobilized through division of lateral peritoneal adhesions to facilitate inspection of the tumor and the mesentery. Tracer, either both Technetium-albumin (Albu-Res) 50MBq/mL and PB (Guerbet, Paris) (six cases) or PB alone (24 cases), were injected into the bowel wall subserosa around the tumor using a 27-Gauge needle. The total amount of RT or dye was 1 mL. Nodes in the mesentery that turned blue within 10-15 minutes were defined as SN and marked with sutures (Fig. 1). Resection of the large bowel with tumor followed standard routines except in cases with SN located outside normal resection area (see below). The specimen was fixed in formalin. The location of the SN was specified. Routine histopathological investigation was performed with hematoxylin-eosin staining on 10 µm sections taken at multiple levels of the sentinel nodes and other non-SN. Tumor status in SN was compared with the status in all other harvested regional nodes for each patient. Patients were followed at regular visits for a minimum of 30 months.

SENTINEL NODE LYMPHOCYTES: TUMOR-REACTIVE LYMPHOCYTES IDENTIFIED INTRAOPERATIVELY FOR THE USE IN IMMUNOTHERAPY OF COLON CANCER (PAPER 2)

Patients
Fifteen patients with colon cancer and no preoperative signs of metastatic disease were studied. There were 7 men and 8 women, with an average age of 71 years. One patient was operated because of a primary tumor in caecum, six patients because of tumors in colon ascendens and one patient in the study because of a tumor in the transverse colon. Two patients had surgery due to tumors in the descending colon and five patients due to sigmoid tumors. The study was approved by the local ethical committee and informed consent was given by each patient.
Fig. 1. Intraoperative identification of sentinel node(s). Identification of sentinel nodes was performed by subserosal injections of Patent blue dye at four sites around the tumor (A). Usually within five minutes, one or more blue-colored lymph nodes appear in the mesentery (B).

Methods
The colonic tumor site was mobilized through division of peritoneal adhesions to facilitate inspection of tumor and mesentery as outlined in the first study. One mL PB was injected subserosally and peritumorally in order to find the SN. After surgery SN and non-SN were immediately put on ice and taken to the laboratory, also one piece of the tumor (including the invasive margin) and peripheral blood (PBL) were handled similarly. Samples from the central and the peripheral part of the nodes were dissected for flow cytometry (FACS) and proliferation analyses, and remaining parts of the nodes underwent routine histopathological examination. The tumor specimen was analyzed by flow cytometry and above all, after being transformed into a homogenate mixture, used as an antigen source for the expansions. PBL were analyzed using flow cytometry. Lymph node cells and tumor cell suspensions at 1 x 10^6 cells/sample were made for expansions (cell cultures) (for laborative details see “Materials and methods” paper no 2) and for the measurements of IFN-γ secretion.
SENTINEL NODE CD4+ TH1-CELLS INDUCE TUMOR REGRESSION IN HUMANS (PAPER 3)

Patients

Sixteen patients participated in the study, 10 men and 6 women with an average age of 62 years. Three patients were operated due to primary tumors in caecum and colon ascendens respectively and one additional patient had a local intraabdominal recurrence in the anastomoses after a previously right-sided hemicolecctionomy. Six patients had primary tumors in the sigmoid colon. Two patients in the study appeared with metastases to the liver from a primary rectal tumor and a primary sigmoid tumor, respectively.

There were five patients with high-risk Dukes´ B (who had tumor growth into nerves or vessels) in the study, together with two patients having Dukes´ C and nine patients classified as Dukes´ D tumors.

The study was approved by the local ethical committee and informed consent was given by each patient.

Methods

Mobilization of the tumor and injection were done according to previous studies. In those three patients with earlier resected primary tumors, their metastases were handled similarly. The latter procedure is described in detail in the fourth paper.

After surgery SN and non-SN were kept sterile in AIM V® Media, together with one piece of the tumor (including the invasive margin) and PBL. The lymphocytes were treated by the same principles as in study no 2 (for details see “Materials and methods” paper no 3), however, all substances that were involved in the laboratory processes now had to be approved for the use in humans. All laboratory work had to be performed in a laboratory according to the principles of GLP (Good laboratory practice).

Expansions were initiated and monitored meticulously. After about 2 weeks cultures a restimulation with PBL from the patient in order to provide fresh dendritic cells were performed to improve the expansion. Following an average period of slightly more than four weeks, expanded cells were counted, evaluated by FACS and carefully investigated for the exclusion of malignant cells and bacteria before they were considered ready for transfusion. The transfusions took place at a surgical ward as an intravenous transfusion during one hour.

Patients were followed according to the regular follow-up program for CRC at Stockholm South General Hospital.

METINEL NODE – THE FIRST LYMPH NODE DRAINING A METASTASIS – CONTAINS TUMOR-REACTIVE LYMPHOCYTES (PAPER 4)

Patients

Nineteen patients (9 men and 10 women) with an average age of 51.5 years were included. All patients had metastases from solid disseminated tumors. The sources to the liver metastases were from CRC in five patients and in one patient each from ovarian cancer, breast cancer, cholangiocarcinoma and leiomyosarcoma. Four patients had subcutaneous lymph node metastases from disseminated malignant melanoma and one patient each from ovarian cancer, breast cancer and squamous cellular cancer of the
tongue. Two patients had intraabdominal local recurrences after surgery for colon cancer and one patient following surgery for pancreatic cancer. The study was approved by the local ethical committee and informed consent was given by each patient.

**Methods**

Lymphatic mapping of liver metastases, intraabdominal local recurrences or subcutaneous metastases were performed by injecting about 1 mL PB subserosally or subcutaneously in four places around the metastasis or in the tissues close to the local recurrences. After the injection of PB, the first draining lymph nodes turned blue within 3–10 minutes and were regarded as metastasis-draining lymph nodes.

In six patients a preoperative LS was performed by a subcutaneous injection of 4 x 10-15 MBq Tc-nanokolloid RT in four quadrants around the subcutaneous metastasis. The LS was done in order to plan the surgical procedure by checking possible lymphatic regions where to localize the metastasis-draining lymph node(s).

After surgery the metastases-draining lymph node(s), one non metastases-draining lymph node together with a piece of the metastasis and PBL were kept sterile in AIM V® Media, put on ice and immediately taken to the laboratory. The specimens were treated by the same principles as demonstrated in the 3rd study (for laboratory details see “Materials and methods” paper no 4).

In order to verify clonal expansion of T lymphocytes, Vβ-repertoire analyses were performed in a few cases. To test for functionality, single cell suspensions were investigated regarding Th1 and Th2 cytokine production, IFN-γ and IL-4 respectively. After on average four weeks, expanded cells were considered ready for transfusion according to the same principles as stated in paper 3.
RESULTS

IDENTIFICATION OF SENTINEL NODES IN PATIENTS WITH COLON CANCER (PAPER 1)

The study demonstrated the feasibility to find first lymph nodes draining primary tumors, SN, in patients with colon cancer, which may improve the diagnosis of lymph node metastases. Blue-colored SN were identified in all 30 cases (28 intraoperatively) and the average number of detected nodes were 2.2 (range 0-6). The mean number of investigated lymph nodes in the specimens were 17.4 (range 4-35). In four patients the SN was the only metastatic node. The histopathological status in the SN was diagnostic for the status in the entire regional lymphatic field in 28 out of the 30 patients (93.3%). Twelve patients had lymph node metastases at surgery and ten of them had metastases to the identified SN, leading to a false negative rate of 16.7% (2/12 patients had a false negative SN). The use of RT in addition to PB demonstrated the same SN in all six investigated cases.

The most frequent position of the SN were among the intermediate nodes in the mesentery, but in four cases the mesenteric resections were modified due to unexpected locations of the SN. Two sigmoid resections were enlarged in distal direction whereas one sigmoid resection and one right-sided hemicolectomy respectively, were extended in a central direction due to the locations of the identified SN.

All patients were followed for at least 30 months, median 45 months, demonstrating that three out of seven deaths had occurred due to metastatic colon cancer. Two of these three patients had metastatic SN and one had false negative SN. Thus, only patients with lymph node metastases at surgery died from colon cancer. The remaining four patients died from other causes.

SENTINEL NODE LYMPHOCYTES IDENTIFIED INTRAOPERATIVELY FOR THE USE IN IMMUNOTHERAPY OF COLON CANCER (PAPER 2)

SN were detected intraoperatively in all patients with an average of 2.3 SN (range 1-4) per patient. Histopathological examinations retrieved between five and 29 lymph nodes (average 19) in the specimens. All tumors contained TIL, both CD4⁺ and CD8⁺ subsets, but upon stimulation with tumor homogenate, they were unresponsive to proliferation, despite that they presented an active CD69⁺ phenotype. TIL were also proliferatively unresponsive to Concanavalin A (Con A) (a general T lymphocyte stimulator). However, in one patient with Dukes’ B, TIL responded to Con A stimulation by the secretion of IFN-γ, and five times the background level upon addition of tumor homogenate.

SN and non-SN both contained similar numbers of activated CD4⁺CD69⁺ lymphocytes, regardless of absence (Fig. 2A middle panel) or presence of metastases (Fig. 2B middle panel). SN lymphocytes from patients classified as Duke’s B proliferated in an antigen dependent manner towards tumor homogenate (Fig. 2A right panel). SN lymphocytes
from patients having lymph node metastases, Dukes’ C, proliferated poorly upon stimulation with tumor homogenate (Fig. 2B right panel). By stimulating SN lymphocytes via Con A, two Dukes’ C patients still remained totally unresponsive. SN lymphocytes from three Dukes’ B patients and from one Dukes’ C patient secreted IFN-γ 4-38 times above background level when stimulated with autologous tumor homogenate. In two patients having Dukes’ D, SN lymphocytes did not produce IFN-γ, upon stimulation with tumor homogenate.

In non-SN proliferation upon stimulation with tumor extract was not detected, with the exception of three patients. Lymphocytes from non-SN did not respond with any IFN-γ secretion above background levels, with the exception of one patient where the secretion was 30 times the background level.

In peripheral blood activated, CD69+ lymphocytes were absent and there were no proliferative responses towards tumor homogenate, with the exception of minor responses in three patients. In two patients secretory responses of IFN-γ of 4-5 times background level were seen in PBL.

**Fig. 2.** Characterization of lymphocytes. In 15 colon cancer patients, tumor-draining, sentinel lymph nodes were identified by peritumoral injection of Patent blue dye. Specimens from the tumor (CC), sentinel node (SN) and non-sentinel node (LN) were stained with hematoxylin-eosin (left panels) (40x). Data from patient no 1 with a Duke’s B tumor (A) and patient no 12 with Duke’s C (B). Arrows indicate the presence of metastatic colon cancer cells in a sentinel node (B, left panel, SN). Single cell suspensions from the specimens were stained with antibodies against CD4 and the activation marker CD69 and analyzed using flow cytometry (middle panels), the percentage of double positive activated CD4 T helper cells are indicated in the upper right corner. Cell suspensions in triplicates were incubated with a 10- and 100-fold dilutions of autologous tumor homogenate in a day 5-7 proliferation assay. Cells were pulsed 18 h before harvesting with 1 mikroCi 3H-Thymidine. Proliferation data from day 5 (A) and day 6 (B), respectively, are shown. Error bars indicate SEM. (right panels).

**SENTINEL NODE CD4+ TH1-CELLS INDUCE TUMOR REGRESSION IN HUMANS (PAPER 3)**

**Immunology**

We show, for the first time, human adoptive autologous immunotherapy derived from CRC SN, without any observed side effects, and with encouraging results.
On average 2.1 (range 1-3) SN were identified in all sixteen. Among the patients treated with regular colonic resections, an average of 15.8 lymph nodes were retrieved in the specimens.

SN acquired lymphocytes contained mainly CD4+ T helper lymphocytes and CD8+ T cytotoxic lymphocytes, with an average quotation (CD4+/CD8+) of 4.8 at start, higher than the CD4+/CD8+ ratio in PBL. In addition a low number of CD19+ B lymphocytes and CD56+ NK-cells were present in SN.

The average number of harvested SN acquired lymphocytes at start of the expansions was 115.2 million cells and the cells were held in expansions for on average 36.5 days. Initially, the total number of cells decreased. B-lymphocytes disappeared almost completely and the number of CD8+ T-cytotoxic cells diminished. The average quotation of CD4+/CD8+ cells at the time for transfusion was 86.6, suggesting that mainly CD4+ T helper cells were promoted in the expansions, expressing the CD45RO memory phenotype. However, in some cases a substantial number of CD8+ T-cytotoxic lymphocytes developed against exogenous antigens, most likely through cross-presentation, a mechanism recently described at the molecular level.170, 171

In six cases, the expanded T-lymphocytes were functionally tested before transfusion by measuring the stimulated levels of the Th1 cytokine IFN-γ and the Th2 cytokine IL-4 in the cell culture supernatants. The average level of IFN-γ was 956 pg/mL and the average level of IL-4 11 pg/mL, indicating that the expanded T-lymphocytes were functional and Th1 responsive.

Restimulation of the expansions with autologous tumor homogenate antigen and APC, was performed after about fourteen days. It resulted in further clonal expansion of tumor-reactive lymphocytes as valued by investigating the TCR Vβ−repertoire of SN acquired lymphocytes before and after in vitro culture.

On average 71 million clonally expanded autologous tumor-reactive T-lymphocytes were transfused to each patient. The transfusions were performed at a surgical ward at Stockholm South General Hospital as an intravenous transfusion during about one hour, and after a few hours of observation the patients were discharged. No toxic side-effects, such as fever, chills, malaise, severe fluid retention, pulmonary oedema or respiratory distress, were observed.

Outcome

All 16 patients displayed signs of benefits from the treatment, with either extended periods of stable disease (n=10), partial response with diminished tumor burden (n=2) or complete response with no detectable remaining tumor (n=4). Most interestingly, all nine patients with distant metastases (Dukes’ D) responded to treatment (Table 1). The patients were followed for 24.1 months on average (range 6-33).

Seven patients did not receive any chemotherapy. Patients no 9 and 14 initially received chemotherapy following primary surgery one year earlier. Nine patients were treated with chemotherapy after immunotherapy, but no regular chemotherapy schedules were applied. Adverse effects related to chemotherapy and limited patient compliance lead to early termination of chemotherapy treatment in 4 patients. Four Dukes’ D patients are tumor free with an overall average survival of almost three years. Life expectancy in Dukes’ D patients is 6-9 months.71 Patient no. 12 (Table 1) with disseminated disease had a large sigmoid tumor resected en bloc, involving the left ovary, the left uterine tube and the upper part of the uterus. She had metastases in both liver lobes. The metastases regressed completely after immunotherapy, as demonstrated
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<th>IFN-γ (pg/ml)</th>
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Complete response (CR), Partial response (PR), Stable disease (SD).
5-Fluorouracil (5-FU), Leucovorin (Leu), Bevacizumab (Bev).
* The numbers represent the percentage of CD4⁺ and CD8⁺ cells detected with FACS.
¹ Initial progress and developed liver metastases, however metastases have decreased in size and the patient is presently in good clinical health and classified as stable disease.
² Stable disease with slightly decreasing size of liver metastasis, decreased CEA levels and improved clinical condition. She died unexpectedly at home in a phase of stable disease where she had recovered dramatically following immunotherapy, with regain of appetite and weight and she was in a state of physical fitness compatible with outdoor activities (WHO class I). Unfortunately no post mortem examination was performed but it is likely that a heart attack or a lung embolus was the cause of death.
³ The patient had multiple lymph node metastases (n=16) and presented after primary surgery with one liver metastasis which was resected. Later he developed several liver metastases. He received further immunotherapy based on expansion of peripheral blood lymphocytes using autologous tumor extract. The patient has then developed a complete response without any remaining liver metastases and he is now free from disease.
⁴ The patient was an 82-year old lady with cardiopulmonary disease and metastases in liver, bilateral lung metastases and spleen at diagnosis. After immunotherapy she lived with stable disease for 5 months without any signs of progressing malignant disease. The patient died from cardiopulmonary disease, no autopsy was done.
⁵ The patient was a young man who presented as an emergency with colonic obstruction and metastatic disease (intraperitoneal and liver). The primary tumor causing the obstruction was resected. The tumor responded initially with regression of liver and peritoneal metastases. In addition ascites disappeared with normalized S-CEA levels, and an enhanced general well being. After 9 months the disease progressed and he died 3 months later.
⁶ Chemotherapy was received after immunotherapy
⁷ Chemotherapy was received after primary surgery. Patients later developed metastases (at least 1 year later) and received sentinel node based immunotherapy, but no further chemotherapy.
Fig. 3 A. Imaging of liver metastases. Computer tomography (CT) of patient 12 showing a metastasis measuring 12.7 x 13.0 x 13.3 mm in the liver at the time of treatment (left panel). Follow up CT scan 3 months after treatment showed no signs of liver metastasis (right panel).

Fig. 3 B. FDG-PET of patient no 12 at the time of treatment revealed additional FDG positive metastases of the liver indicated by arrows (left panel). Follow-up FDG-PET scan 6 months after treatment without any signs of FDG positive metastases indicating a complete remission. The only remaining FDG uptake is seen in the kidneys (right panel).

Fig. 3 C. Imaging of lung metastases. In patient 16 bilateral lung metastases were detected with computer tomography (CT) prior to surgery (Before). Follow-up CT scan 3 months after cell transfusion, revealed complete remission of some metastases and a significant decrease in the remaining metastases (After).
primary tumor was resected because of obstructive symptoms, and immunotherapy followed after 5 weeks. A partial response with marked regression of liver metastases and disappearance of several lung metastases occurred within three months (Fig. 3C).

Three patients have deceased, but they were all classified as responders with partial response or stable disease. These patients all received lymphocytes in the lower range, suggesting overwhelming immunosuppression from their residual metastatic disease.

To further support the T cell mediated response we have investigated the peripheral recall response upon reactivation of peripheral blood using autologous tumour extract. We have been able to identify proliferative and IFN-γ anti tumoral recall responses 10 months after immunotherapy suggesting an increased number of circulating responder Th1 memory cells present after transfusion.

**METINEL NODE – THE FIRST LYMPH NODE DRAINING A METASTASIS – CONTAINS TUMOR-REACTIVE LYMPHOCYTES (PAPER 4)**

**Metinel nodes**

We conclude that it is possible to perform lymphatic mapping from metastases, intraabdominal recurrences and subcutaneous metastases in order to find the first metastasis-draining lymph node(s), and that these nodes contain tumor-reactive lymphocytes. We named these metastasis-draining lymph nodes “Metinel nodes”. Metinel nodes (MN) were identified in all 19 cases with an average number of 2.6 nodes/patient, range 1-5. Forty percent (17/42) of the analysed MN were positive for metastatic disease. In 25% (4/16) of the patients, where data were available, all MN were positive for metastatic disease (range 2-5) and in 50% (8/16) all MN (range 2-3) were negative for metastatic disease.

PB and/or RT were injected around liver metastases (n=9), intraabdominal recurrences (n=3) or close to subcutaneous metastases (n=7) and MN appeared visually blue within 3-10 minutes or positive in LS (Fig. 4).

![Image](image-url)

**Fig. 4.** Preoperative percutan injection of 4 x 15 MBq Tc-Nanokolloid around an ovarian cancer groin local recurrence (No 8) demonstrates after 10 minutes a medially and distally situated metinel node.
Nine patients in the study were operated due to liver metastases, among them 5/9 (55%) from CRC and one patient each from ovarian cancer, breast cancer, cholangiocarcinoma and leiomyosarcoma. In all cases lymph nodes draining the area of the metastases were found within the liver hilum or hepatoduodenal ligament. The average number of MN to liver metastases were 2.4 and 7/22 (32%) of the analysed MN were positive for metastatic disease.

Two patients were operated due to intraabdominal local recurrences from colon cancer with resections of the recurrences and resections of the bowel en bloc. One patient with pancreatic cancer had tumor-reducing surgery due to a local intraabdominal recurrence after a previous Whipple operation.

Four patients were operated with lymph node biopsies due to subcutaneous metastases from disseminated malignant melanoma and one patient each due to subcutaneous metastases from disseminated breast cancer, groin lymph node metastasis due to ovarian cancer and subcutaneous metastases related to squamous cellular carcinoma of the tongue.

**Immunology**

MN were predominated by CD4⁺ T helper cells with an average CD4⁺/CD8⁺ ratio of 2.3 (range 0.1-6.6), but five patients displayed an increase in the fraction of CD8⁺ cytotoxic T cells. Two of these patients suffered from malignant melanoma. The average quotient of CD4⁺/CD8⁺ cells were 2.8 for patients with successful expansions and 2.1 for patients with unsuccessful expansions. Consequently the CD4⁺/CD8⁺ quotient were 33% higher for patients having successful expansions (p<0.001 according to Wilcoxon rank sum test).

An average of 97 x 10⁶ cells were harvested and the cells were expanded on average for 31 days before transfusion. According to the previously described technique (paper 3) we succeeded in expanding the cells until transfusion in 47% (9/19) of the patients. However, in one of these cases cells were successfully expanded and prepared for transfusion but the patient suddenly died, without having received any cells.

At the time of transfusion a mean of 44 x 10⁶ tumor-reactive T cells were returned. No side effects from transfusion were seen and the patients were discharged from the hospital at the day of the transfusion. The expansions were successful for 5/7 patients who had colorectal primary tumours, 1/2 in patients with ovarian cancer and breast cancer and for 1/1 in patients with cholangiocarcinoma and leiomyosarcoma. All expansions from patients with malignant melanoma (4/4), pancreatic cancer (1/1) and squamous cellular cancer (1/1) failed.

By analyses of the CD4⁺ T cells, the specific TCR in the proliferating lymphocytes, the Vβ−repertoire, were identified in two patients. In the first case analyses of the Vβ−repertoire at the day of operation and after 43 days in two fused MN demonstrated clonal expansions of some TCR families towards TAA (Vβ families 7.1, 13.2 and 20). Analysis of the Vβ repertoire in the second patient, in two independently cultured MN derived from one breast cancer metastasis, demonstrated clonal expansion after 12 days short term culture of the same T cell receptor families (Vβ 4, Vβ 9, Vβ 20 and Vβ 21.3) (Fig. 5).

Cells from one patient, stimulated at the end of the expansion, demonstrated a very high production of IFN-γ >1000 pg/ml and no detectable IL-4, indicating a Th1 response. In an additional patient the stimulated IFN-γ secretion was 155 pg/ml whereas only a low
Fig. 5. In patient 13 the T-cell receptor Vβ repertoire was investigated by flow cytometry in two sentinel nodes at the day of operation and after in vitro cell culture (43 days). Clonal expansion of Vβ families 7.1, 13.2 and 20 were detected in the CD4+ T-cell population.

IL-4 production of 30 pg/ml was found, again demonstrating a Th1 predominant response pattern.
DISCUSSION

SUMMARY

We have shown that it is possible to find first lymph nodes draining colon cancer, “sentinel nodes”, and similar nodes also exist to drain metastases, intraabdominal recurrences and subcutaneous lymph node metastases, from colorectal cancer and other solid tumors. We named these latter nodes “metinel nodes”. In colon cancer the sentinel nodes (SN) were prognostic for the entire regional lymphatic field in 93.8%. Lymphocytes derived from SN and metinel nodes (MN) contain tumor-reactive lymphocytes, which can be further clonally expanded in vitro. We have used such lymphocytes as adoptive immunotherapy in patients with colorectal cancer (CRC) and also in patients with other solid tumors or metastases. No side-effects were related to this therapy. Follow up after SN based immunotherapy demonstrated that all patients responded to the treatment, and that four out of nine patients with disseminated CRC displayed complete regress of metastases. However, despite increased epidemiological knowledge, modern surgery and adjuvant therapy, about half of all patients will die within five years from diagnosis. Why is this possible?

Several explanations are feasible and have to be considered, These are correct staging procedures, presence of micrometastases, types of chemotherapy and the importance of the innate immune system.

STAGING

The lymphatic system

The importance of lymphogenic spread and its relation to hematogenic spread has been debated, but an increasing amount of facts supports its importance. Studies have shown that primary tumor and metastases secretes different vascular endothelial growth factors (VEGF-A, VEGF-C, VEGF-D), stimulating both peritumoral lymphangiogenesis and angiogenesis. Lymphatic capillaries are much more permeable for diffusion of molecules and cells than blood capillaries. Animal studies have shown that a tumor may disseminate millions of cells into the circulation from a one cm³ tumor, but only few metastases are produced. The tumor cells are most likely to spread through the lymphatics to regional lymph nodes, where about 50% of the lymphatic flow are directed into the systemic circulation by lympho-venous shunts. The existence of circulating tumor cells in a variety of malignancies is known but of uncertain significance in individual cases. It cannot be predicted whether sparse tumor cells found in lymph nodes are destined for immune destruction or tumor progression. Using radiolabeling studies in a murine system, it is observed that fewer than 0.1% of tumor cells that enter the systemic circulation survive to form secondary tumors. However, if arrested in the lymph node they may develop into a metastasis, but are also exposed for APC and are running the risk of being exposed to the cellular and humoral immune system. Probably, most solid tumors and metastases are spreading via the lymphatics in the same way.
Staging in colorectal cancer

Lymph node metastases

The most important prognostic sign in CRC patients, besides distant metastases, is the status of the regional lymphatic field and in cases of lymph node positive disease the 5-year survival is about 50-60%\(^24\). Several studies state a minimum of 10-17 histopathologically examined lymph nodes in a postoperative specimen for correct staging. They clearly demonstrate an improved survival when a sufficient total number of tumor-negative nodes are investigated. The number of positive lymph nodes divided with the total number of examined lymph nodes, the LNR, in Dukes’ C patients was a better prognostic parameter than N-stage. The lower quotation, the better chances of long-term survival.

Correct staging is mandatory for decisions regarding further oncological treatment and prediction of long-term prognosis. It is a well-known fact that CRC patients are at risk of understaging due to the occurrence of undetected regional lymph node metastases. Possible explanations are limited surgical resections or insufficient histopathological examinations. The search for a maximum number of lymph nodes in the specimen may be a labor-intense, expensive and time-consuming work, especially in searching for micrometastases by multilevel sectioning techniques, immunohistochemistry or RT-PCR. Several studies have revealed a large variation in lymph node evaluation between different pathology departments, leading to considerable differences in stage distribution and predicted survival.\(^29,33\)

Micrometastases

The definition of micrometastases is metastases <2 mm and which cannot be detected by standard histopathological microscopical or clinical staging techniques.\(^173\) In many studies micrometastases are mentioned as “a few cells” or “clusters of cells”. The TNM classification defines and embraces the term of micrometastases within e.g. breast cancer, but not within CRC. The prognostic significance of micrometastases in CRC is controversial and there is no established consensus of its importance. Some studies demonstrate a main impact on prognosis,\(^35,36,174\) while other studies have failed to demonstrate any association.\(^38,175\) This ambiguity also concern the clinical benefits of adjuvant chemotherapy in Dukes’ B patients, and there are no general consensus regarding its use. However, in 2006 the Swedish National Board of Health and Welfare presented a “state of the art” document on CRC and concluded that regimens based on 5-FU decrease the risk of recurrences and death with 3-4% by adjuvant chemotherapy in Dukes’ B patients.\(^57\)

SENTINEL NODE

Technical aspects on the sentinel node technique

Physiology

The tracers are transported in the lymph capillaries and accumulates in the SN through phagocytosis by macrophages. RT has theoretical advantages in that isotopes have a slower diffusion through the lymphatics than dye and there are no need for direct visualisation of blue-coloured lymphatic vessels. Lymphatic mapping by RT makes a preoperative LS possible, which was performed endoscopically in a Japanese study evaluating the SN technique in rectal cancer. LS facilitates the intraoperative
identification of the SN, especially when it is located without the expected resection area.

Blue dye entails quicker examinations as it consists of small molecules which travels fast through the lymphatics. While using dye techniques intraoperatively, the SN are often identified within 5-10 minutes. Thus, soon the second echelon of lymph nodes will be blue-coloured and these nodes may falsely be identified as SN. RT-detected nodes are more likely to identify the true SN and thereby more likely to show significance for the regional lymphatic field.

**Tracers and techniques**

The choice of tracers varies between PB, lymphazurine or isosulfan blue dye and the use of RT. None of them are considered superior to the others in single use and the most used tracer is PB.

Allergic and anaphylactic reactions to PB have been described in up to 2.7% of treated patients. As the importance of the SN technique increases, an increase in anaphylactic reactions may be expected due to PB and the medical personnel must be prepared for this advent. All injected dyes may interfere with the pulsoxymetrics used during surgery, in that the peripheral blood oxygen saturation registration may decrease substantially during about one hour or more, or completely disappear.

Within breast cancer and malignant melanoma the use of dual-agent mapping (blue dye and RT together) increases the identification rate and accuracy of the procedure. The few studies performed with dual-agent mapping in colon cancer have shown contradictory results, in that some studies more accurately identifies the SN, while other studies have not demonstrated any improvements. In our own study no differences were seen.

Beside the in vivo technique there is the ex vivo technique, in which the tracer immediately postoperatively is injected into the specimen and distributed into the lymphatics through gentle massage of the tumor by hand. These two techniques have shown comparable results, but in the ex vivo technique the lymphatic vessels may have been damaged by the surgery and an unexpectedly located SN will not be identified.

In accordance with the increased laparoscopic colorectal surgery the SN technique has been evaluated laparoscopically, where the tracer has been injected subserosally via endoscopy. During laparoscopic colectomies the root of the mesentery may be difficult to dissect and visualize due to obesity or adherences and the SN technique may therefore be advantageous in staging.

The learning curve is considered to be rather short in the SN technique. However, the sources of error in the SN technique may be intraluminal injection, incomplete unsymmetrical peritumoral injection of the tracer, previous surgery or disturbances in the tumor lymphatic drainage because disruptions in the lymphatic vessels due to locally invasive growth and obvious lymph node metastases. If the tracer is injected into the bowel lumen instead of subserosally, no SN will be found, and if the tracer is injected too deep into the bowel wall, the tracer will spread along the bowel wall and paracolic nodes will be identified as SN.
Identification of the sentinel node

Sentinel node in colorectal cancer staging

The main advantage of the SN technique in CRC is in achieving a correct staging by illustrating the true directions of the lymphatic drainage and in finding the first lymph node(s) draining the tumor. The prognostic significance of the SN are also underlined by that histological parameters indicating a T-cell mediated immune response in mesocolic lymph nodes have been found to correlate with increased survival rates.184

The reported results of successful identifications of SN varies between 71% to 100%.183, 185, 186 The success rate of that the identified SN is representative in its histopathological status for the entire regional lymphatic field, varies between 78% and 100%, 185-187 and the false negative rates varies between 0% to 54%.187, 188 In three laparoscopic studies the identification rate were 100% and the same studies also identified the most aberrant lymphatic drainage in finding unexpected locations of the SN in 27% to 36%.183, 186, 189 In addition, a small laparoscopic study of nine patients reported success rates of 100% in identifying the SN with an accuracy of 100%.190 In our study on 30 patients with colon cancer the SN was predictive for the entire lymphatic field in 28 patients (93.8%) and there were a false negative rate of 16.7%. In four cases the SN were the only metastatic node. Median follow-up after 45 months demonstrated that seven patients had died and three of them had died of metastatic cancer. All of these three patients had lymph node metastases at surgery. The study supported that the SN technique may be of use in colon cancer for improved staging and prognosis.

Another advantage for the SN technique is in the search for micrometastases, although the prognostic value of micrometastases in CRC is not demonstrated. It has been demonstrated that thorough analyses of the SN in CRC by multilevel sectioning,191 or preferentially by immunohistochemistry or RT-PCR, reveals the presence of micrometastases. These techniques have been performed in about 2/3 of all studies and a true upstaging from Dukes’ B to C have been showed in between 0% to 26%.36, 104, 185, 192,193, 194

SN mapping may also be useful when limited surgery must be performed or when the SN are identified in aberrant positions, which may be explained by the phenomenon of skip metastases. Studies have showed the presence of skip metastases in colon cancer in that the intermediate mesenteric lymph nodes often are the first draining lymph nodes to the tumor, the SN.195, 196 Most studies have not been able to demonstrate any cases of aberrant findings in the lymphatic drainage, despite relatively large studies with 48 patients197 or 72 patients,185 or have not mentioned any cases in their publications. In our study on 30 patients the colonic resections were modified and extended in four cases (13%) due to unexpected locations of the SN.

Conclusions of the sentinel node technique in colorectal cancer

The SN technique has been evaluated within many different types of tumors and has become of routine within some surgery for breast cancer and malignant melanoma. About 20 studies have analyzed the SN concept in CRC, however, with diverging results. The varying results in the SN studies reflects the lack of a standardized technique and an univocal definition of which stained lymph node(s) should be considered as SN.198 Further, the studies have different designs, patient populations,
different numbers of lymph nodes evaluated and paucity of prospective data. A more standardized SN technique would be useful for prospective studies determining the prognostic value of nodal micrometastases.

Since reported accuracy rates in identifying the SN are between 93% to 100%, the sensitivity of predicting the regional lymphatic field is between 90% to 100% and true upstaging rates is in average between 5% and 14% , it can be concluded that SN mapping have a great potential in improving staging in colon cancer.

It is unrealistic for the pathologists to always investigate the whole of all lymph nodes in all specimens for the presence of metastases as this would be very expensive, laborious and time-consuming. Meticulous analyses of the SN for correct staging are a more favorable alternative. In addition, many nodal metastases in CRC are less than five millimetres in diameter.  

We believe that the SN technique may be of use in CRC as a staging tool leading to more patients receiving adjuvant therapy, correct prognosis and routine of follow-up. When indisputable evidence supporting the connection between micrometastases and prognosis patients are available, together with a clinical benefit in the regular use of adjuvant therapy in Dukes’ B patients, the value of the SN technique in CRC will increase rapidly. Still, the practice of the SN technique within CRC is not recommended for clinical use outside studies.

**IMMUNOLOGY**

**Tumor physiology**

There is a rapid turnover of cells in a tumor, lack of oxygen and nutrition, causing a hostile environment which attracts antigen presenting cells (APC). The APC phagocytose debris from tumor cells and then migrates via the lymph vessels to the first draining lymph nodes, the SN or the metinel node (MN). Within the lymph node the APC present tumor-derived peptides, thus activating tumor-reactive T lymphocytes. Whole tumor cells shedded into the lymphatics may also stimulate the lymphocytes in the lymph nodes through TAA. These stimulated tumor-reactive lymphocytes proliferates clonally and are homing towards the tumor to become tumor infiltrating lymphocytes (fig. 6).

![APC carrying tumor antigen](image)

**Fig. 6. Hypothetical scheme of activation of tumor reactive lymphocytes in the sentinel node.** In the tumor there is a rapid turnover of cells, lack of oxygen and nutrition, causing a hostile environment which attracts macrophages and dendritic cells. These professional antigen presenting cells (APC) phagocytose debris from tumor cells and then migrate via the lymph vessels to the draining sentinel node. In the sentinel node the APC present tumor-derived peptides, thus activating tumor-reactive T lymphocytes. The sentinel node can be detected intraoperatively by peritumoral injections of Patent blue dye.
The diverse results in reactions of the tumor-reactive lymphocytes upon stimulation displayed in the study, may be due to differences in the biology of the primary tumor. Tumor cells may decrease its antigens on the cell surface by down regulation of MHC-class I on their cell surfaces, leading to that cytotoxic T cells does not recognize the tumor cells and they avoid being killed by them. The amount of adhesion-proteins on tumor cell surfaces are being down-regulated, leading to that dendritic cells, NK-cells and cytotoxic T cells do not get a grip of them. Tumor cells also has the capacity to secrete different types of cytokines, e.g. TNF, which suppresses the immune defense. The tumor that finally emerges is poorly immunogenic and immune-resistant, consistent with the escape mechanism of the immunoediting theory.

**Sentinel node immunology**

SN derived lymphocytes are endogenously sensitised towards TAA, derived from whole tumor cells or by presentation of antigen presenting cells, and are therefore defined as tumor-reactive lymphocytes. Tumor-reactive lymphocytes are mainly clonally expanded CD4+ T helper cells, but also CD8+ cytotoxic T cells with specific activity towards specific tumor antigens.

Adoptive transfer of in vitro cultured CD4+ Th1-cells has been demonstrated to destruct specific β-cell destruction in mice. CD4+ Th1-cells are necessary for the effective functioning of cytotoxic CD8+ T-cells and for the development of memory cells. CD4+ Th1-cells mature due to the expression of the transcription factor T-bet, which also induces production of IFN-γ, necessary for the immune response towards tumors. We believe that tumor-activated CD4+ Th1-cells have a crucial function in eliciting the immune response towards a tumor, thereby eradicating cells who expresses TAA. Marits et al have identified tumor-reactive lymphocytes in SN to urinary bladder cancer, proliferating dose-dependently upon stimulation with IL-2 and tumor homogenate.

These facts supports the idea of the SN being regarded as the primary site for activation of the immune defence towards tumors and therefore the SN entails a suitable source for adoptive immunotherapy. We have demonstrated the presence of tumor-reactive lymphocytes in SN from colon cancer patients, but also in lymph nodes draining metastases, MN.

SN derived tumor-reactive lymphocytes were cultured in vitro and regularly stimulated by tumor homogenate, low doses of IL-2 and APC. Analyses by time course 3H-Thymidine incorporation assays demonstrated that tumor-reactive lymphocytes proliferated dose-dependently in the absence of lymph node metastases, but were unresponsive to proliferation in the presence of lymph node metastases. The lymphocytes were mainly T helper cells type 1, which produced IFN-γ, and proliferated upon stimulation. The production of IFN-γ by the tumor-reactive lymphocytes corresponded closely, with a few exceptions, with the proliferative responses. TIL were unresponsive to stimuli in vitro.

In a murine system tumor-reactive lymphocytes were identified within tumor draining lymph nodes and proliferated in vitro expansions by stimulation of anti-CD3 MAb and IL-2. This stimulation converted them into effector cells capable of mediate regression of metastases after adoptive immunotherapy. However, human malignancies and immune system differs considerably from animals, requiring broadening research. Adoptive transfer of in vitro expanded lymph node cells have been demonstrated within humans. 23 patients with colorectal cancer were included, but
the results were moderate, with one patient showing partial response and four patients minor responses.

The positive prognostic importance of TILs within ovarian cancer and CRC has been demonstrated. The higher levels of CD8+ T cells and CD45RO+ memory cells evaluated immunohistochemically in those tumors, the less signs of metastatic invasion and less advanced pathological stage, but increased survival.124

We presume that the CD4+ Th1-lymphocytes mainly occurring in the SN or the MN, are too few to elicit an efficient immune response against the tumor, possibly due to immunosuppression. T cells may be expanded and proliferated in vitro and used in adoptive immunotherapy, and therefore, we wanted to investigate the possibilities of such SN based immunotherapy. Immunotherapy offers an appealing alternative to traditional chemotherapy, in that the patients own cells entails the treatment and that a possible long-term protection against tumor recurrences through immunological memory may develop.

**Immunotherapy**

We managed to isolate and expand SN derived tumor-reactive lymphocytes in vitro, by the stimulation of tumor homogenate, low-dose interleukin-2 and APC. The expansions lead to proliferation of tumor-reactive lymphocytes producing IFN-γ, which after in average 36.1 days were ready for transfusion.

During expansions the CD4+/CD8+ ratio changed from in average 4.8 at start, to 86.6 at the time of transfusion, consequently the expansions supported the proliferation of CD4+ Th1-cells, expressing the CD45RO memory phenotype.

Investigations of the T-cell receptor (TCR) Vβ-repertoire demonstrated clonal expansion of a few Vβ-families in every expansion, indicating tumor-reactive lymphocytes towards specific TAA.

The effect of immunotherapy using SN acquired CD4+ Th1 lymphocytes seemed to be dose dependent, since the Dukes’ D patients with complete responses received a significant higher number of T cells, 140 million versus 26 million T-cells (p<0.05).

In our 3rd paper we performed autologous adoptive transfusions of SN derived T-cells in all sixteen patients with colon cancer and in our 4th paper we performed adoptive immunotherapy of MN derived lymphocytes in 7/19 patients. No side-effects were seen.

**Sentinel node based immunotherapy**

All patients responded to the therapy by showing stable disease or objective responses.

Out of the nine patients having Dukes’ D, two patients displayed partial responses and four patients complete responses of metastases. Four Dukes’ D patients are tumor free and two display stable disease, with an overall average survival of 29.8 months, which is encouraging as average life expectancy is 6-9 months in Dukes’ D patients.71

Since this was a pilot study, nine of the patients received chemotherapy, but two of these received their treatments one year earlier. The other patients received their chemotherapy after the immunotherapy, but no regular schedules of treatment were followed due to side-effects or lack in compliance. One patient who achieved a complete response did not have any chemotherapy treatment.

In addition, chemotherapy kills dividing cells and may therefore have an adverse effect in adoptive immunotherapy, by suppressing T-cells and decreasing the immune response. Two of the patients achieving complete response displayed initial progress of
metastases, which may have been the result of chemotherapy induced immunosuppression, but an induced tumor cell death may also lead to an increased amount of available TAA, thereby improving the immune response.

The study cohort are small, but all included patients are considered to be responders, without anyone having received any regular chemotherapy treatments after immunotherapy, and in addition, patients with complete responses received a significant higher amount of T-cells, all factors supporting the idea of a T cell mediated effect. A secondary booster dosage may be preferred.

**Metinel node based immunotherapy**

Metastases have the capacity of shedding tumor cells and creating remetastases and studies have also demonstrated the presence of a lymphangiogenesis similar to surrounding primary tumors. We performed lymphatic mapping from metastases, intraabdominal local recurrences and subcutaneous lymph node metastases and identified the first draining lymph nodes in all cases. We named these nodes “metinel nodes” (MN). Beside the use of PB, we performed a preoperative lymphoscintigrapy in six cases of subcutaneous lymph node metastases, a technique, to our knowledge, never used before in lymphatic mapping of metastases. The second aim of the study was to see if the MN contained tumor-reactive lymphocytes suitable for immunotherapy.

Out of the nineteen included patients, nine performed lymphatic mapping based on liver metastases and five of them originating from primary colorectal cancer. In all cases of lymphatic mapping based on liver metastases, the MN were identified within the anatomical distribution of the hepatic lymphatic drainage.

While studying the literature we could only find one previous study performing lymphatic mapping based on metastases, despite the major impact on prognosis of remetastases to perihepatic lymph nodes within liver surgery. Most studies identify few 5-year survivors following liver surgery involving positive perihepatic lymph nodes and within surgery for CRC liver metastases, many authors recommend a restrained manner to surgery if positive nodes.

Analyses of the Vβ-repertoire in two patients demonstrated clonal expansion of a few selected Vβ-families. In a few samples of patients we investigated the ability of the T-cells to produce IFN-γ and found high levels of IFN-γ and correspondingly low levels of IL-4, favouring the presence of tumor-reactive Th1-lymphocytes. The fact that it was possible to expand the MN derived lymphocytes in *in vitro* expansions over several weeks of time together with our analysis that they contain lymphocytes which show clonal expansion towards tumor antigens and produce high levels of IFN-γ in the expansions, demonstrates the presence of tumor-reactive lymphocytes. These cells are mainly T-helper 1 cells which have developed an immunological response towards the metastatic cells.

7/19 patients received autologous adoptive immunotherapy, but these patients were a very inherent group with diversified diagnosis and follow up time. The average quotation of CD4+/CD8+ (2.8/2.1) were significantly higher (p<0.001 according to Wilcoxon rank sum test) in cases of successful expansions versus unsuccessful expansions. Five out the seven patients who received immunotherapy are still alive,
although the follow-up time are very varying. So far our results are encouraging since
the prognosis in these type of metastatic disease usually are poor.
We believe that the MN technique in the future may be as useful as the sentinel node
technique in staging and as a source for lymphocytes suitable for immunotherapy.

OTHER STUDIES IN ADOPTIVE IMMUNOTHERAPY
Several minor studies have presented occasional results of adoptive immunotherapy in
CRC and have mainly been discussed earlier in the text. However, the inevitable most
successful research group in adoptive autologous immunotherapy is the group of
Steven A Rosenberg, Surgery branch, National Cancer Institute, Bethesda, Maryland.
Rosenberg et al have performed autologous adoptive or humoral immunotherapy in
several different ways, derived from leukopheresis or TIL, with or without
lymphoablative neoadjuvant treatment. Results are encouraging within disseminated
renal cancer or malignant melanoma, especially when combined with neoadjuvant
lymphoablative treatment with cyclophosamid, where 18/35 patients in one study
experienced an objective clinical response (>50% reduction in tumor). However, there
are some differences compared to our studies. By harvesting cells from peripheral
blood, the lymphocytes are unlikely to be enriched in tumor-reactive lymphocytes. A
main advantage for the SN/MN technique are that the lymphocytes are individualized and
customized for each and every patient, since it is the patients own tumor cells that have
initiated the tumor-reactive lymphocytes. Most other immunotherapies consists of more
diffuse specificities or are prefabricated to react on one specific molecule or protein.
Rosenbergs patients are treated with high-doses of interleukin-2, leading to severe side-
effects like fever, malaise, severe fluid retention with heart failure or pulmonary
oedema and nursed at intensive care units for weeks. The patients in our studies
received their treatments within one hour and were thereafter discharged within a few
hours, without any observed side-effects. The long-term side effects of lymphoablative
treatment are unknown, but since the main reason for the treatment is to eliminate Treg
cells, there are a theoretical risk of increased morbidity of autoimmune diseases and
decreased acquired immunity.

CONCLUSION
We have demonstrated the feasibility of identifying sentinel nodes in colon cancer and
that they may improve staging and prognosis. We have also demonstrated the
feasibility to find draining lymph nodes to metastases, intraabdominal local recurrences
and subcutaneous metastases, metinel nodes. Both the sentinel node and the metinel
node are enriched in tumor-reactive lymphocytes and entail an excellent source of
lymphocytes suitable for adoptive immunotherapy. So far the sentinel node technique
has been used as a diagnostic tool to improve staging or to direct the extent of surgery.
To our knowledge, we are the first to use the sentinel node technique in a therapeutic
setting.
The tumor-reactive lymphocytes from sentinel nodes and metinel nodes can be
proliferated in vitro and used in immunotherapy for colorectal cancer patients and other
solid tumors and metastases. Preliminary results of the sentinel node based
immunotherapy are encouraging with responses in all 16/16 patients and complete
responses in four patients with regress of metastases to the liver or lungs. The sentinel
node and metinel node based autologous adoptive immunotherapies are well tolerated,
without observed side-effects, and should be evaluated in future clinical trials.
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Cancer inom tjocktarmen (kolon) och ändtarmen (rektum) är den tredje vanligaste cancerformen i världen och cirka en miljon personer insjuknar årligen. I Sverige insjuknar ungefär 5 500 människor i kolorektal cancer varje år, varav 2/3 pga cancer i tjocktarmen. Förekomsten av kolorektal cancer varierar mycket mellan olika länder och är vanligare i västvärlden. Utöver årtiliga faktorer, anses detta till stor del bero på levnadsfaktorer såsom kost och vikt.

Boten för kolorektal cancer är kirurgi. Om tumören eller dess dottertumörer (metastaser) inte opereras bort så kan patienten inte botas från sin cancer. Den genomsnittliga femårsöverlevnaden vid kolorektal cancer är ungefär 50%. Vid diagnosstillfället har ungefär 1/3 av patienterna en spridd sjukdom med metastaser, vanligen till lever eller lungor, och lever därefter i genomsnitt ytterligare 8-12 månader.

För övriga patienter är det viktigaste om tumören har spridit sig till lymfkörtlarna i tarmväster, eller ej. Med tarmväster avses den del i tarmarnas närhet som består av fett, blodkärl och lymfatisk vävnad. När det finns metastaser i dessa lymfkörtlar lever 40-60% av patienterna i ytterligare fem år. Om tumören har spridit sig till dessa lymfkörtlar erbjuds patienterna cellgiftsbehandling (cytostatika), vilket botar cirka 10% av patienterna. Påträffas inte några metastaser inom tarmvästers lymfkörtlar, så är dock femårsöverlevnaden inte 100%, utan endast cirka 60-95%.

Förklaringen till detta är ej helt klarlagd. Man har visat att det är viktigt att undersöka så många lymfkörtlar som möjligt efter operationerna för att sedan kunna erbjuda patienterna rätt behandling. Undersökningar har visat att noggranna mikroskopiska undersökningar som görs av patologerna ökar femårsöverlevnaden. Det finns också metastaser som är så små att de inte kan upptäckas vid rutinundersökningar, mikrometastaser, men betydelsen av dessa är inte helt klarlagd. Vissa studier visar att man kan hitta mikrometastaser i upp till 22.4% av alla ”sentinel nodes” (vaktpostkörtlar) hos patienter där man tidigare inte kunnat hitta några metastaser till lymfkörtlarna, och att dessa patienter lever kortare tid.

Med vaktpostkörtel avses den lymfkörtel i tarmväster som först tar emot den lymfvätska som kommer från en cancer tumör. Kolorektala tumörer sprider sig via det lymfatiska systemet och därför är vaktpostkörteln av stort intresse. Studier visar att mikroskopiska undersökningar av vaktpostkörteln är generellt representativa för patientens övriga lymfkörtlar, dvs finns det t ex inga metastaser i vaktpostkörteln så är sannolikheten låg att det finns några metastaser i andra lymfkörtlar i tarmvästen.

Kroppens immunsystem har en stor betydelse vid cancersjukdomar, vilket kan illustreras av att människor med nedsatt immunförsvar har en ökad risk för cancersjukdomar, t ex vid AIDS, hög ålder och vid medicinering som nedsätter immunförsvarsverket. I vaktpostkörteln finns bl a en stor mängd vita blodkroppar, lymfocyter, och körteln har både ett centralt läge och en central funktion vid tumörsjukdomar.
Trots dagens moderna medicinska teknik avlider fortfarande ungefär hälften av alla patienter som fått diagnosen cancer inom tjocktarm eller ändtarm och det finns ett behov av förbättrade behandlingsmetoder. Vi vill med denna avhandling studera om det är möjligt att hitta vaktpostkörtlar vid tjocktarmscancer och om dessa körtlar innehåller lymfocyter som kan användas i tumörbehandling, sk immunoterapi.

STUDIE NR ETT
I detta arbete studerades möjligheterna att finna portvaktskörtlar till tjocktarmscancer. 30 patienter ingick i studien. Under operationerna sprutade vi 1 ml blått bläck, ”Patentblått”, på fyra platser omkring tumören. Bläcket transporterades sedan snabbt från tumören vidare in i lymfkärlen och efter cirka 5-10 minuter sågs de första blåfärgade lymfkörtlarna, portvaktskörtlarna, i tarmkäxet. Vi identifierade en till sex portvaktskörtlar hos alla patienter. Hos 28 av de 30 patienterna (93.8%) visade den mikroskopiska undersökningen av portvaktskörtlarna samma bild som hos övriga lymfkörtlar i tarmkäxet. Tolv patienter hade metastaser i lymfkörtlarna vid undersökningen av preparatet, varav tio patienter även hade metastaser i sina portvaktskörtlar, dvs hos två patienter (16.7%) visade inte portvaktskörteln en sann bild för alla lymfkörtlar. I fyra fall var portvaktskörteln den enda körteln med fynd av metastas hos patienterna.

Vid kontroll efter i genomsnitt 45 månader efter operationerna kunde vi se att endast patienter som hade lymfkörtelmetsaser vid operationerna avlidit i tjocktarmscancer. Studien visar att det går att identifiera portvaktskörtlar till tjocktarmscancer och att dessa körtlar i stor utsträckning utgör en sann bild för patientens alla andra lymfkörtlar i tarmkäxet.

STUDIE NR TVÅ

Studien visar att i portvaktskörtlar ansamlas tumörspecifika lymfocyter, som kan känna igen och döda patientens tumörceller, och att dessa celler kan överleva utanför kroppen, in vitro, i cellodlingar.
**STUDIE NR 3**


Nio patienter erhöll cytostatika, men av dessa hade två patienter erhållit sin medicinering ett år tidigare och de övriga sju patienterna genomförde inte sin medicinering. Samtliga patienter reagerade på behandlingen. Vid kontroller efter i medeltal 30 månader noterades att fyra av de nio patienterna med generellt spridd cancer var utan tecken till kvarvarande sjukdom. Två patienter uppvisade en total tillbakagång av sina lever- och lungmetastaser. De övriga patienterna i studien uppvisade mindre tillbakagångar av metastaser eller avsaknad av tumörtillväxt.

Studien visar att det är möjligt att genomföra en immunoterapi med lymfocyter som kommer från portvaktskörtlar och att inga biverkningar kunde ses. Resultaten av immunoterapin verkar lovande då samtliga patienter i studien reagerade gynnsamt på behandlingen och att två patienter upplevde total tillbakagång av sina lever- och lungmetastaser.

**STUDIE NR 4**

Avsikten med det fjärde arbetet var att undersöka möjligheten att identifiera portvaktskörtlar till metastaser och att studera om dessa körtlar också innehåller tumörspecifika lymfocyter. 19 patienter med metastaser ingick i studien. Den ursprungliga tumören fanns i tjocktarmen hos sju patienter, malignt melanom fyra patienter, äggstockscancer och bröstcancer två patienter vardera, samt en patient vardera med ursprungstumörer inom bukspottkörteln, gallvägarna, glatt muskulatur och tungan. Metastaserna fanns i lever hos 9 patienter, i form av återfallet av cancer i buken efter tidigare operation av tjocktarmcancer hos 3 patienter och i underhudens lymfkörtlar hos 7 patienter.

I alla 19 fallen identifierades en till fem portvaktskörtlar efter att vi sprutat Patentblått omkring metastaserna. Vi kallar dessa portvaktskörtlar till metastaser för "metinel nodes". I sex fall sprutade vi innan operationerna in ett radioaktivt ämne kring lymfkörtelmetastaserna i underhuden för att se portvaktskörtelns läge. Lymfocyter från metinel nodes förvarades i celloidlingar, in vitro, och tillfördes regelbundet tumörceller, signalsubstanser och eget blod från patienterna. I sju fall lyckades vi att genomföra immunoterapi. Inga biverkningar kunde observeras.

Studien visar att det är möjligt att identifiera portvaktskörtlar till metastaser, "metinel nodes", och att dessa innehåller tumörspecifika lymfocyter som kan användas som immunoterapi.
DISKUSSION


Noggranna undersökningar av portvaktsskoértlarna med specialmetoder kan hitta mikrometastaser hos 15-20% av de patienter där man med vanliga metoder inte funnit några metastaser. Betydelsen av dessa mikrometastaser är osäker. Färskna studier visar att om patienter utan lymfkoértelometastaser behandlas med cytostatika, så överlever 3-4% fler av dessa patienter. Man tror att denna överlevnadsvist kan bero på att patienter med mikrometastaser hindrats från att utveckla stora metastaser.

Man kan med stor säkerhet identifiera portvaktsskoértlar till cancer i tjocktarmen och dessa återspeglar i stor utsträckning bilden av alla lymfkoértlar i tarmväven. Vi anser att analyser av portvaktsskoértlar förbättrar möjligheterna att få en korrekt uppfattning om patients eventuella metastaser i lymfkoértlarna. Om det i framtiden visas att patienter med mikrometastaser kan botas av en tillägsbehandling, så kommer portvaktsskoértlarnas betydelse öka dramatiskt.


Vi har sett att i våra cellodlingar så tillväxer en typ av tumörspeficka lymfocyter som kallas för hjälparcell. Den spelar en viktig och central roll i början av immunförsvar mot t ex en tumör, och behövs för att starta immunförsvar. En tumör kan sprida miljontals celler och celldelar i lymfystemet varje dag som sedan transporteras vidare till portvaktsskoérteln. Portvaktsskoérteln borde därför ha en mycket central roll i immunförsvar mot tumörer. Vi anser att portvaktsskoérteln utgör en lämplig källa för lymfocyter att användas som immunoterapi mot cancer. Immunoterapi är en intressant behandlingsmetod då hjälparcellerna bl a stimulerar bildandet av minnesceller. Dessa minnesceller cirkulerar sedan runt i kroppen under mycket lång tid och aktiveras snabbt om de skulle träffa på några likadana tumörceller i framtiden. Mot bakgrund av detta ville vi studera möjligheterna till immunoterapi med tumörspeficka lymfocyter från portvaktsskoértlar hos patienter med tjocktarmscancer.
Studier har visat att metastaser från olika tumörsorter har samma förmåga som de ursprungliga tumörerna att själva sprida sina tumörceller och orsaka metastaser. Vi ville därför även studera möjligheterna till immunoterapi med tumörspecifika lymfocytter från portvaktskörtlar hos patienter med metastaser.

Vi lyckades att genomföra immunoterapi i båda grupperna. Hos patienterna med tjocktarmscancer lyckades vi alla 19 fallen och i den andra gruppen i sju av nitton fall. I båda grupperna kunde vi se en tillväxt av få hjälpareceller i cellodlingarna. Andra analyser visade att de celler som tillväxte mest i en cellodling var av samma sort, dvs de var tumörspecifika lymfocytter som kan känna igen en celltyp och döda den. Undersökningarna visade att det kunde finnas tre till fyra olika celltyper, kloner, i samma cellodling.


Vi lyckades att identifiera portvaktskörteln till metastaser i alla fallen och till vår kännedom har bara en liknande studie gjorts tidigare. Vi kallar dessa portvaktskörtlar för "metinel nodes". Vi tror att denna metod kan bli lika användbar som den andra redan etablerade metoden där man utgår ifrån ursprungstumören. Metoden borde ha ett stort värde vid t ex kirurgi för levermetastaser, där betydelsen av lymfkörtelmetastaser omkring levern från själva metastasen, är stor.

Hittills har portvaktskörteltekniken endast använts för att studera ev metastaser i lymfkörtlar eller för att kunna justera omfattningen av operationerna. Till vår kännedom är vi de första att använda portvaktskörteln som en källa för immunoterapi. Denna immunoterapi visar lovande resultat, utan observerade biverkningar och måste utvärderas ytterligare i större studier.
REFERENCES


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