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SENTINEL NODE BASED IMMUNOTHERAPY OF CANCER

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny ...'

Isaac Asimov

ABSTRACT

The tumor draining lymph node, the sentinel node, is regarded as a valuable station for staging and evaluating prognoses for patients with cancer. The object of this thesis was to investigate the immune response against solid cancers and to explore the sentinel node as a source of naturally occurring tumor reactive lymphocytes, for further activation and expansion *in vitro* for the purpose of immunotherapy. Since mortality rates in colon cancer and urinary bladder cancer are high, in spite of surgery and chemotheraphy, immunotherapy may offer an appealing complement with possible long-term protection against tumor recurrence through immunological memory.

We have demonstrated that sentinel nodes from patients with urinary bladder cancer and colon cancer contain tumor reactive immune responses. Sentinel node acquired lymphocytes proliferated dose dependently when subjected to autologous tumor extract and produced the Th1 cytokine IFN-γ, whereas tumor infiltrating lymphocytes responded poorly. However, the responses in sentinel node acquired lymphocytes harvested from metastatic lymph nodes were dampened or absent, suggesting tumor induced immunosuppression. Using long term cultures in the presence of IL-2 and autologous tumor extract, the state of anergy was overcome, since proliferation and functional production of IFN-γ were restored. Thus, the sentinel node seems to be the predominant location for activation and expansion of tumor-reactive lymphocytes, cells that can be cultured and expanded *in vitro*.

Since the sentinel node is the predominant location for metastases, we have developed a flow cytometry based assay, using antibodies directed against the cell surface markers EpCAM and CA19-9 to ensure tumor free expansion. We were able to detect very low levels of tumor cells in single cell suspensions. By using the combination of two cell surface markers, the problem of downregulated protein expression by tumor cells could be avoided.

Sentinel nodes were recovered intraoperatively from 16 patients with colon cancer for adoptive immunotherapy. Sentinel node acquired lymphocytes were collected, activated and expanded against autologous tumour extract. The procedure promoted mainly the clonal expansion of T helper 1 lymphocytes, producing IFN-γ upon stimulation with tumor extract. On average, 71 million activated and clonally expanded autologous T lymphocytes were transfused back to each patient. No toxic side-effects or other adverse advents were observed. Four Dukes' D patients displayed complete responses with clearance of tumor burden, they have an overall average survival of 38 months. Interestingly we could demonstrate a dose response since the patients with partial response or stable disease received significantly fewer lymphocytes than patients with complete responses. The cumulative survival of immunotherapy treated patients compared with all Dukes'D cases in the Stockholm region during the year of 2003, demonstrates a significant increased survival in the immunotheraphy treated group, with an average survival of 2.6 years compared to 0.8 years for the control group (P=0.048). In conclusion, sentinel node acquired lymphocytes can be harvested, cultured and clonally expanded *in vitro* with maintained functionality. Immunotherapy using expanded sentinel node acquired lymphocytes is feasible, safe without apparent side effects and seems to induce tumor regression in patients with colorectal cancer.

LIST OF PUBLICATIONS

I. Karlsson M, Marits P, Sherif A, Garske U, Thorn M, Winqvist O.
Detection of immune responses against urinary bladder cancer in sentinel lymph nodes.
European Urology, 2006, 49: 59-70

II. Karlsson M, Marits P, Dahl K, Larsson P, Wanders A, Thorn M, Winqvist O.
Sentinel node lymphocytes: tumour reactive lymphocytes identified intraoperatively for the use in adoptive immunotherapy of colon cancer.
British Journal of Cancer, 2006, 94: 1478-1484.

III. Karlsson M, Dahl K, Marits P, Dagoo T, Enerback S, Thorn M, Winqvist O.
Sentinel node CD4+ Th1-cells induce tumor regression in humans.
Submitted manuscript.

IV. Karlsson M, Nilsson O, Thorn M, Winqvist O.
Detection of metastatic colon cancer cells in the sentinel node by flow cytometry.
Submitted manuscript.

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen presenting cell
BCG	Bacillus Calmette-Guerin
BCR	B cell receptor
CA19-9	Carbohydrate antigen 19-9
CD	Cluster of differentiation
CEA	Carcino embryonic antigen
CK20	Cytokeratin 20
CRC	Colorectal carcinoma
CTA	Cancer-testis antigen
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
CV	Coefficient of variation
DC	Dendritic cell
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EpCAM	Epithelial cell adhesion molecule
FAP	Familial adenomatous polyposis
GM-SCF	Granulocyte monocyte colony-stimulating factor
HLA	Human leukocyte antigen
HNPCC	Hereditary non-polyposis colorectal carcinoma
HNSCC	Head and neck squamos cell carcinoma
IDO	Indoleamine 2, 3-deoxygenase
IFN	Interferon
IL	Interleukin
LAK cells	Lymphokine activated killer cells
MHC	Major histocompability complex
NK cells	Natural killer cells
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PRR	Pattern recognition receptor
SLN	Sentinel lymph node
TAA	Tumor associated antigen
TAP	Transporter associated with antigen processing
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper cell
TIL	Tumor infiltrating lymphocyte
TLR	Toll like receptor
Tregs	Regulatory T cells
UBC	Urinary bladder cancer
VEGF	Vascular endothelial growth factor
ELISA	Enzyme linked immunosorbent assay

BACKGROUND

CANCER

Cancer remains a major health problem worldwide, with more than 10 millions of new cases and 7 million deaths each year. The disease is characterized by uncontrolled division of cells, and the ability of these cells to spread to other sites and form metastases. There are many different forms of cancers, the majority of tissues can be subject to malignant transformation, and classification is based upon the tissue of origin. In this thesis the focus will be to explore the possibility of therapeutic intervention using immune cells, in two common solid tumors, urinary bladder cancer and colorectal cancer.

Urinary bladder cancer

Incidence

Worldwide, urinary bladder cancer (UBC) is the ninth most common cause of cancer with 360 000 new cases per year (Parkin et al., 2005). Bladder cancer is more prevalent in developed countries, and the incidence is increasing in most Western countries. The majority of tumors in the Western world are transitional cell carcinomas, which originate from the transitional epithelium of the bladder. The prevalence of UBC is also very high in Egypt and other parts of Northern Africa (el-Mawla et al., 2001). In these areas UBC is mostly of the squamos cell type and can be attributed to underlying infections with schistosomiasis. The incidence is approximately four times higher in men than in women, which reflects the more frequent exposure of men to the two major recognized environmental risk factors, tobacco smoking and aromatic amines (Negri & La Vecchia, 2001). In addition there appears to be some genetic involvment since the the risk significantly increases if first-degree relatives are affected (Plna & Hemminki, 2001).

Two divergent pathways

The overall mortality of urinary bladder cancer worldwide was 40% in 2002 (Parkin et al., 2005). When considering mortality it is important however, to distinguish between the two main phenotypic variants of urothelial carcinoma. Most tumors are thought to progress along a single pathway from benign to malignant by successive mutations in tumor suppressor and oncogenes (Hanahan & Weinberg, 2000), (Kinzler & Vogelstein, 1996). Whereas in urothelial carcinoma there appears to be two divergent pathways of tumorigenesis, and where the biological behavior and prognosis differ drastically (Wu, 2005).

Muscle invasive bladder cancer

About 20% of the patients suffer from high-grade, muscle-invasive tumors which are aggressive and associated with significant mortality (Wu, 2005). These tumors are characterized by defects in the p53 and retinoblastoma protein pathways. The golden standard of treatment in muscle invasive bladder cancer is radical cystectomy and

pelvic lymph node dissection. However, despite this aggressive treatment, almost 50% of the patients have recurrences and progress to systemic disease (Garcia & Dreicer, 2005). The use of neoadjuvant and adjuvant chemotherapy has shown limited benefits, but further research is needed before definitive conclusions can be drawn (Cruz M, 2005). The prognosis for systemic disease is very poor with a 5-year survival of little more than 5%.

Papillary tumours

The remaining 80% of urothelial carcinomas are low-grade, non-invasive papillary tumors, which develop along a p53 independent pathway. These tumors rarely invade the bladder wall or metastasize and are associated with a very good prognosis, and a 5-year survival of 90% (Wu, 2005). Standard treatment consists of transurethral resection, often combined with intravesical instillation of Bacillus Calmette-Guerin or chemotherapy, which has been shown to decrease the risk of recurrence (Sylvester et al., 2004), (Bohle et al., 2003).

Colorectal cancer

Incidence

Each year, more than 1 million cases of colorectal cancer (CRC) occur, making it the third most common form of cancer worldwide (Parkin et al., 2005). No gender specific differences are seen, the incidence is equally distributed in men and women. Approximately 50% of the patients diagnosed with colorectal cancer eventually die from the disease. Adenocarcinomas, originating from the glandular epithelium of the colon, constitute the vast majority of all colorectal cancers.

Etiology

It is believed that both genetic and environmental factors contribute to the development of colorectal cancers. A family history of colorectal cancer increases the life-time risk of developing the diseases, suggesting involvement of a genetic factor (Fuchs et al., 1994). Tumorigenesis has been thoroughly studied in colorectal carcinomas. In part this is due to that the sequential transition of cells from normal colonic epithelium, to adenoma and then to adenocarcinoma, easily can be followed. Furthermore, the existence of high penetrance genetic syndromes that predispose to colorectal carcinomas, has aided in the identification of several genes involved in tumor development.

Familial adenomatous polyposis (FAP) is a disorder with extensive adenomatous polyp formation in the colon, almost inevitable one or more of these polyps eventually progress and become malignant. This syndrome has been linked to inactivation or deletion of a tumor suppressor gene, an event which also has been shown to be common in sporadic CRC (Kinzler & Vogelstein, 1996). In hereditary non-polyposis colorectal carcinoma (HNPCC) the underlying genetic defect was found in one or more DNA mismatch repair genes, a genetic alteration encountered also in non-hereditary CRC (Kinzler & Vogelstein, 1996). However, even though FAP and HNPCC have contributed strongly to the understanding of genomic instability in colon cancer, only about 5% of CRC can be ascribed to genetic syndromes (Bodmer, 2006).

Environmental factors

The environment also plays an important role in the development of CRC. A Western life style is considered to increase the risk, it has been estimated that as much as 50% of the sporadic cases are caused by a Western type diet (Ahmed, 2006). A considerable number of factors have been investigated with regards to the risk for developing CRC, a high intake of tobacco, alcohol or meat as well as a high body mass index seem to increase the risk. In contrast a diet rich in vegetables, non-steroidal anti-inflammatory drugs, hormon replacement therapy and physical activity are factors associated with a decreased risk of CRC (Potter, 1999). Studies on migrants from low- to high risk areas support the importance of environmental factors. The incidence of CRC among immigrants and their descendants rapidly increase to the level of the host country, sometimes already in the first generation (Haenszel & Kurihara, 1968), (Le Marchand et al., 1997), (Stirbu et al., 2006).

Treatment

The main treatment modality is surgery, during which the tumor bearing bowel segment is removed together with draining lymph vessels and regional lymph nodes. For patients with localised disease (stage I-II) this treatment is curative in 80-90% of the cases, but when the disease has spread to regional lymph nodes the 5-year-survival-rate decreases to about 60% and when distant metastases are present the rate is less then 10% (O'Connell et al., 2004).

The use of chemotherapy in stage II patients has not been shown to give any statistically significant survival benefit (Benson et al., 2004) but in stage III patients 5-Fluorouracil-based therapy is considered the standard care and renders a survival benefit of 10% (Arkenau et al., 2003), (Haydon, 2003). In stage IV disease the use of chemotherapy can give both longer time to progression and overall survival, however life expectancy is still less than 12 months for the large part of patients and it is also important to consider the toxicity of chemotherapy and possible decrease in quality of life (Best, 2000).

THE IMMUNE SYSTEM

Innate and adaptive immunity

Our immune system protects us from infections with disease-causing microorganisms, ubiquitously present in our surroundings, and can be divided into innate and adapative immunity. The innate compartment of the immune system evolved first, and can be found in most multicellular organisms. If the physiological barriers, ie the skin, and the respiratory- and gastrointestinal epithelium, are breached the innate immune system provides the first line of defense. Cells of the innate immune system recognize invading organisms in a generic way, with so called pattern recognition receptors (PRR) for a number of highly conserved molecular structures. Toll like receptors (TLR) are an important family of such receptors that activates innate immunity (Pasare & Medzhitov, 2004). Phagocytosis by macrophages and neutrophilic granulocytes, is one defense mechanism which is initiated, and complement activation an other. Triggering

of PRRs also induces secretion of cytokines, inducing protective acute inflammation. In case the innate response is insufficient to clear the infection, PRR also provide a link to the adaptive immune system by activation of dendritic cells (DC). DCs, which are essential in the initiation of an effective adaptive immune response, express TLRs and binding of ligands induces DC maturation.

In vertebrates there is also a second line of defense, the adaptive immune system. It is distinguished by its specificity and long-term memory rendering protective immunity. T and B cells are the mediators of adaptive immunity, these cells are equipped with highly specific somatically rearranged receptors, capable of recognizing a large spectrum of antigens. The B cell receptor (BCR) is a membrane-bound immunoglobulin, and antigen recognition induces the secretion of soluble immunoglobulins with the same specificity. However, specific immune responses against tumours are largely attributed to cellular immunity, and the main aim of this thesis is to evaluate the feasibility of T cell based adoptive immunotherapy, therefore focus here will be T cells.

T lymphocytes

T cell precursors derive from the the bone marrow, but maturation into functional naïve T cells takes place in the thymus. Due to T cell receptor (TCR) rearrangements during T cell development, each T cell express a unique TCR which recognize a specific antigen. However, unlike the BCR which can bind to soluble, intact proteins, the T cell only recognize protein fragments, peptides. Furthermore, the peptide has to be presented by another cell on a particular molecule, the major histocompability complex (MHC). Two major subsets of T cells can be defined depending on the expression of two different coreceptors, CD4 or CD8. T cells which co-express CD8 recognize peptides presented on MHC class I, whereas CD4+ T cells are restricted to MHC class II.

There are three major subsets of T cells, cytotoxic T lymphocytes (CTL), T helper (Th) cells and T regulatory cells (Tregs). CTLs are restricted to antigens presented on MHC class I, which are expressed on all nucleated cells in the body, loaded with peptides from the cytoplasm. When an activated CTL recognise its antigen on the surface of a cell, cytotoxic effector molecules such as perforin, are released and disruptions in the cell membrane will induce cell death. In addition, CTLs express FasL on their surface, and by binding the Fas receptor on the target cell, apoptosis is induced.

Th cells are restricted to MHC class II, which are expressed only on professional antigen presenting cells (APC) of an hematopetic origin, ie DCs, B cells and macrophages. Traditionally Th cells are classified as Th1 or Th2, depending on which cytokines that are secreted. Th1 cells skew an immune response towards cellular immunity, characteristic cytokines are interleukin 2 (IL-2) and interferon γ (IFN- γ). Th2 cells induces an humoral immune response, isotype switching and maturation of B cells is stimulated, by secretion of cytokines as IL-4, IL-5 and IL-13. Recently also a third subset has been identified, this lineage has been called Th17 since it produces the proinflammatory cytokine IL-17 (Weaver et al., 2006).

Tregs are a relatively newly discovered subset of CD4+ T cells, which actively suppress activation of self-reactive T cells, thus preventing autoimmunity (Yamaguchi & Sakaguchi, 2006). In addition to the naturally occurring Tregs which develop their phenotype in the thymus, there are adaptive Tregs which are induced from naïve CD4 cells in the periphery (Wing et al., 2006).

Antigen recognition in the lymph node

The general view today is that activation of naïve lymphocytes takes place in secondary lymphoid organs, such as spleen and lymph nodes (Fig. 1). More particular in the secondary lymphoid organ draining the site of antigen entry (Itano & Jenkins, 2003). Blood borne antigens will thus mainly be detected in the spleen, whereas lymph nodes are the recipients of antigens from peripheral tissues. The lymph node provides a specialised microenvironment for the complex interaction between immune cells that occurs during the initiation of an immune response (Castellino & Germain, 2006).

Antigen presenting cells, in particular DC, are of great importance during the initiation of an adaptive immune response. When a DC is activated, a maturation process starts during which the phagocytic and antigen presenting capacity is initially increased. Phagocytosis then decreases, and antigen loaded DCs migrate to the draining lymph nodes where processed antigens are presented to Th cells (Shakhar et al., 2005) (Hugues et al., 2004). Activated Th cells promote further activation of APCs, which then secrete chemokines and cytokines attracting cytotoxic T cells (CTL) (Castellino & Germain, 2006). CTLs can also be activated against exogenous antigens by DCs, due to their capability of crosspresentation (Guermonprez et al., 2003), (Houde et al., 2003).

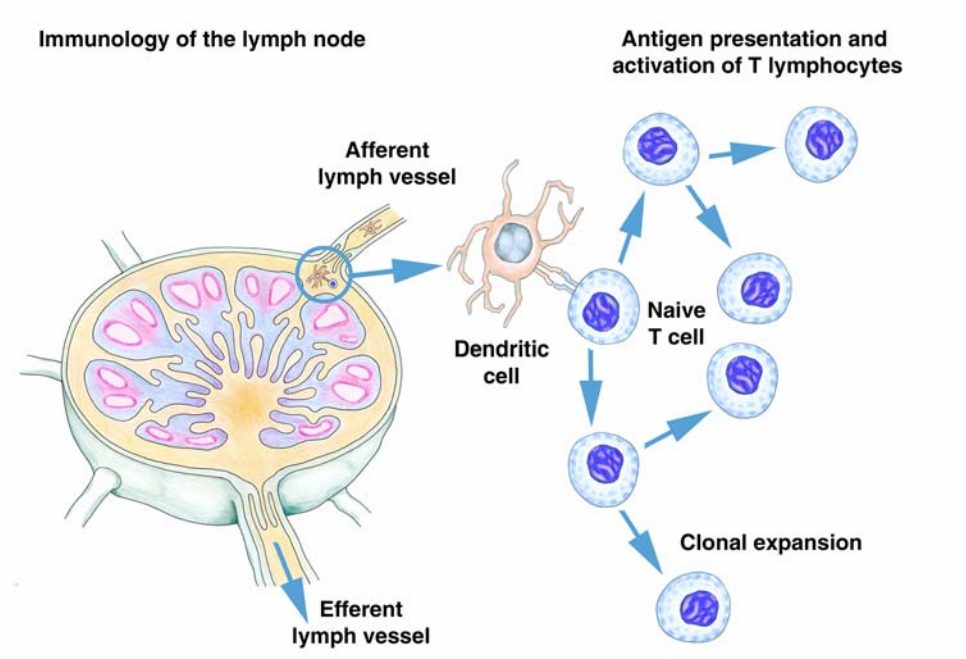

Figure 1. *Dendritic cells enter the lymph node with the afferent lymph vessels, and presentation of endocytosed antigens takes place in the organised microstructure of the lymph node. The presence of foreign antigens and an activated state of the antigen presenting cell will induce activation and proliferation of T cells specific for the presented antigen.*

The state of the APC is vital for the outcome of the meeting between the lymphocyte and the presented antigens. An APC with an activated phenotype, activate naïve T cells, in contrast to APC with immature phenotype which promotes a state of tolerance. This is due to that activated DCs display high levels co-stimulatory molecules, such as B7.1 and B7.2, which interacts with CD28 on T cells. For a T cell to become activated both a primary signal from recognition of the antigen via the TCR, and a second signal by binding of CD28 is required. However, when a T cell recognizes its cognate antigen, but does not receive a secondary signal the antigen is instead tolerized. Tolerance can be achived by rendering T cells apoptotic or by the induction of an anergic state, characterized by unresponsiveness to restimulation. Furthermore, tolerance can also be induced by the activation of Tregs.

Immunological memory

After clearance of the foreign antigens, the immune response subsides, most effector cells undergo apoptosis. However, an adaptive immune response also give rise to memory cells, of both the T helper and cytotoxic T cell subset. These cells circulate and upon re-encounter of the antigen, a secondary immune response which is stronger and faster than the primary is initiated. It appears that the presence of CD4+ T cells are important for the creation of an adequate immunological memory. Lack of Th cells can lead to that an immunological memory does not correctly emerge (Janssen et al., 2005), (Antony et al., 2005). The importance of CD4+ T cell help has been demonstrated in an animal model, where the adoptive transfer of Th1 CD+ T cells could induce tumor regression (Surman et al., 2000).

TUMOR IMMUNOLOGY

Tumor immunosurveillance

The general idea, that intrinsic mechanisms of the immune system could protect against the development of malignant disease, was initially proposed a century ago (Ehrlich, 1909). The term immunosurveillance was phrased later in the 20th century by Thomas and Burnet (Thomas, 1959), (Burnet, 1970). According to the immunosurveillance hypothesis transformed cells are recognized by the immune system as foreign, and can induce an immune response. Tumors can occasionally arise, and be eliminated, without ever becoming clinically detectable. For several decades the hypothesis was controversial, much due to that the initial experiments in nude mice did not support an increased susceptibility to neither chemically induced nor spontaneous tumors (Stutman, 1974), (Rygaard & Povlsen, 1974).

Experimental support of immunosurveillance

However, it has since been shown that the adaptive immune system is not altogether obliterated in nude mice, and moreover that the intact innate immune system probably also renders a certain degree of protection against tumors. New molecular techniques, have provided possibilities to study much more specific models of immunodeficiency, in a variety of knockout mice. Experiments in RAG2$^{-/-}$ and/or IFNGR1$^{-/-}$ mice, ie lacking functional T and B cell receptors and/or IFNγ-insensitive, have provided strong experimental support of the immunosurveillance hypothesis. Knockout mice were more

susceptible to chemically induced carcinogenesis and most important, an increased incidence of carcinomas in the breast, intestine and lung was seen compared to wild type mice (Shankaran et al., 2001). The importance of functional effector cells has been demonstrated in mice lacking perforin ($pfp^{-/-}$), a cytolytic protein secreted by cytotoxic T cells and NK cell. $Pfp^{-/-}$ knockout mice were more susceptible to tumor formation, when challenged with tumor cell lines or carcinogenic chemicals (van den Broek et al., 1996).

Cancer immunosurveillance in humans

Evidence is accumulating that data collected from experimental settings in animal models also have relevance in humans. Several follow-up studies of transplant patients, receiving immunosuppressive drugs, have shown an increased risk of not only virally induced tumors but also of malignancies of a non-viral etiology. In a follow-up study of more than 28,000 patients with kidney transplants, a significantly increased incidence was seen in virus related malignancies as well as in spontaneous tumors (Vajdic et al., 2006). In agreement with this a long-term study of renal transplant recipients in Nordic countries between 1964-1985, showed a 2- to 5-fold excess risk for cancers of the colon, larynx, lung and bladder (Birkeland et al., 1995).

The existence of tumour infiltrating lymphocytes (TILs) further indicates that an immune response is initiated aginst the tumour. A correlation between TILs and improved prognosis, was seen in transitional cell carcinoma in the bladder (Lipponen et al., 1992). In patients with ovarian carcinoma, the presence of TILs correlated to an significant increase in five-year overall survival, 38 percent compared to 4.5 percent when TILs were absent (Zhang et al., 2003). A more detailed examination, with regards to the phenotype, was performed of the TILs in colorectal cancer and it was reported that the presence of activated and memory CD3+ T cells was advantageous for survival (Pages et al., 2005).

Spontaneous tumor recognition

An important milestone in the field of tumor immunology has been the identification of tumour associated antigens (TAA), providing further support for the idea of a naturally occurring immune response against malignancies. The first tumour antigen, MAGE-1 was identified from malignant melanoma in1991 by van der Bruggen et al (van der Bruggen et al., 1994). Since then a large number of antigens has been identified, many of these in malignant melanoma, and the majority MHC I restricted. However, an increasing number of antigens have been identified in other malignancies up to date, as well as several MHC class II restricted peptides (Novellino et al., 2005).

TAAs are commonly divided into four categories:
1) Cancer-testis antigens (CTA). These antigens are expressed in tumors of many various types, but normal expression in adult somatic tissues is restricted to male germ cells in the testis. The first identified TAA MAGE-1 is a cancer-testis antigen.
2) Differentiation antigens. TAAs in this category are associated with the differentiation of a particular cell type, and are shared between the tissue of origin and the tumor, for example tyrosinase in melanoma.

3) Widely occurring overexpressed tumor associated antigens. Certain proteins, which are expressed in a wide variety of normal tissue, are expressed to a much higher degree in tumors. Examples are epithelial cell adhesion molecule (EpCAM) and HER2/neu.
4) Unique and shared tumour specific antigens. These antigens are unique for an individual malignancy and result from mutations, and alterations, in genes ubiquitously expressed during transformation.

The number of antigens identified in CRC and UBC are less abundant than in malignant melanoma but some progress has been made. Immunohistochemical analyses of 94 samples of invasive bladder tumors showed that 77% expressed at least one, and 61% more than one, of the seven cancer-testis antigens which were investigated (Sharma et al., 2006). In a subsequent study, CTLs specific for the cancer-testis antigen NY-ESO-1, were isolated from peripheral blood (Sharma et al., 2007). The presence of cancer-testis antigens has been investigated also in CRC. In accordance with previous studies, the frequency of CRC tumors and metastatic lesions expressing CTAs, were low (Alves et al., 2007). However in two patients, whose tumors expressed NY-ESO-1 or MAGE-A3, T cell responses towards the respective antigen were detected. The antigen most commonly associated to CRC is carcinoembryonic antigen (CEA). During follow-up of patients with CRC monitoring of CEA levels in the serum is an important clinical parameter. Initially CEA was classified as a CTA, but it has since been found in normal tissue and is currently classified as a differentiation antigen (Novellino et al., 2005).

Immunoediting

Tumors do, however, arise in immunologically competent hosts, which means that transformed cells have developed mechanisms to circumvent immunosurveillance. Moreover, evidence is accumulating that a selective pressure from the immune system promotes the growth of tumor escape variants and promote less immunogenic tumors. The broadened term immunoediting has been proposed to better describe the interactions between the immune system and the tumor (Dunn et al., 2002). This concept includes three possible outcomes of immunosurveillance: elimination, equilibrium or escape.

Experimentally the theory that a functional immune system will select for less imunogenic tumors has been tested in knockout mice, deficient in functional T and B cell receptors and/or IFN-γ insensitive (Shankaran et al., 2001). Tumours from wild-type mice grew progressivly when transplanted to either wild-type or knockout mice. In contrast tumours generated in the immunodeficient knockout mice were rejected when transplanted to immunocompetent wild-type mice, indicating a more immunogenic state.

Nude mice have also been used as a model, chemically induced sarcomas from either T cell-deficient nude mice or congenic T cell-competent mice, were transplanted to T cell competent nu/+ mice (Boesen et al., 2000). The rejection rate of tumors from immunodeficient hosts was almost twice that of sarcomas originating from T cell competent mice. Furthermore, depletion of cytotoxic T lymphocytes in the nu/+ immunocompetent hosts, prior to transplantation lead to acceptance of sarcomas otherwise rejected.

Immunoselection for escape variants in humans was observed in a patient with malignant melanoma receiving vaccination with gp100, MART-1 and tyrosinase peptides (Khong et al., 2004). The initial response to treatment was extensive, with regression of metastases in multiple sites, but after a year the patient got recurrent disease. In one recurrent lesion, several tumor antigens were still expressed but complete loss of MHC class I was found. Another new tumor instead had continued expression of MHC class I but no longer expressed melanoma antigens, previously found in tumors from the patient.

Escape mechanisms

It thus appears that tumor cells displaying different mechanisms of tumor escape are selected for during tumor development. Tumor immune escape has grown into a large field of research, and numerous ways in which transformed cells avoid immune rejection, has been described.

Induction of tolerance and dendritic cells

Induction of tolerance is one possible cause of tumor escape. Inflammation does not normally occur at the tumor site until later stages, when fast growth and insufficent bloodflow leads to necrosis. Thus, due to lack of inflammation during initial stages of tumor development DCs ingesting tumor debris, will not receive any activation signals and remain immature. Experimentally this has been investigated in sporadic tumor model, transgenic mice were created by the insertion of a construct containing the tumor inducing SV40 T antigen silenced by an attenuator (Willimsky & Blankenstein, 2005). Rare, random events in a cell occasionally quench the attenuator leading to cell transformation, and expression of an highly immunogenic antigen. Transplantion into syngenic, immunocompetent mice resulted in tumor rejection, indicating a state of tolerance rather than loss of immunogenicity.

Tumor burden also appears to directly affect DC function in several ways. A study of patients with breast, head and neck, and lung cancer tumor draining lymph nodes and peripheral blood were investigated with regards to the presence of and function of DCs (Almand et al., 2000). A dimished capacity to activate T cells were identified in both lymph nodes and blood, in addition the amount of DCs present were decreased and an increased fraction displayed an immature phenotype.

Immunosuppression induced by regulatory T cells

Activation of Tregs appears to play a role in tumor immune escape. A significant correlation between the presence of Tregs in the tumor and survival was shown in patients with ovarian carcinoma (Curiel et al., 2004). Transfer experiments of wild-type splenocytes to nude mice support the hypothesis that Tregs might mediate tumor immune evasion (Shimizu et al., 1999). If the splenocytes were depleted of CD4+CD25+ cells prior to transplantation the athymic nude mice were capable of rejecting the following tumor challenge, whereas mice reconstituted with nondepleted splenocytes developed tumors.

Loss of antigen presentation and processing

Varying defects in antigen presentation have been identified in numerous malignancies. Alterations in the expression of MHC class I is one impairment which frequently has

been observed, including total loss of HLA or merely loss or down-regulation of specific haplotypes (Algarra et al., 2004). However, total loss of MHC does render cells vulnerable to lysis by natural killer (NK) cells (Ljunggren & Karre, 1990). The non-classical MHC-like molecule HLA-G NK cell lysis. Indeed, expression of HLA-G has been detected in several types interacts with inhibitory receptors of NK cells, and expression of HLA-G could infer protection against of cancer, including colorectal cancer, where expression correlated to a significantly shorter survival time (Ye et al., 2007).

Other levels in the antigen processing might also be affected. In small cell lung carcinoma cell lines mRNAs coding for transporter associated with presentation (TAP) 1 and TAP-2, as well as for parts of the immunoproteasome, were absent which was consistent with failure to process antigens (Restifo et al., 1993). Decreased immunogenicity, by loss of tumor antigens, is another way of avoiding tumor reactivity. An investigation of expression of the melanoma differentiation antigens, tyrosinase, gp100 and MART-1, showed that decreased expression was associated with disease progression (de Vries et al., 1997). Downregulation of antigen expression has also been observed in residual tumor burden after peptide vaccination with melanoma differentiation antigens (Jager et al., 1996), (Lee et al., 1998).

Secretion of immunosuppressive factors

Tumor immune escape has also been attributed to secretion of immunsuppressive cytokines, for example interleukin 10 (IL-10) and tumor growth factor β (TGF-β). Melanoma cells, preferentially in metastatic lesions, secret IL-10 and this has been correlated to impairments in DC functionality (Gerlini et al., 2004). In a transgenic animal model, expressing IL-10 under the control of the IL-2 promotor, the immunosuppressive mechanism of IL-10 in lung cancer was dissected. IL-10 was found to negatively effect the function of both DCs and CTLs, and reduce the capacity to produce Th1 cytokines (Sharma et al., 1999).

TGF-β is an immunoregulatory cytokine with great importance in the control of an immune responses, and maintenance of homeostasis and tolerance. (Li et al., 2006). High levels of TGF-β has been observed in several malignancies, and correlated to a worse prognosis (Gorsch et al., 1992), (Robson et al., 1996). Indoleamine-2,3-dioxygenase (IDO) is an enzyme which degrades tryptophan, an amino acid essential for the activation of T cells. Expression of IDO was found in large variety of human tumors, and in mice previously immunogenic tumors were no longer rejected if production of IDO was induced in the tumor cells (Uyttenhove et al., 2003).

Furthermore, impairments in the T cell signalling cascade has been seen in patients with malignant disease. Downregulation of the CD3ζ chain, a constituent of the TCR receptor signalling complex essential for T cell activation, is a common defect. This is in part believed to be caused by factors secreted by the tumor, but direct interactions between tumor and T cells is also believed to contribute (Whiteside, 2004).

THE SENTINEL NODE

The sentinel node concept

The sentinel node is defined as the first true tumor draining lymph node and it is often the first site of metastases (Wiese et al., 2000), (Leong, 2004). Since the presence of metastases is an extremely important prognostic factor, the sentinel node is of great interest for accurate tumor staging. It is not possible to predict how a tumor connects to the lymph flow, and the localisation of the sentinel node needs to be determined in each separate case (Fig. 2). It can be detected pre-and/or peroperatively, by injections around the tumor of either radioactivity or vital dyes, which then is transported by the lymphatics to the draining node.

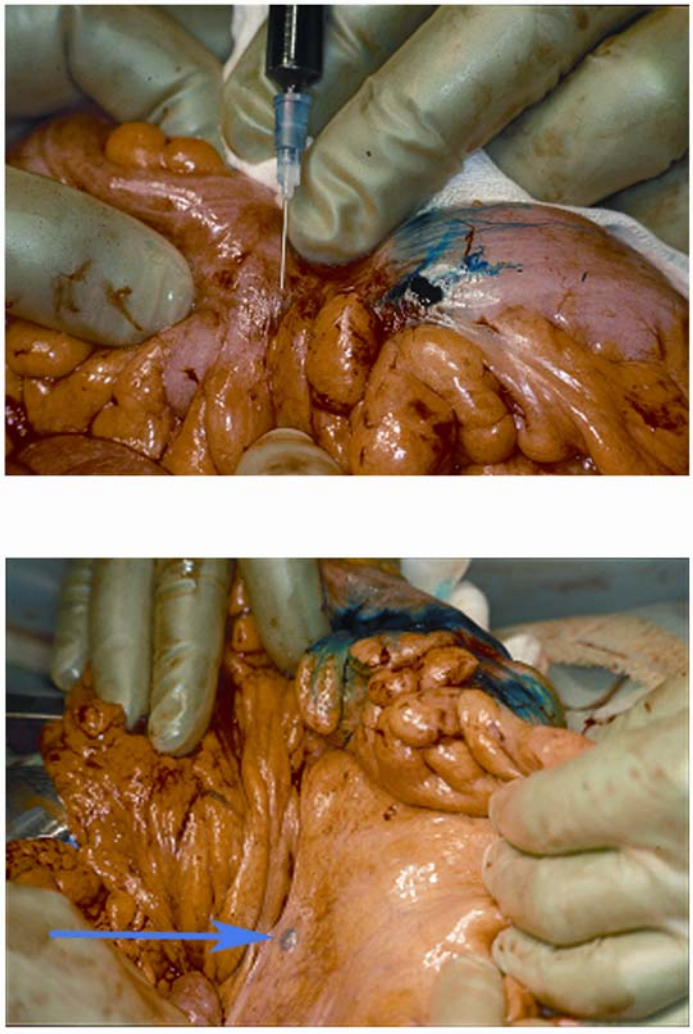

Figure 2. *Intraoperative injection of patent blue dye, during surgery of an patient with colorectal carcinoma (upper panel). The tracer dye follows the lymph flow from the tumor, and after approximately 5 minutes blue colored sentinel nodes can be identified in the mesentery ((lower panel). The sentinel nodes are marked by a suture and removed following resection of the bowel segment.*

Identification of the sentinel node was introduced by Cabanas in 1977, with the aim of increasing accuracy of staging in penile carcinoma (Cabanas, 1977). The technique was further developed, and put into clinical practice by pioneering work in malignant melanoma (Morton et al., 1992). Currently, the method is well established in malignant melanoma and breast cancer (Leong, 2004). In these malignancies the metastatic status of the SLN is regarded as representative for the entire lymphatic field and form the base for deciding whether or not an extended lymph adenectomy will be done.

In colorectal- and urinary bladder carcinoma

The sentinel node technique has also been shown to be applicable in colon cancer and urinary bladder cancer (Saha et al., 2004) (Thörn, 2000) (Sherif et al., 2001). In colorectal cancer, unlike in malignant melanoma and breast cancer, sentinel node identification is not made in an attempt to reduce the extent of the lymphadenectomy. In contrast, if aberrant or unexpected lymphatic drainage is detected, the lymphadenectomy might be more radical.

In urinary bladder cancer the lymph node dissection is traditionally limited to the obturator fossa bilaterally. However, when sentinel node identification was applied three out of four SLNs were detected outside of this region, indicating the need for an extended lymphadenectomy (Sherif et al., 2001). Thus, sentinel node identification in CRC and UBC mainly aim at increasing the likelihood of curative surgery and correct staging. Identification of the sentinel node show great potential to increase accuracy of staging in both malignancies (Dahl et al., 2005) (Liedberg et al., 2006).

Immunology of the sentinel node

The increasing knowledge of how an adaptive immune response is initiated, has also created an interest in the sentinel node from an immunological point of view. DCs loaded with tumor derived peptides, and soluble tumor antigens, follow the lymphatic drainage to the sentinel node, where it is presented to circulating lymphocytes. Primary tumor draining lymph nodes are therefore the site of encounter between the adaptive immune system and tumor antigens, and the natural location for an anti-tumoral response to be initiated (Fig. 3).

Since immunomodulators and other molecules produced by tumor cells and tumor stroma, also are transported with the lymph, the sentinel node is extensively exposed to tumor induced immunosuppresssion (Cochran et al., 2006). The sentinel node is thus subject to both activation and suppression, and in accordance with this studies show varying results with regards to the activation status of the sentinel node. A comparison of the cytokine profile in SLNs draining melanomas, versus non-draining lymph nodes, showed that levels of interferon-γ (IFN-γ), IL-2 and granulocyte monocyte colony stimulating factor (GM-CSF) were upregulated in the sentinel nodes (Leong et al., 2002). In contrast, there were no difference in the levels of IL-10, and furthermore when micrometastases were present in SLNs the differences levelled out.

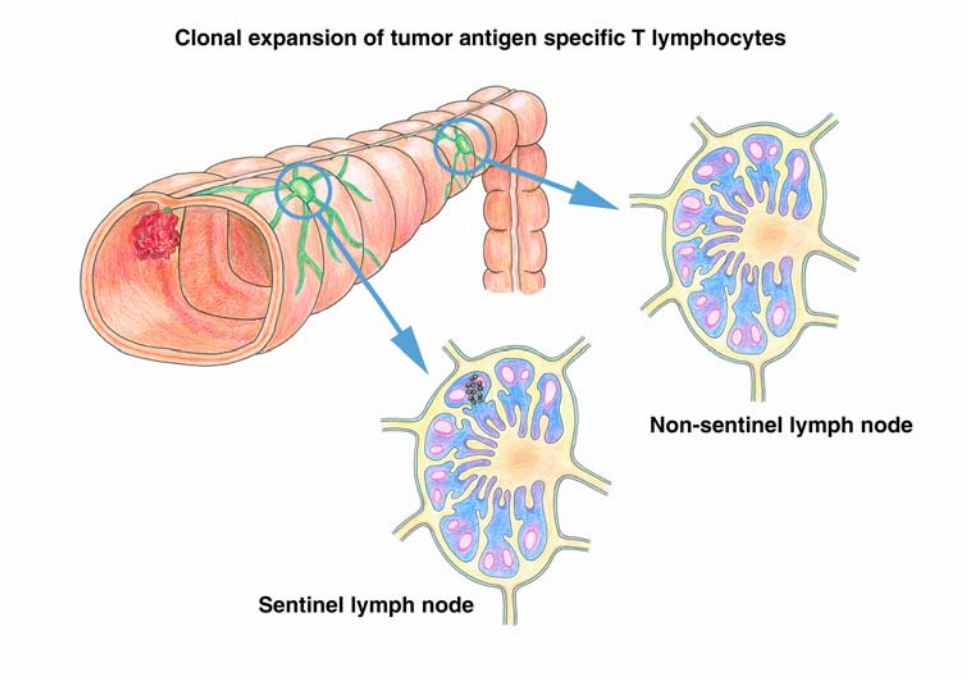

Figure 3. *The tumor draining lymph node will recieve tumor debris and dendritic cells loaded with tumor antigens and constitutes the initial site of encounter between the adaptive immune system and the tumor. Non-sentinel nodes will not contain any tumor antigens and tumor-reactive lymphocytes will not be activated.*

IMMUNOTHERAPY

Over the years, numerous attempts to recruit the intrinsic power of the immune system in the fight against cancer have been made. The different approaches are ususally classified as non-specific versus specific, and active versus passive. The instillation of Bacillus Calmette-Guerin (BCG) used in low-grade, non-invasive urothelial carcinomas is an example of non-specific, passive immunotherapy (Bassi, 2002). In animal models, it has been demonstrated that the effect of BCG is T cell dependent (Ratliff et al., 1993). Distribution of the T cell growth factor, interleukin 2 (IL-2), is another clinically used non-specific, passive immunotherapy, mainly applied in malignant melanoma and renal carcinoma. IL-2 therapy alone has shown significant antitumor effects, but only in a limited number of patients (Rosenberg et al., 1994).

Passive specific immunotherapy

Distribution of antibodies is a specific, passive immunotherapy. In colon cancer some success with prolonged disease free- and overall survival has been seen. Bevacizumab is a monoclonal antibody against vascular endothelial growth factor (VEGF), with antiangiogenic properties. In a study with 813 patients receiving chemotherapy in combination with either bevacizumab or placebo, the overall survival increased from 15.6 to 20.3 months (Hurwitz et al., 2004). Increased overall survival were also seen in a study with an antibody specific for the endothelial growth factor receptor (EGFR) (cetuximab) (Cunningham et al., 2004). In urinary bladder cancer animal models suggest that the use of antibodies could be advantageous, particularly against EGFR and Her/Neu2, which are commonly expressed in transitional cell carcinomas of the bladder (Bellmunt et al., 2003).

The use of immunomodulatory antibodies is another interesting approach, one example of this is antibodies directed at cytotoxic T lymphocyte-associated antigen 4 (CTLA-4). Expression of CTLA-4 is induced upon activation of T cells. Ligand binding of CTLA-4 mediates downregulation of T cell activity, one important mechanism of controling the extent of an immune response. Blockade of this receptor is believed to prevent inhibition of activated T cells. After promising results in animal models CTLA-4 blockade was tried in 14 melanoma patients with progressive disease, in combination with peptide vaccinations (Phan et al., 2003). Autoimmune manifestations were induced in six patients, in three cases the autoimmunity concurred with objective responses of tumor regression. It appears that CTLA-4- blockade is a possible way to overcome tolerance, two complete and one partial response was seen.

Active immunization

Active immunotherapy aim at eliciting or amplifying a prevalent but insufficent, immune response against a specific antigen, and the induction of an immunological memory. Numerous variants of active immunization has been tried including use of peptides, whole tumor cells, tumor cell extract, DNA coding for tumor antigens and antigen loaded DCs. The relatively recent identification of tumor antigens has had a large impact on this field, since it made specific vaccinations possible.

Unfortunatly the clinical success has been limited, in spite of ample attempts. In a review by Rosenberg et. al., the number of objective responses in 35 vaccination trials were evaluated (Rosenberg et al., 2004). Together the 35 studies represented treatment of 765 patients, with metastatic disease but of different primary origins, and objective responses were only detected in 29 of these, corresponding to a response rate of 3.8%.

The lack of clinical reponses can be explained by a number of factors. In the light of recent findings about thymic expression of tissue restricted antigens, including non-mutated shared tumor antigens, the choice of antigen is one important factor to consider (Kyewski & Klein, 2006). Thymic expression of these antigens will induce central tolerance, by negative selection of T cell clones with high avidity, and possibly by induction of naturally occurring Tregs. In addition, cancer vaccination is often attempted in patients with compromised immune responses due to tumor burden, but also often due to old age. It is possible therefore that more efficient adjuvant settings, can help overcome tumor escape and initiate a sufficient cellular immune response against the tumor.

Increasing insights to the complex function of the immune system, and the balance between tolerance and activation can hopefully improve the outcome of cancer vaccination. In a small trial, 12 patients with colorectal- or non small cell lung cancer displaying progressive disease refractory to chemotherapy, were vaccinated with DCs loaded with an altered, more immunogenic carcino embryonic antigen (CEA) peptide (Fong et al., 2001). It appeared that tolerance aginst the self antigen CEA was broken, CTLs recovered after vaccination could lyse CEA positive tumor cells. Furthermore, tetramer staining showed that CTLs specific for the altered peptide also recognized the unaltered CEA peptide, but more importantly, complete responses were seen in two patients.

Adoptive immunotherapy

Adoptive immunotherapy entails the collection of tumour-reactive lymphocytes, which after *ex vivo* expansion and activation, are given back to the patient as an intravenous infusion. The use of adoptive immunotherapy is particularly well explored in malignant melanoma, by the pioneering work of Rosenberg and colleagues. Prior to the discovery of the T cell growth factor IL-2, it was difficult to maintain lymphocyte cultures. The identification and subsequent cloning of the gene coding IL-2, made the growth factor available in large amounts. The discovery that normal lymphocytes after co-cultivation with IL-2 were capable of lysing fresh autologous tumor samples created a surge of interest in these lymphokine activated killer (LAK) cells (Lotze et al., 1981). When tried in the clinic, a limited number (8/106) patients with severe metastatic melanoma responded with complete tumor regression, to treatment with adoptive transfer of LAK cells in combination with IL-2 (Rosenberg et al., 1987).

These results were encouraging, and by applying the increasing knowledge of basic immunology and new techniques improved responses has been achived. In murine models it was found that TILs were much more capable of inducing tumor rejection and that lymphodepletion prior to T cell transfer further increased the effect of treatment (Rosenberg et al., 1986). A combination of lymphodepletion, adoptive transfer and expanded TILs and high dose IL-2 was tried in a clinical setting in patients with metastatic melanoma refractory to treatment with high-dose IL-2 and chemotherapy (Dudley et al., 2005). Objective responses were seen in 18/35 patients, out of which three were complete responses.

In invasive urinary bladder carcinoma adoptive immunotherapy is basicly unexplored, whereas in colorectal carcinoma there has been attempts at adoptive immunotherapy. In parallell to clinical trials in malignant melanoma, 19 patients with metastatic or inoperable colorectal carcinoma were treated with LAK cells and high dose IL-2 (Hawkins et al., 1994). The overall objective response rate was no more than 5%, one patient had a complete response, with a duration of eight months. Gardini et al investigated the possible anti-tumoral effect of adoptive immunotherapy with TILs, in patients undergoing liver resection due to colorectal recurrences (Gardini et al., 2004). Similar to the results from treatment with LAK cells the response was poor, no significant overall or disease free survival were found in the group of patients receiving immunotherapy.

Several stategies are under evaluation in order to improve the outcome of adoptive immunotherapy. The possible use of T cells transduced with a transgenic, tumor specific TCR, was investigated in patients with malignant melanoma (Morgan et al., 2006). It was demonstrated that the method is technically feasible and that transgenic T cells remained a long time in the recipient. The use of T cells transduced with a chimeric immunoglobulin TCR gene, expressing the specificity of a monoclonal anti-CEA antibody fused with CD28 and CD3ζ-chain, so called T-bodies is another attempt to create tumor specific T cell responses (Sasaki et al., 2006). PBMC from a patient with colon cancer was transduced and cytolytic activity and induction of IFN-gamma was seen in response to an autologous tumor cells.

AIMS

The overall aim of this thesis was to explore the feasibility of using sentinel node aquired lymphocytes, of the T helper cell subset Th1, for adoptive immunotherapy of cancer.

The specific aims were:

- To investigate the presence of anti-tumoral responses in sentinel nodes from patients with urinary bladder cancer.

- To investigate the presence of tumor reactive lymphocytes in sentinel nodes from patients with colorectal cancer.

- To evaluate flow cytometric detection of lymphatic metastases in patients with colorectal cancer, using direct fluorophore conjugated antibodies against multiple extracellular tumor associated antigens.

- To perform a pilot study, using sentinel node based immunotherapy with the main objectives, to explore the feasibility, safety, adverse events and monitor effects of treatment on tumor progression.

MATERIAL AND METHODS

PATIENTS

Fourteen patients undergoing cystectomy due to urinary bladder cancer were included in the study in paper I. The average age at diagnosis was 65 years, 11 patients (78%) were male and 3 female. Adjuvant therapy had not been administered to any of the included patients. The motivation for performing a radical cystectomy in patient number one, staged as T1G3, without prior attempts to treat with BCG or mitomycin instillation, was the additional presence of multiple carcinoma in situ. At the time of surgery none of the patients had known distant metastases.

In paper II fifteen patients diagnosed with colon cancer, and suitable candidates for sentinel node idenification, were included. None of the patients had signs of distant metastases or lymph node involvement prior to surgery. The included patients had an average age of 71 years, seven male and eight female.

Sixteen patients diagnosed with colorectal cancer, undergoing surgery either to remove the primary tumor or due to recurrence of disease, were included in paper III. The average age of the treated patients was 62 years, there were six women and ten men. The patients were staged after histopathological evaluation, there were five cases of Dukes' B, two cases of Dukes'C and 9 cases of Dukes' D. The included Dukes' B patients all had aggressive tumors as characterized by the presence of ulcerations, perineural or perivascular invasion. Three patients (No.s 9, 10 and 14) had recurrence of the disease, after previous resection of the primary tumor, abdominally (No. 9), metastases in the liver (No.s 10 and 14).

In paper IV sentinel nodes from two patients, diagnosed with colon cancer and suitable candidates for the sentinel node technique, were examined. The first patient, a man age 65 years, had metastases in the liver and underwent a liver resection. Patient two, a woman age 77 years, was undergoing surgery due to a primary tumor in the colon.

All studies were approved by the local ethical committee and informed consent was given by included patients.

Identification of the sentinel node

The sentinel node is identified pre- and/or intra operatively, by the use of tracer substances, injected around the tumor. The exact procedure is adjusted to the anatomical location of the tumor.

In urinary bladder cancer both pre- and intra operative detection was applied. Before surgery, a radioactive tracer (Albures, Scanflex, Nycomed Amersham), was injected transurethrally, in the muscular wall of the bladder around the tumor margin. One to four hours later patients underwent lymphoscintigraphy. In addition to the preoperative sentinel node detection, Patent blue dye (Guerbet, paris) was injected transurethrally immediately before surgery staining draining lymph nodes blue. Sentinel nodes were

thus identified during lymph node dissection, by their blue color and/or detection of radioactivity with a handheld γ-tube.

Identification of sentinel nodes in colorectal carcinoma was performed peroperatively. After mobilisation of the tumor site according to standard surgical procedure, Patent blue dye was injected in the serosa surrounding the tumor. Usually within 5-10 minutes the blue tracer stained tumor draining lymph nodes blue. These lymph nodes were marked with sutures and dissected after resection of the bowel segment.

Preparation of specimens

Sentinel- and non-sentinel lymph nodes were cut in half, and 1 mm thick slices were sampled from the central and peripheral parts. The remaining part of the lymph nodes were sent for routine histological evaluation. A part of the tumor or metastases, including a sample of the invasive margin, was provided for analyses and as antigen source. The tumor, or metastasis, was sent for examination by a pathologist to determine if the removal was radical and for morphological classification.

Follow-up and clinical evaluation

Patients were clinically evaluated according to the regular follow-up program after diagnosis and treatment of colon cancer in Sweden. Follow-up visits usually took place 3, 6, 12 and 18 months after cell transfusion. On each of these occasions the general condition was estimated and the amount of carcino embryonic antigen (CEA) in blood measured. Every six months the patients were also examined for the presence of liver and lung metastases by ultrasound, X-ray or computer tomography (CT) scan, or fluorodeoxyglucose positron emission tomography (FDG-PET). Assessment of the clinical response was made by comparison of radiographic measurements and physical examinations before and after treatment using WHO criteria. The complete disappearance of all evaluable disease was defined as a complete response. A partial response was defined as a decrease, equal to or greater than 50%, in the sum of the products of perpendicular diameters of all measurable lesions. Stable disease was defined as no signs of clinical or radiological tumour progression.

CELL PREPARATION AND CULTURE

Preparation of single cell suspensions

The sentinel nodes, tumor, non-sentinel lymph nodes (I and II) and samples of venous blood were taken care of immediately. Single cell suspensions from tissue specimens were obtained by gentle pressure in a loose fit glass homogenizer. During preparation the material was kept on ice. Peripheral blood monnuclear cells (PBMC) were extracted from the venous blood by density centrifugation (Ficoll-paque, GE Healthcare). After preparation cells were either put in long term culture or subject to analyses.

Ex vivo culture of sentinel node aquired lymphocytes (I and III)

For long term ex vivo cell culture of sentinel node lymphocytes, single cell suspensions were prepared in the manner described above, by homogenisation in a loose fit glass homogenizer.

In paper I, the feasibility of long-term ex vivo cell culture of sentinel node lymphocytes from patients with urinary bladder cancer, was investigated. The cells were kept in RPMI 1640 (Life technologies), supplemented with 10% human serum (Sigma), 100 units/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma). Recombinant interleukin-2 (IL-2) (Sigma), a major T cell growth factor, was added every 3-4 days. Autologous tumor extract was prepared by homogenisation with an Ultra Turrax in 5 volumes (w/v) 2 x PBS followed by denaturation for 5 minutes at 97˚C. To provide antigenic stimulation and promote tumor reactive lymphocytes autologuos tumor homogenate was added to the culture.

The objective in paper III was to investigate the possible use of sentinel node aquired lymphocytes in adoptive immunotherapy of colon cancer. A serum free medium of clinical grade, AIM V (Invitrogen), was therefore used for preparation of single cell suspensions as well as for culture. Every 3-4 days a low dose of IL-2 (Proleukin®, Chiron) was added to the cell culture. The cells were normally maintained ex vivo for 4-5 weeks, during which time autologus tumor extract (prepared as described above) was added at the start of culture and after 2-3 weeks. At restimulation after 2-3 weeks radiated autologous PBMC were added simultaneously to provide antigen presentation. Previous to transfusion viable cells were retrieved by density centrifugation (Ficoll-paque, GE Healthcare) and investigations for microbial presence were performed. On the day of transfusion cells were washed in saline solution (Sodium clorid Baxter Viaflo 9 mg/ml, Baxter)and resuspended in saline solution supplemented with human serum albumin (Baxter) in a transferbag. Infusion of the cells were performed during 1-2 hours under professional medical supervision.

The DLD-1 colon cancer cell line (IV)

In paper III we aimed at evaluating flow cytometric detection of tumor cells in single cell suspensions. For this purpose the DLD-1 colon cancer cell line (ATCC number CCL-221) was purchased from American tissue culture center (ATCC). The cell line was grown in RPMI 1640 (Sigma) supplemented with 10% (v/v) bovine growth serum (BGS) (Hyclone), 100 units/ml penicillin and 100 µg/ml streptomycin (Hyclone) and 2 mM L-glutamin (Hyclone) under an atmosphere of 95% air and 5% CO2 at 37ºC. The cells were detached with 0.25% trypsin and 1mM EDTA (Invitrogen).

IMMUNOLOGICAL EVALUATION

Flow cytometry

Characterization of cells using flow cytometry was applied in paper I-IV, for specific details regarding staining procedures and specific antibodies used see respective paper. In short, staining for cell surface markers was performed as follows. Cell samples of the sentinel- and non-sentinel lymph nodes, tumour cells or PBMC were stained at $1x10^6$ cells per sample. Cells were washed in PBS supplemented with 2% FCS and 0.05% NaN_3 (staining buffer), followed by 30 min incubation with antibodies against the immune cell or tumor marker of interest.

For intracellular staining of cells were either first treated with Cytofix/Cytoperm (Becton Dickinson) according to the manufacturers instructions and then kept in staining buffer supplemented with 0.3% saponin, or directly permeabilized with saponin. Acquisition of cell samples was done on a FACS Calibur (Becton Dickinson)

or a FACSAria (Becton Dickinson) and analysis performed with the CellQuest or FACS Diva computer software.

T proliferation assay (I, II and III)

The proliferative response against autologous tumor extract was measured using a time course tritium labelled thymidine incorporation assay. Lymphocytes from sentinel- and non-sentinel lymph nodes, infiltrating the tumor and from peripheral blood were distributed in 96 well plates at 300,000 cells per well, all samples in triplicates. Cells were kept in RPMI 1640 medium (Life technologies) supplemented with 10% human serum (Sigma), 100 units/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma). Autologous tumor extract, prepared by homogenisation with an Ultra Turrax in 5 volumes (w/v) 2 x PBS followed by denaturation for 5 minutes at 97˚C, was added at 1/100 and 1/10 (v/v). Background proliferation was assessed in triplicate in medium without addition of antigenic stimulation and the proliferative response was further tested upon stimulation with the mitogen ConcanvalinA (ConA) 10µg/ml (Sigma). On day two (ConA) and day 4-8 (tumor homogenate) 1µCi ^{3}H-thymidine (Amersham) was added to each well, eighteen hours prior to harvesting. Samples were then subject to scintillation counting.

Enzyme linked immunosorbent assay (ELISA) (II and III)

The amount of interferon-γ (IFN-γ) secreted into the supernatant upon stimulation with autologous tumor extract was measured with ELISA. For paper II stimulation of sentinel- and non-sentinel lymphocytes, PBMC and tumor infiltrating lymphocytes, was performed in a 96 well plates with 300,000 cells per well. Cells were resuspended in RPMI 1640 medium (Life technologies) supplemented with 10% human serum (Sigma), 100 units/ml penicillin (Sigma), 100 µg/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma). Autologous tumor extract was added at two concentrations, 1/100 and 1/10 (v/v). Secretion was further investigated in medium alone, and upon addition of ConA (10 µg/ml), all samples in triplicates. In paper III the secretion of IFN-γ was also investigated during ex vivo long term cell culture of sentinel node lymphocytes, by collection of supernatant. The amount of IFN-γ was measured using an ELISA-kit (Human IFN-γ duoset) from R&D Systems according to the manufacturer's instructions.

RESULTS AND DISCUSSION

DETECTION OF IMMUNE RESPONSES AGAINST URINARY BLADDER CANCER IN SENTINEL LYMPH NODES (I)

Sentinel node identification and staging

Fourteen patients undergoing cystectomy due to urinary bladder cancer were included in the study. In twelve of these patients the sentinel node could be identified preoperatively, and/or during surgery by peritumoral injections of radioactive tracer and/or blue dye. In the two cases where no sentinel nodes were identified, this was due to extensive fibrosis in the true pelvis (patient 1) and a locally very advanced tumor (patient 7). Consistent with previous findings several sentinel nodes were detected outside of obturator fossa bilaterally (Sherif et al., 2001), often the only area dissected during radical cystectomy, underlining the need for individual identification of the tumor draining lymph nodes. Pathological investigation of excised lymph nodes revealed metastatic spread in seven patients.

Characterization of cell populations

Cells from the sentinel- and non-sentinel lymph nodes, the tumor, and peripheral blood were characterised by staining for lymphocyte- and tumour markers and analysed by flow cytometry. A total of 44 lymph nodes were obtained for analysis, 23 of these were detected using the sentinel node identification technique and the remaining 21 were considered non-sentinel lymph nodes. However, flow cytometric analysis revealed the presence of cytokeratin 20 (CK20) positive cells in two non-sentinel nodes. CK20 is an intermediate filament normally present in urothelial cells but not in lymph nodes, detection in lymph nodes indicates metastases, thus the two non-sentinel nodes were regarded as false negative sentinel lymph nodes. It is possible that these false negative sentinel nodes escaped detection due to altered lymphatic drainage caused by the metastases. The detection of CK20 was in agreement with the results of the histological evaluation in all examined cases.

The lymphocyte populations were investigated with regards to T cell subpopulations and activation markers. Lymph nodes were enriched in T helper cells, there were 2 to 6 times more CD4+ cells than CD8+ cytolytic T cells, this is in accordance with values obtained by others (Battaglia et al., 2003). Cells were also investigated for the expression of CD69 and CD62L. The very early activation marker CD69 is upregulated upon activation, in contrast CD62L, an adhesion molecule involved in lymph node homing of lymphocytes is downregulated after activation in the lymph node. Activated lymphocytes were identified to an equal degree in sentinel and non-sentinel lymph nodes. This is not unexpected since the immune defense continually encounters various foreign antigens. In peripheral blood virtually no activated lymphocytes were detected. Among the lymphocytes infiltrating the tumor, the CD4 and CD8 subsets were present in a ratio of approximately one, and about half of these displayed an activated phenotype. This is in contrast to the non-responsive behaviour this cell population display in the functional assay discussed in the following section.

Anti-tumoral reactivity in sentinel node lymphocytes

The proliferative response, against autologous tumour extract and the mitogen ConA, was tested in a time course proliferation assay based on tritium labelled thymidine incorporation. Dose dependent proliferation upon stimulation with autologus tumour extract was detected in six of the fourteen patients, two of these are displayed in Fig. 4. The magnitude of the response varied between patients. Considering the many individual factors that determine the immune response, such as antigen processing, HLA haplotypes and immunogenicity of the tumor, this is not surprising. Two non-sentinel nodes responded in the proliferation assay. However, both these patients had regionally advanced tumours and in one patient no sentinel nodes were detected. It is possible that these lymph nodes represent secondary tumor draining lymph nodes, but still exposed to tumor antigens, or that the progression of the tumor altered the lymphatic drainage.

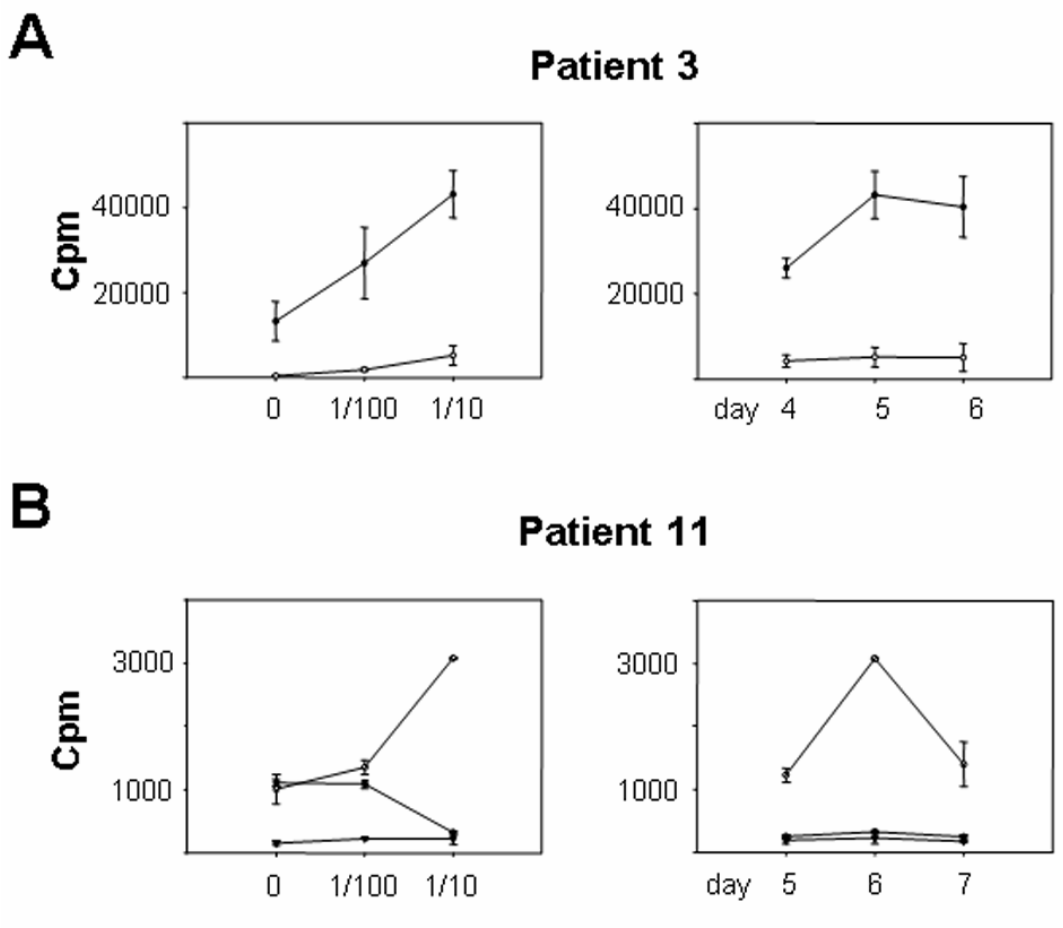

Figure 4. *Stimulation with autologous tumor extract induces proliferation in sentinel node aquired lymphocytes. The proliferative responses from the in vitro recall assay from patient 3 (A) and patient 11 (B) are shown. Cells from the sentinel nodes (filled or shaded circles), non-sentinel nodes(open circles) and tumor infiltrating lymphocytes (filled triangles), were stimulated with tumor homogenate diluted 1/100 or 1/10 (v/v) or kept in medium alone (0). Proliferation in a dose dependent matter was detected in sentinel node aquired lymphocytes, but absent in non-sentinel node and in tumor infiltrating lymphocytes (A and B left panels). Time courses are shown for proliferation upon addition of 1/10 (v/v) tumor homogenate (A and B right panels). The values represent the mean counts per minute (cpm) of triplicates ± 1 SD.*

In two patients (no 7 and 8) immune responses of a lower magnitude was detected in PBMC, upon stimulation with tumor homogenate, in addition to proliferation in lymph nodes. The addition of ConA resulted in vigorous proliferation in cells from sentinel and non-sentinel nodes and PBMC. Investigated TILs on the other hand showed no proliferative responses in the *in vitro* recall assay, neither upon addition of tumor extract nor stimulation with ConA, even though about half of the TILs expressed an activated phenotype.

Furthermore the possibility to maintain sentinel node aquired lymphocytes *in vitro* long-term, providing no antigen but autologous tumor homogenate, was investigated. Adequate amounts of lymphocytes for cell cultures were obtained from three patients. All cultures were viable for several weeks, despite the fact that sentinel node lymphocytes in one case were unresponsive, and another showed a weaker response in the in vitro recall assay. During the course of cell culture cells displayed increased expression of CD69 and decreased amounts of CD62L, indicating the presence of tumor reactive lymphocytes. The cell culture conditions favored the expansion of T helper cells, as displayed by an increased CD4/CD8 ratio.

Conclusion

Based on our findings that sentinel node aquired lymphocytes, proliferated and could be maintained in long-term cultures, when stimulated with autologous tumor extract we conclude that tumour reactive lymphocytes are present in the tumour draining lymph node of urinary bladder cancer.

SENTINEL NODE LYMPHOCYTES: TUMOUR REACTIVE LYMPHOCYTES IDENTIFIED INTRAOPERATIVELY FOR THE USE IN IMMUNOTHERAPY OF COLON CANCER (II)

Identification of sentinel nodes and classification

Sentinel nodes could be identified in all fifteen included patients, on average 2.3 sentinel nodes were detected (range 1-4). In addition to identified SLN, the resected bowel segment were examined for lymph nodes, between 5-29 were identified and histologically evaluated. Nine patients (no 1-9) were classified as Dukes' B since the tumor invaded the muscular layer but no metastases were detected (Fig. 5, left column). In four patients (no 10-14) tumor deposits were identified in the SLN, along with other lymph nodes from the resected specimen, consequently these patients were classified as Dukes' C. Patient 15 was classified as Dukes' D, since there in addition to one metastatic sentinel node were metastases in the liver, identified during surgery.

Immune cell characterization by flow cytometry

Single cell suspensions of lymphocytes from sentinel and non-sentinel lymph nodes, the tumor specimen and peripheral blood were triple stained for CD4, CD8 and CD69. CD4 and CD8 is expressed by two subsets of T cells, T helper and T killer cells respectively, CD69 is a lymphocyte activation marker. No notable differences between sentinel and non-sentinel nodes were found with regards to the distribution of T cell subsets nor the amount of activated lymphocytes (Fig. 5, middle column). Lymph nodes were enriched in T helper cells, sentinel nodes contained on average 6 times

more CD4+ than CD8+, about half of both subsets displayed an activated phenotype. Tumor infiltrating lymphocytes (TIL) were detected in all investigated cases, CD4 as well as CD8 positive cells were present, a majority of both subsets expressed CD69 (Fig. 5, middle column). As expected, T helper and T killer cells in peripheral blood did not display an activated phenotype, no CD69 expression was detected.

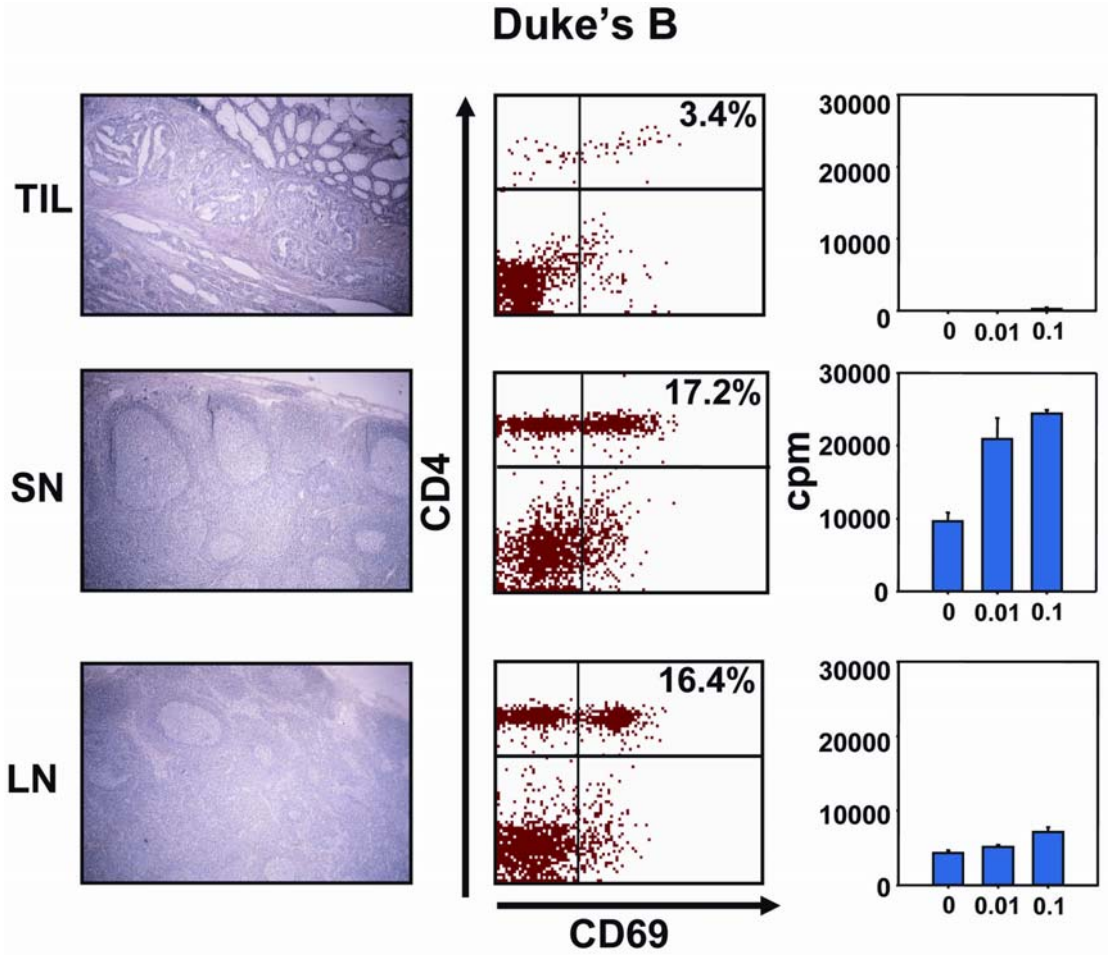

Figure 5. *Characterization of lymphocytes. The data shown is from patient 1, staged as Dukes' B since the tumor had invaded the muscular layer, but metastases were absent. The left panels show sectional hematoxylin-eosin stainings of the primary tumor, the sentinel node(SLN) and the non-sentinel node (NSLN). The degree of activation in the T helper population was investigated by staining for CD4 and the very early activation marker, CD69. No difference in activation was detected between SLN and NSLN. Tumor infiltrating lymphocytes were present in the tumor, about half of which displayed an activated phenotype (middle panels). Lymphocyte functionality was assessed in a time-course assay (day5-7), based upon the incorporation of tritium labelled thymidine (right panels). Stimulation with autologous tumor extract, diluted 1/100 or 1/10 (v/v), resulted in dose dependent proliferation in sentinel node lymphocytes. In NSLN some spontaneus background proliferation occurred, but no increase upon addition of tumor antigen was seen. Tumor infiltrating lymphocytes were unresponsive. Proliferation data are from day 5, samples were in triplicates and the the error bars represent s.e.m.*

Functional characterization of lymphocytes

The capacity of lymphocyte populations from the different localisations, to proliferate upon stimulation with autologous tumor extract or the mitogen Concanavalin A (ConA) was investigated. In time-course thymidine incorporation assays, lymphocytes recovered from tumor tissue were unresponsive (Fig. 5, left column), showing that these cells had been affected by the hostile microenvironment in the tumor. SLN lymphocytes, on the other hand, displayed dose dependent proliferation in responds to tumor homogenate (Fig. 5, left column). Notably, the detected responses was weaker or absent in 5 out of 6 patients with metastatic lymph nodes (no 10-12 and 14-15). One Dukes' C patient (no 13), however, displayed vigorous background proliferation which was further enhanced by tha addition of tumor extract. It could be hypothesized that such a strong immune response might correlate to an improved prognosis.

Lymphocytes from non-sentinel nodes did not proliferate in response to tumor antigens (Fig. 5, left column), with the exceptions of patient 6, 7 and 8. In patient 6 and 8, the lymph nodes were located as likely secondary tumor draining lymph nodes. In patient 7, the exact location could not be determined, but all 22 identified lymph nodes in the resected specimen were enlarged indicating a strong ongoing immune response. In PBMC responses towards tumor extract were generally absent, but three patients (no 7, 13 and 14) displayed some proliferation. Tumor reactivity has previously been identified in pheripheral blood in CRC patients (Nagorsen et al., 2000). SLN, NSLN and PBMC lymphocytes all responded with vigorous proliferation upon addition of ConA. This is in contrast to TIL, and SLN lymphocytes from two patients with lymphatic metastases (no 11-12), that remained unresponsive un response to mitogenic stimulation.

Further functional characterization was done by measuring the secretion of IFN-γ upon addition of autologous tumor extract or ConA, enough cells were obtained in six patients (no 4, 5, 8, 13-15). Interestingly, TILs from patient 4 responded to tumor homogenate with secretion of IFN-γ, five times the background level. In addition, a strong secretory response to ConA was detected, contrasting to the unresponsive behaviour in the proliferation assay. The two more cases of investigated TILs (no 5 and 15) displayed no IFN-γ secretion, neither in response to tumor extract nor ConA.

In agreement with the proliferative responses, SLN lymphocytes from Dukes' B patients 4, 5 and 8, and Dukes' C patient 13 secreted IFN-γ upon addition of tumor extract (Fig. 6). In patient 14 and 15, no proliferation was detected in the metastatic SLN and neither was there an secretory response. NSLN lymphocytes did not produce any IFN-γ above background level in response to tumor homogenate, a part from patient no 8, coinciding with proliferative response. Two cases (no 4 and 8) of IFN-γ secretion was detected in PBMC. A vigorous secretory response were obtained in lymphocytes from the sentinel and non-sentinel nodes, as well as in PBMC in response to ConA.

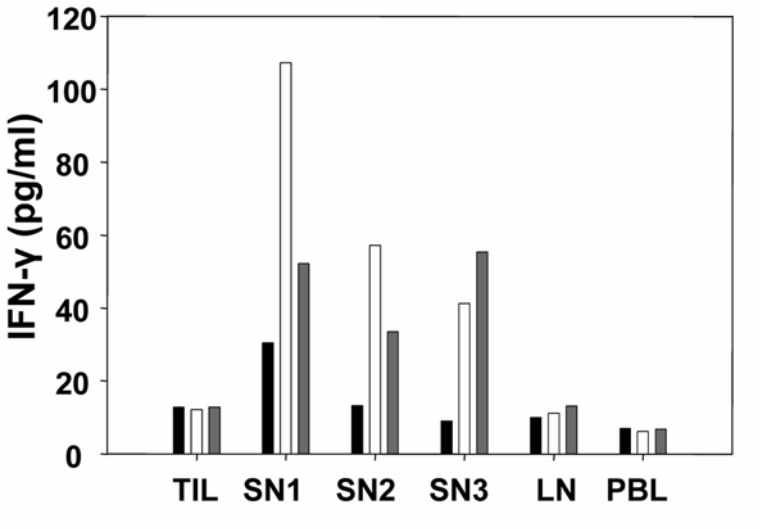

Figure 6. *Secretion of Th1 type cytokine interferon γ (IFN-γ) is induced by addition of autologous tumor extract to sentinel node aquired lymphocytes. The amount of IFN-γ present in the supernatant was measured by ELISA in pooled samples of triplicates. Cells were cultured in medium alone (black bars), or stimulated with tumor homogenate 1/100 (open bars) or 1/10 (black bars), (v/v). The data shown is from patient no 5, supernatant collected on day 5. Secretion was induced in sentinel node lymphocytes (SN), whereas the levels secreted from non-sentinel node(LN), tumor infiltrating lymphocytes(TIL) and peripheral blood leukocytes (PBL) remained at background level.*

Conclusion

Sentinel node aquired lymphocytes can be induced to proliferate and secrete directly ex vivo upon stimulation with autologous tumor. The magnitude of the responses varied and the presence of metastatic deposits in the lymph node generally hampered the response. However, it has been shown that such tumor induced immunosuppression can be overcome by in vitro culture supplemented with IL-2. We conclude that the tumor reactive lymphocytes, that can be obtained from the sentinel node, constitutes a promising source for adoptive immunotherapy.

SENTINEL NODE CD4$^+$ TH1-CELLS INDUCE TUMOR REGRESSION IN HUMANS (III)

We were inspired by the immunological findings from patients with urinary bladder cancer (Paper I) and colon cancer (Paper II) demonstrating that the sentinel nodes contained tumour-reactive lymphocytes proliferating dose-dependently with secretion of interferon-gamma upon stimulation with tumor homogenate. The finding that freshly isolated sentinel node lymphocytes from patients with solid tumors proliferate when stimulated with autologous tumor extract indicates that they have been naturally sensitized and have undergone *in vivo* expansion towards tumor-derived antigens in the local draining lymph node. This prompted us to explore an adoptive immunotherapy based on the isolation and *ex vivo* expansion of autologous tumour-reactive lymphocytes isolated intraoperatively from the the sentinel node.

Patient and cell characterization

Sentinel nodes were recovered intraoperatively from 16 patients with disseminated or locally advanced, high-risk colon cancer in order to investigate the feasibility of using sentinel node acquired lymphocytes for adoptive immunotherapy. Nine patients were staged as Dukes' D patients due to distant metastases and two were Dukes' C patients with lymph node metastases. The remaining five were Dukes' B patients, with aggressive tumors infiltrating vessels and nerves. Sentinel node acquired lymphocytes were collected, activated and expanded against autologous tumour extract.

Cells were characterised by FACS before, during and after expansion. The ratio between $CD4^+$ and $CD8^+$ cells at start was on average 4.9 indicating a natural expansion of $CD4^+$ T helper lymphocytes in sentinel nodes compared to the CD4/CD8 ratio in peripheral blood (range 1.0-2.5). The cells were held in culture in average 36.1 days. Initially the total number of cells decreased in the cell expansions. B lymphocytes disappeared almost completely and the number of $CD8^+$ T killer cells diminished. This procedure promoted mainly the expansion of T helper lymphocytes with an average CD4/CD8 ratio at transfusion of 92.5. When functionally tested the expanded T lymphocytes secreted high levels of the Th1 cytokine IFN-γ (average IFN-γ secretion 956 pg/ml) whereas only small amounts of the Th2 cytokine IL-4 (average IL-4 secretion 11 pg/ml) was detected in the cell culture supernatant. Thus the expanded T lymphocytes were functional and Th1 responsive. Restimulation after about 14 days with autologous tumour antigen resulted in further clonal expansion of tumour reactive T lymphocytes as assessed by investigating the T cell receptor Vβ repertoire of sentinel node acquired lymphocytes before and after *ex vivo* culture.

On average 71 million activated and clonally expanded autologous T lymphocytes were transfused back to each patient. Since the expansions contained populations of memory cells we investigated the fate of transfused T lymphocytes in peripheral blood by recall assays of T lymphocyte proliferation and IFN-γ secretion against tumour extract. We were able to detect T lymphocyte responses against autologous tumour antigens in peripheral blood ten months after transfusion indicating the presence of clonally expanded circulating tumour-reactive T lymphocytes.

Clinical outcome

No toxic side-effects or other adverse effects were observed. The patients were followed for 26 months on average (range 6-41), and monitored in accordance with the Swedish colorectal cancer follow-up protocol. 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). All nine patients with Dukes' D responded to treatment with complete responses (n=4), partial responses (n=2) and stable disease (n=3). The four Dukes' D patients displaying complete resonses have an overall average survival of 38 months. In the Stockholm region the life expectancy in Duke's D patients is 8-12 months. We compared the cumulative survival of our 9 immunotherapy treated Dukes'D patients with all Dukes'D cases in the Stockholm region during the year of 2003 (n=174) (Fig. 7). The Kaplan Meier plot demonstrates a

significant increased survival in the immunotheraphy treated group with an average survival of 2.6 years compared to 0.8 years for the control group (P=0.048).

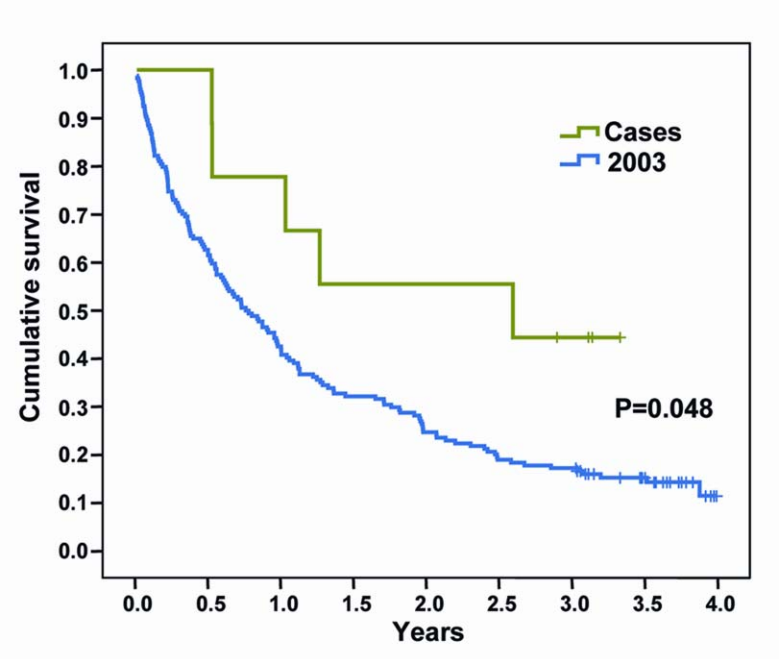

Figure 7. *Significant increased overall survival was observed after treatment with sentinel node based immunotherapy, in stage four colorectal cancer patients. The actuarial survival curves were obtained by comparison of survival among the Dukes' D patients receiving adoptive immunotherapy (9 cases) versus historical controls diagnosed during the year 2003 (174 cases).*

Five Duke's D patients have deceased, but they were all classified as responders. It seems that even when an anti-tumoral response is induced by transfer of tumor-reactive $CD4^+$ Th1-lymphocytes, tumor cells may escape elimination by losing targeted antigens, rendering T-cells anergic by down regulation of co-stimulatory molecules, by inducing regulatory T-lymphocytes, or by specifically deleting responding T-lymphocytes. Thus, an iterated therapy would have been preferred, since anti-tumoral responses were recognized in these patients, and a repeated transfusion might have been beneficial to overcome immunosuppression. Interestingly, we could demonstrate a dose response since, the patients with PR or SD received significantly fewer lymphocytes than the patients with complete response.

Five patients classified as Dukes' B (stage II), with high-risk tumor characteristics such as growth of tumor cells along nerves and in vessels, were included in the study. One Dukes' B patient developed liver recurrences, which responded to immunotherapy with regression of metastases. He is now classified as having stable disease. The other patients with high risk Dukes' B disease (n=4) and the patients with Dukes' C (n=2) show no signs of recurrences and are thus classified as stable disease. Although not significant, due to the small number of treated patients (n=7), the Stockholm region control group of Duke's B and Duke's C patients show a 20% mortality compared to 0 % mortality in the immunotherapy treated group.

Most patients received only immunotherapy. However, since this was the first attempt to use sentinel nodes in treatment, some patients also tried chemotherapy after

immunotherapy. The procedure tended to select patients hesitant to subject themselves to chemotherapy. Therefore the overall amount of chemotherapy used was very low. Seven patients did not receive any chemotherapy. Two patients received chemotherapy after primary surgery, but developed metastases at least one year after chemotheraphy. They subsequently received sentinel node based immunotherapy, without any further chemotherapy. Seven 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. Therefore, we believe that chemotherapy has had limited impact on the study results. Presumably, chemotherapy decreased the proliferative capability of tumor-reactive T-cells and if any thing results may have been underestimated.

In a recently performed retrospective analysis of resected tumors and lymph nodes from patients with colorectal cancer, the influence of immune cells on the advancement and outcome of disease was investigated (Pages et al., 2005). Increased numbers of memory effector cells, Th1-activation as indicated by increased mRNA for IFN-γ, as well as presence of the Th1-inducing transcription factor T-bet within the tumor were all associated with absence of early metastatic invasion and correlated with prolonged survival. Interestingly, the main objective for our study was to use expanded $CD4^+$ T helper 1 effector and memory cells, the cells that seem to contribute to increased survival.

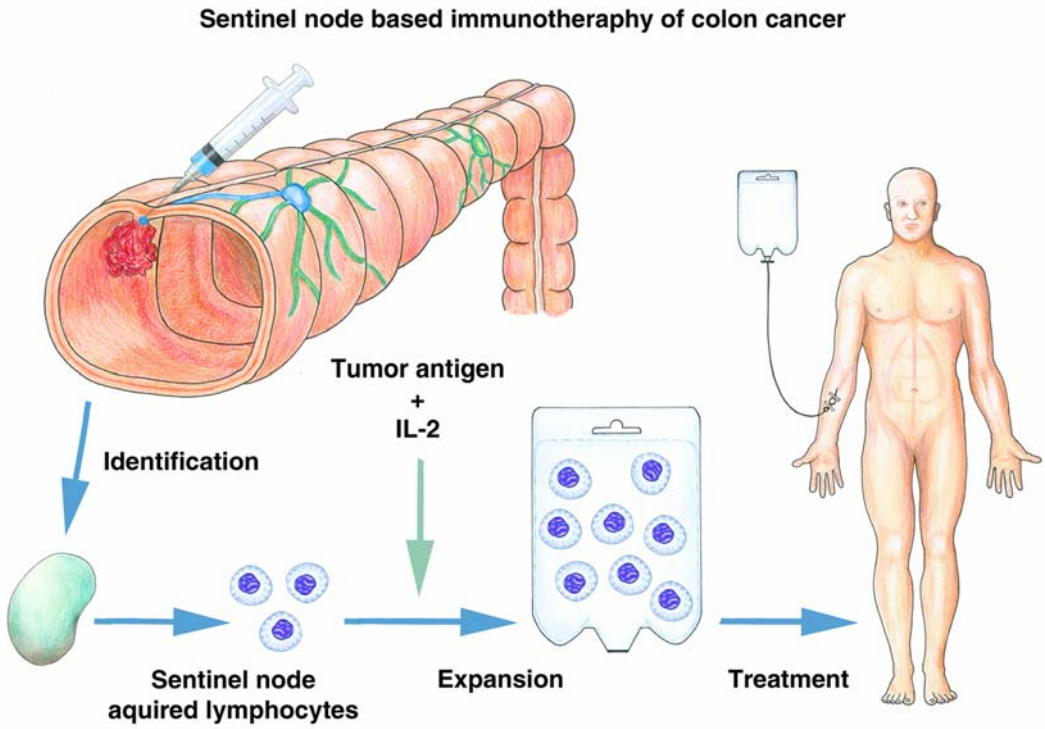

Figure 8. *A schematic view of sentinel node based adoptive immunotherapy. The sentinel node is identified during surgery by injections of a tracer dye (Patent blue). Lymphocytes from the sentinel node are kept in ex vivo cell culture for approximately four weeks, supplemented with the T cell growth factor interleukin 2. Clonal expansion of tumor-reactive T cells is stimulated by addition of autologous tumor homogenate. The expanded lymphocytes are given back to the patient in a transfusion.*

Conclusion

Freshly isolated sentinel node acquired lymphocytes can be expanded and safely transfused back into the patient without complications (Fig. 8). Immunotherapy using expanded sentinel node acquired lymphocytes improved outcome in patients with high-risk or disseminated colon cancer and warrant further efficacy testing in larger clinical trials.

DETECTION OF METASTATIC COLON CANCER CELLS IN THE SENTINEL NODE BY FLOW CYTOMETRY (IV)

Intracellular staining of cytokeratin 20 in a colon cancer cell line

The intermediate filament cytokeratin 20 (CK20) is a constituent of the cytoskeleton in epithelial cells, but not normally present in lymph nodes. When immunohistochemical evaluation of lymph nodes resected from CRC patients show CK20 staining, this is regarded as metastatic presence. We set out to investigate the use of CK20 in detection of colon cancer cells in single cell suspensions by flow cytometry. PBMC samples were spiked with decreasing amounts of the DLD-1 colon cancer cell line. In the highest number of cells added (9%) all colon cancer cells could be detected. It was possible to distinguish tumor cells all the way down to the lowest concentration (0.012%) in the seven points three-fold dilution series. The background staining in PBMC alone was no more than 0.009%, approximately the same as the average staining of the isotype control (0.011%).

There has been previuos reports of CK20 positive cells in PBMCs from healthy individuals, indicating that epithelial cells can be present in blood (Tsavellas et al., 2002). Our findings are in accordance with this, as exemplified by the detection of low levels of cells expressing CK20 in PBMCs, compared to the isotype control, from one colon cancer patient and one healthy individual. We conclude that intracellular staining for CK20, and flow cytometric analysis is a possible manner of detecting colon cancer cells in PBMCs. However, low amounts of CK20 positive cells are at times present in peripheral blood in healthy individuals.

Flow cytometry using surface markers in the DLD-1 cell line

Even though staining for CK20 successfully was used to identify colon cancer cells, the use of this intracellular antigen had some disadvantages. Low levels of CK20 positive cells were identified also in healthy individuals and the staining procedure for cytoplasmic antigens are more cumbersome than for surface antigens. We therefore wanted to investigate the use of direct conjugated antibodies against surface markers present on colon cancer cells. Two tumor associated antigens, epithelial cell adhesion molecule (EpCAM) and carbohydrate antigen 19-9 (CA19-9), were selecected for evaluation (Fig. 9). The antibodies recognizing EpCAM and CA19-9 were direct conjugated with Alexa fluor 488 (AF488) and peridinin-chlorofyll-protein complex (PerCP) respectively.

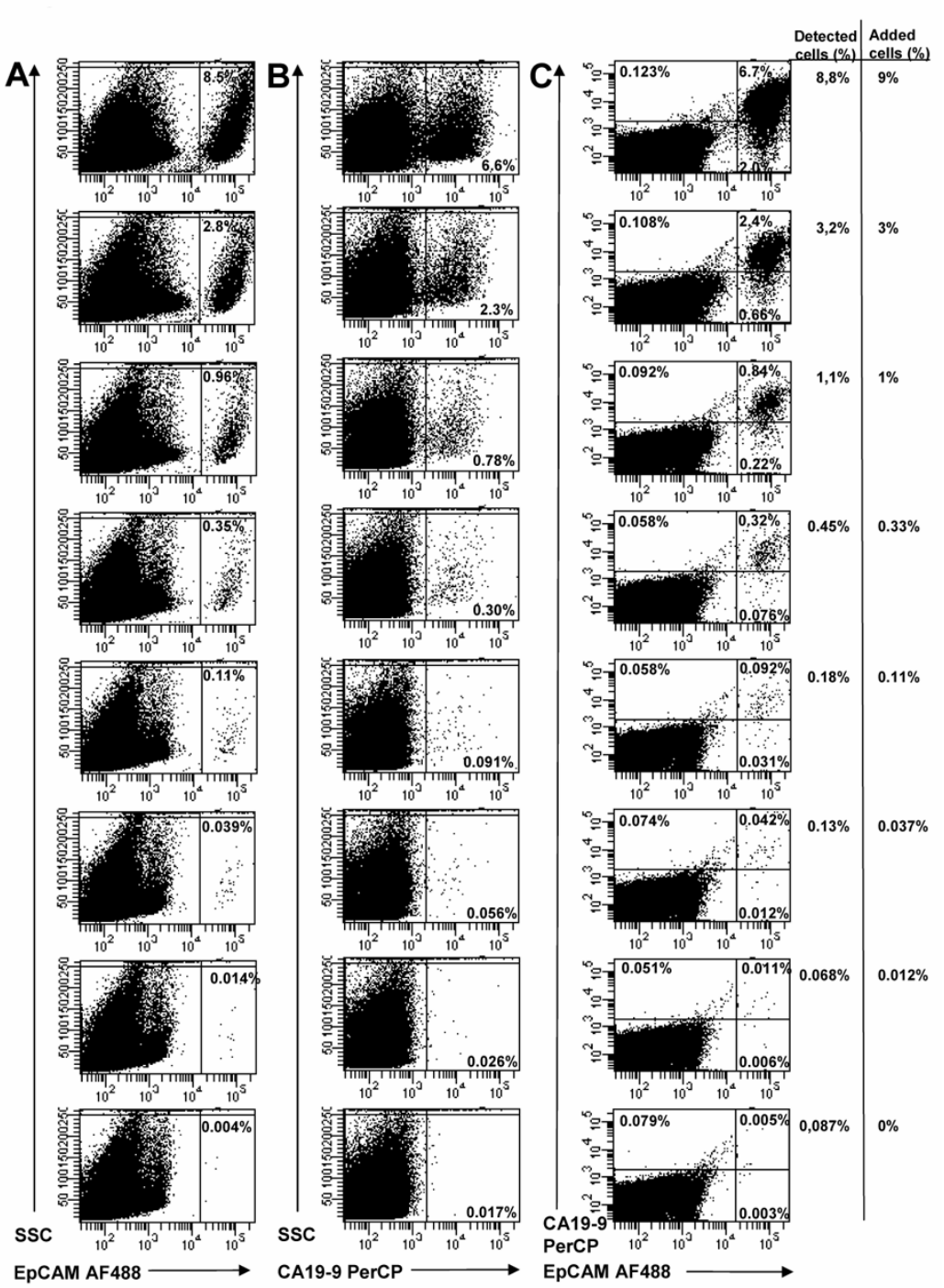

Figure 9. *Peripheral blood leukocytes were spiked with the colon cancer cell line DLD-1, in a seven points three-fold dilution serie with a starting concentration of 9% tumor cells. Close to all tumors cells were recovered by staining for EpCAM (A). CA19-9 appears to be expressed on approximately 80% (B). Analysis of co-expression showed that the majority of tumor cells (84%) stain positive for both EpCAM and CA19-9. Less than one percent is positive for CA19-9 alone, but approximately one fifth of the cells have downregulated CA19-9 and are single positive for EpCAM. It is common that tumors as well as cancer cell lines vary in their expression of antigens, which underlines the need for staining with multiple markers.*

EpCAM

A dilution series of the DLD-1 colon cancer cell line in PBMC was prepared, equivalent to the dilution series stained for CK20. The EpCAM staining resulted in recovery of close to all tumor cells, 8.5% detected in the starting concentration with 9% added (Fig. 8A, top panel). The EpCAM expressing cells were clearly separated from the negative population (Fig 9). The background was very low in PBMCs with no addition of tumor cells, 0.004% stained positive, and tumor cells were easily identified in the sample with the lowest number (0.012%) of added tumor cells (Fig. 8A, bottom panels). EpCAM appears to be a useful surface marker for detection of colon cancer cells by flow cytometry.

CA 19-9

CA19-9 seems to be downregulated on approximately 20% of the DLD-1 colon cancer cell line, whereas the remaining 80% stained positive (Fig. 9B). The background staining in PBMCs alone was slightly higher (0.017%) than for EpCAM, and the distiction between negative and positive cells somewhat less marked. Even so, tumor cells were readily detected down to a concentration of 0.11% added colon cancer cells and CA19-9 seem to be a relevant marker for flow cytometric analyses of tumor cell presence. Possibly the separation between the positive and negative poulation, as well as the background, could be improved by exchanging the fluorophore.

Co-expression

The use of multiple parameter flow cytometry, combining antibodies conjugated to different fluorophores, makes it possible to examine co-expression of tumor markers (Fig. 9C). When EpCAM was plotted versus CA 19-9, the majority of cells were double positive (average 84%), a proportion expressed only EpCAM and a very low amount were CA19-9 single positive cells (average 0.5%). There was a good correlation between the number of added and detected cells in the range of 0.11-9% added tumor cells. The use of fluorescence on both axes increased the background, particularly increasing the number of false positive CA19-9 events. However, analyses of co-expression can be done in alternative ways by gating, and multiple parameter flow cytometry also has the advantage of reducing the amount of cells needed for analysis. Furthermore, as is stressed below when sentinel nodes are analysed, surface molecules are differentially expressed on tumor cells, the use of several markers decreases the risk for false negative results.

Assay performance

The intra assay variability, when staining for tumor surface markers, was investigated by dividing samples into ten aliquots before flow cytometry analyses. From the dilution series, samples with the concentrations 0.037%, 0.11%, 0.33%, 1% and 3% added colon cancer cells were examined, along with samples of PBMC alone. Samples were analysed in the same manner as the samples portrayed in Fig. 9. For EpCAM and CA19-9, when presented separately, the number of positive events were determined in dot plots of respectively fluorescence in log-scale versus linear SSC properties. The total number of detected cells was determined by plotting log green fluorescence versus

log red fluorescence, and the events recorded as either $EpCAM^{+}CA19\text{-}9^{-}$, $EpCAM^{-}CA19\text{-}9^{+}$ or $EpCAM^{+}CA19\text{-}9^{+}$ were summed up.

The coefficient of variation was, with four exceptions, in the range 3.7 to 10.4 indicating a very low intra assay variation. The four exceptions were CVs of 12.9, 15.5, 16.1 and 90 were all found in either the two lowest concentrations of added tumor cells or in PBMC alone. However, since the mean values in these samples were close to zero, the coefficient of variation is extremely sensitive to changes in the standard deviation and of limited use.

Regression analyses was applied on the dynamic range of 0-3% added DLD-1 colon cancer cells. The sample cofficient of determination was one for EpCAM vs SSC, CA19-9 vs SSC, as well as for the sum of $EpCAM^{+}CA19\text{-}9^{-}$, $EpCAM^{-}CA19\text{-}9^{+}$ and $EpCAM^{+}CA19\text{-}9^{+}$ cells, demonstrating an invariable and reliable performance within the investigated range.

Detection of metastatic presence in sentinel nodes

The usefulness of flow cytometric detection of tumor cells in patients with colon cancer was confirmed in single cell suspensions from fresh specimens of sentinel node Double staining for EpCAM and CA19-9 was performed in parallel with intracellular staining of CK20 to enable a comparison.

In the first patient three sentinel nodes were identified during surgery, flow cytometry revealed metastatic presence in two of these, there was agreement between the CK20 and CA19-9 staining. However, in contrast to the DLD-1 cell line EpCAM expression appeared to be downregulated on two thirds of the cells, this underlines the need for multiple markers to avoid that tumor cells escape detection. The low amounts of positive events in the third sentinel node were close to detection limit and this lymph node was regarded as negative.

In the second patient investigated, two sentinel nodes were detected In SN1 the presence of tumor cells was indicated by the presence of CK20, EpCAM as well as CA19-9 positive cells. In the second sentinel node all three markers were close to the levels of detection and SN2 was thus considered free from tumor burden.

Conclusions regarding flow cytometric detection of tumor cells

In this manuscript we have investigated the feasibility of detecting metastatic colon cancer cells in lymph nodes by multiparameter flow cytometry. Staining was performed with direct fluorophore conjugated antibodies against multiple extracellular tumor associated antigens. Our results suggest that the method is a reliable, fast and easy way of detecting colon cancer cells in single cell suspensions. Multiparameter flow cytometry might constitute an alternative to the time-consuming and labor-intensive standard histopathological examination of lymph nodes. Further testing, with flow cytometric analyses performed in parallel with standard histopathological evaluation is warranted.

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

The perspective of utilizing the immune system to fight malignant disease is very appealing. The primary tumor rarely causes mortality, it is the capacity of a tumor to metastazise that makes it lethal. Active immunotherapy could create an immunological memory thus preventing recurrencies and possibly creating a cure.

Adoptive immunotherapy of autologous T cells is one of the more successful strategies so far. An advantage with this approach is that it circumvents the issue of tumour induced immunosuppression in cancer patients. The sources for T cells have mainly been peripheral blood or tumor infiltrating lymphocytes. In peripheral blood the challenge is to obtain the tumor-reactive lymphocytes. There are approximately 10^{12} different T cells in the human body (Arstila et al., 1999), so the precursor frequency of tumour reactive T cells are likely to be low in peripheral blood. TILs can also be difficult to obtain in large numbers, and have been subject to to the immunosuppressive milieu in the tumour and display functional defects in the TCR signaling (Whiteside, 2004).

The sentinel node detection was originally designed to increase accuracy of staging. The work of my thesis has focused on the immunological response in the sentinel node. We have demonstrated the presence of tumor-reactivity in tumor draining lymph nodes from two common solid tumors, invasive urinary bladder cancer and colorectal cancer. Sentinel node aquired lymphocytes were directly *ex vivo* stimulated with autologous tumor extract, and responded with proliferation and secretion of the Th1 cytokine IFN-γ. In addition, sentinel node aquired cells could be maintained in long-term cultures, provided with antigenic stimulation only from autologous tumor homogenate.

Contradictory results with regards to the activation status of the sentinel node have been found. In some cases an activated state has been reported, whereas in others the sentinel node has been found to be immunosuppressed. It is quite possible that the interactions between the tumor and the immune system go through different stages during tumor development. Initially the tumor does not provide any danger signal since there is no inflammation, which means that the tumor might be tolerized. With tumor growth, necrosis and inflammation arises, initiating an immune response. Tolerance can be broken, due to activation signals from the innate immune system and increasing amounts of available antigen. Either the immune system succeeds in eliminating the tumor, and it will never be clinically detected, or the tumor escape recognition and continue to grow.

In accordance we found that in colorectal cancer patients, the proliferative responses in metastatic sentinel nodes were dampened or absent. It appears however, that this immunosuppression can be overcome. Lymphocytes from metastatic as well as non-

metastatic sentinel nodes, from both CRC and UBC patients, could be kept in long-term cultures. The cells were supplied with low doses of IL-2 and antigenic stimulation was provided by the addition of autologous tumor homogenate. The sentinel node, the natural site for activation of tumor reactive T cells, seemed to be a promising source of T cells to use in adoptive imunotherapy. UBC is particularly interesting in this context, since it is one of the few tumor types where there already is an established immunotherapy. BCG instillation is well established and proven to have effect. Furthermore, it has in animal models been demonstrated that this effect is dependent on the presence of T cells (Ratliff et al., 1993).

We turned our interest to the $CD4^+$ T helper subset, whereas previous adoptive immunotherapy mainly has focused on the $CD8^+$ cytotoxic T cell subset. We where inspired by animal studies where antigen specific $CD4^+$ T helper 1 cells (Th1) in a transgenic mouse model were sufficient for the induction of antigen specific cell destruction in an immunocompetent mouse (Camacho et al., 2001). Furthermore, the presence of T helper cells is important for the recruitment of antigen specific $CD8^+$ cytotoxic T cells, and as it appears necessary for the creation of an adequate immunological memory.

We investigated the feasibility, safety and possible effect on tumor regression, in a pilot study of sixteen patients with colorectal cancer using sentinel node based adoptive immunotherapy with T helper cells. The results were most encouraging. No side-effects of the treatment was observed. Furthermore the adoptive transfer of sentinel node aquired lymphocytes appeared to reduce tumor burden as complete responses were seen in four patients with distant metastases. Finally, Dukes' D patients treated with immunotherapy demonstrated a significantly increased overall survival compared to historical controls. Thus, it appears that the transfussion of tumor reactive $CD4^+$ T cells may change the balance of the immune system in favor of the patient. Interestingly, supporting our findings, favourable prognoses of Duke's B patients with signs of microinvasiveness was reported to correlate with the presence of tumor infiltrating $CD4^+$ memory cells expressing the Th1 hallmark transcription factor Tbet (Pages et al., 2005).

The method as such, with identification of the sentinel node, *ex vivo* expansion of tumor reactive lymphocytes and retransfusion, may be applicable in other forms of solid tumors. The promising results from the pilot study, warrant further testing in patients with colorectal cancer, as well as in other solid tumors. Our studies have focused on the T helper cell. Perhaps parallel cultures of optimized $CD4^+$ and $CD8^+$ expansions should be benificial providing tumor specific effector $CD8^+$ cytotoxic T cells and $CD4^+$ T helper cells simultaneously. Moreover, there was a dose response correlation with tumor regression seen in our patients suggesting that improved means of numerical expansions are necessary and that the patients may benefit from repeated transfusions. The accumulating knowledge in molecular immunology as well as insights of tumor biology and the mechanisms of metastases will hopefully provide clues in order to improve the efficacy of adoptive imunotherapy and overcome tumor induced immunosuppression.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Tjocktarmscancer är en av de vanligaste cancerformerna i västvärlden, ungefär en miljon människor drabbas årligen och ca hälften dör varje år i sjukdomen. Kirurgi är den främsta behandlingsmetoden och botar ungefär hälften av patienterna. Urinblåsecancer är den nionde vanligaste tumörformen och ca 40% av patienterna kommer att avlida i sin sjukdom. Behandling med cellgifter har avsevärt förbättrats överlevnaden för patienter med spridd sjukdom, men alternativa kompletterande behandlingsmetoder är eftersökta. Den tumördränerande portvaktskörteln, sentinel node, är den lymfkörtel som först tar emot metastaserande celler. Undersökning av sentinel node har tidigare framför allt använts för prognos och varit vägledande för val av behandling. Den här avhandlingen syftar till att undersöka det immunologiska svaret mot tumören i sentinel node och undersöka om immunceller ifrån sentinel node kan aktiveras och mångfaldigas med syftet att ge immunterapi till patienter med spridd cancersjukdom.

Vi utvecklade ’sentinel node’-tekniken för att utvinna och studera immunologiskt tumör reaktiva T celler ifrån patienter med urinblåsecancer och med tjocktarmscancer. Vi fann att lymfocyter från sentinel node kunde fås att dela på sig och att bli aktiverade vid båda dessa tumörformer. Vidare reagerade T cellerna med produktion av IFN-γ som tecken på igenkänning av tumören. Lymfocyter ifrån sentinel node som var metastaserade svarade dock svagt eller obefintligt, ett resultat av att tumörceller hämmar immunsystemets celler. Genom att odla och mångfaldiga cellerna i närvaro av tillväxtfaktorn IL-2 och extrakt ifrån patientens egna tumör lyckades vi få de hämmade cellerna att åter igen dela på sig och producera IFN-γ.

För att kunna långtidsodla T celler i kultur och försäkra sig om att inga tumörceller finns närvarande har vi utvecklat en flödescytometrisk undersökningsmetod där två proteiner som uttrycks på ytan av cancerceller undersöks. Metoden har visat sig vara så känslig att även enstaka cancerceller närvarande i en suspension av lymfocyter kan upptäckas.

I en efterföljande pilotstudie har vi behandlat 16 patienter med tjocktarmscancer med lymfocyter skördade från sentinel node. Dessa lymfocyter har mångfaldigats genom stimulering med tumörextrakt följt av återtransfusion. Från pilot-studien kan vi dra slutsatsen att behandlingen är säker då inga biverkningar förekom. Fyra patienter med metastaser uppvisade komplett respons då ingen kvarvarande sjukdom kunde detekteras. Vid jämförelse med konventionell behandling har patienterna behandlade med immunterapi en signifikant ökad överlevnad, 2.6 år jämfört med 0.8 år.

Sammanfattningsvis visar det sig att sentinel node är den naturliga källan för att finna tumörreaktiva T celler. Dessa T celler kan skördas, aktiveras och mångfaldigas i laboratoriemiljö. Patienter med spridd colon cancersjukdom som erhållit immunterapi med expanderade T celler har uppvisat effekt med minskat tumörbörda och förlängd överlevnad.

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