Difficulties and dangers of CEA-targeted immunotherapy against colorectal cancer

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Introduction

Immunotherapy of cancer

Infectious diseases have been prevented by vaccination as a standard procedure for many years already. Due to a better understanding of molecular biology and tumor immunology, vaccines are now also being developed for treatment or prevention of different types of cancers. Immunotherapy of cancer began about one hundred years ago when Dr. William Coley showed that he could control the growth of some cancers and cure a few advanced cancers with injections of a mixture of streptococcal and staphylococcal bacteria known as Coley's toxin. These data showed that non-specific stimulation of the immune system could positively influence the anti-tumor response. However, the finding that tumor cells are characterized by numerous changes in a variety of genes, and therefore differ from normal cells, started the development of tumor specific immunotherapies. Over the years many different approaches to immunotherapy that are more selective for tumor tissue have been tested. Together, this research indicated that treatment of cancer through immunotherapy is possible, but it also showed that it can be very complicated due to immune tolerance and auto-immunity.

Target antigens for immunotherapy

To achieve effective immunotherapy it is crucial to identify suitable target antigens that will be recognized as tumor-specific by the immune system. Virus-induced tumors express virus-encoded antigens that are shared by all tumors induced by the same virus. A number of viruses are known to cause tumors in animals (SV-40 virus, adenovi-

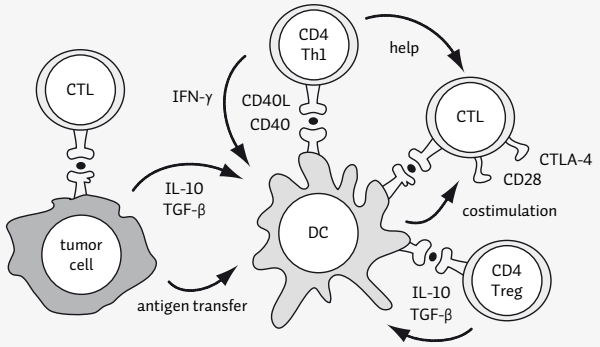
rus, Rous sarcoma virus, Friend erythroleukemic virus, Moloney Rauscher and Gross viruses) or human beings (HTLV-1 in leukemia, hepatitis-B and C viruses in hepatic carcinoma, Human Papilloma Virus (HPV) in cervical cancer). For antigens expressed by such tumors, the induction of an effective immune response is not hampered by self-tolerance. Nevertheless, these cancer viruses manage to establish persistent infections and cause cancer in susceptible people. This problem of immunological tolerance is even more prominent in the induction of immune responses against tumors that lack foreign antigens such as viral antigens. In this case, immunotherapy needs to target tumor-associated auto-antigens (TAAs) that might be weakly immunogenic because of self-tolerance. T-cell tolerance can be initiated early in the development in the thymus where expression of peripheral antigens leads to negative selection of T cells, but T-cell responses can also be suppressed in the periphery by multiple mechanisms.

Immune regulation

At the initiation of each T-cell response, numerous mechanisms contribute to the eventual quality and magnitude of the response. It is clear from previous reports that effective T-cell activation needs 2 signals. Antigen presenting cells (APC) display antigens in MHC molecules on their cell surface, that will be recognized by the T-cell receptor, leading to signal 1 for the activation of the naïve T cell. In addition to the antigen specific signal, co-stimulation (signal 2) is provided in the progress of naïve T cells into fully activated effector T cells. Several co-stimulatory molecules expressed on APC interacting with T cells have been identified, including ICAM-1, LFA-3, CD70, CD80, CD86, OX40L and 4-1BBL [1,2]. Interaction of these molecules with their receptors on T cells plays a crucial role in the promotion of cytokine secretion, T-cell differentiation and proliferation. When co-stimulation is lacking, naïve T cells will not be fully activated and might become ignorant or tolerant. This is generally the case for naïve T cells that encounter antigens on tumor cells that do express antigens in MHC molecules but lack the expression of co-stimulatory molecules. To overcome this problem and improve T-cell activation by antigen specific immunotherapy, the induction of the immune response can be positively regulated. This can be achieved by the use of vector-based vaccines that in addition to the transgene-encoded antigen also encode for co-stimulatory molecules or stimulatory cytokines [3]. Cytokines such as GM-CSF, IL-2 or IL-12 can also be used as an adjuvant for peptide/protein based vaccines to further enhance T-cell activation [4]. However, when a T-cell response is successfully induced, it might not always be as effective as expected, because also at this level immune regulation continues. Immunoregulatory mechanisms that are normally active to prevent autoimmune pathology can in addition hamper anti-tumor immune responses (Box I). Regulatory T cells mediate one of the major mechanisms that play a crucial role in the suppression of tumor specific CD8+ T-cell responses by direct cell-cell contact and/or the production of inhibitory cytokines [5-7]. Another important regulator is the cytotoxic T lymphocyte-associated antigen (CTLA)-4, which shares its ligands (CD80 and CD86) at the APC with CD28 and downregulates T-cell responsiveness [8]. Also tumor cells can acquire an immunosuppressive phenotype by secretion of the inhibitory cytokines IL-10 and TGF-β [9,10]. The awareness of these regulatory capacities of the immune system

has led to the development of strategies that can specifically hamper these inhibitory mechanisms. The effect of regulatory T cells can be diminished by depletion of this CD25+ T-cell subset by injecting CD25-specific antibodies. Administration of antibodies that block the inhibitory effects of CTLA-4 have been shown to enhance anti-tumor responses and also the effect of IL10 and TGF-β can be inhibited by the use of specific blocking antibodies. These strategies have mainly been tested in mouse models [11-14], but also clinical trials have been performed with CTLA-4 blocking antibodies. These studies not only demonstrated the effectiveness of this treatment, but also showed that interference with regulatory mechanisms can result in the induction of autoimmune responses [15,16]. This should be taken into account when applying these strategies.

BOX 1. General immune regulation and inhibitory mechanisms that hamper anti-tumor immunity.



The immune system is very complex and has many regulatory capacities that contribute to the induction or inhibition of T-cell responses directed against tumor cells. Antigens derived from tumor cells can be captured by antigen-presenting cells (APCs), presumably dendritic cells (DCs), which process these antigens and present peptides on their MHC class I and II molecules that will be recognized by T cells. After recognition of the peptide, CD4+ T cells will up-regulate CD40-ligand, which interacts with CD40 molecules expressed on the DC. This interaction will lead to final maturation of the DC, resulting in high expression of MHC-I and co-stimulatory molecules, which is necessary for efficient CTL priming. Also most tumor cells express MHC class I molecules and can therefore present peptides to CTLs. However, because tumor cells lack expression of co-stimulatory molecules, CTLs might not get fully activated and will fail to produce cytokines, will be unable to sustain proliferation, and often undergo apoptosis or become non-responsiveness to subsequent stimulation. In addition, tumor cells can also suppress immunity by secretion of the inhibitory cytokines IL-10 and TGF-β, which suppress APC function by inhibiting expression of MHC molecules, CD80, CD86 and IL-12. These cytokines can also be produced by regulatory T cells, which is a T-cell population that can functionally suppress an immune response by influencing the activity of another cell type. Besides cytokine production, other suppressive mechanisms of these cells like perforin and Granzyme B release, induction of IDO expression by APCs or CTLA-4 interactions with CD80/CD86 might cause APC and T-cell apoptosis, APC dysfunction and/or T-cell anergy.

Colorectal cancer

Colorectal cancer is one of the most common cancers and is the second leading cause of cancer deaths in industrialized countries. It usually begins as a polyp, which is a precancerous lesion of the colon or rectum epithelium. Polyps can be benign, but over the years they can develop into more dysplastic abnormalities that eventually progress to

invasive cancer. The staging of the tumor is evaluated by the TNM (Tumor, Node, Metastasis) staging system (Box II) [17]. This system looks at the level of wall invasion of the primary tumor, the presence or absence of regional lymph node involvement and the status of distant metastasis. According to the American Cancer Society [57] the estimated 5-year survival rate is 90% for patients in whom cancer is detected at an early, localized stage (stage I). Unfortunately, only 39% of colorectal cancers are diagnosed at this stage. The survival for patients with metastatic colorectal cancer ranges from a few months to more than 30 months with current treatment options. The most common site of metastases in patients with colorectal cancer is the liver and hepatic metastases are responsible for at least 2/3 of the deaths of these patients [18]. The standard treatment of colorectal cancer involves resection and it can be cured when polyps are found and removed in early stages. In more advanced stages, chemotherapy or chemotherapy plus radiation is given before and/or after surgery. Systemic chemotherapy with fluoro-uracil (FU) has been the standard treatment for many years. After the introduction of new chemotherapeutic agents, the prognosis has improved dramatically over the years. New agents like oxaliplatin and irinotecan have been shown to improve survival in combination with FU-based therapies [19]. Recently, two other agents for treating colorectal cancer have been approved by the American Food and Drug Administration. This so-called targeted therapy exploits monoclonal antibodies or small molecule based drugs that attack the tumor through growth factor receptor pathways. Cetuximab is a human epidermal growth factor receptor targeted monoclonal antibody that has a direct effect on the tumor. Bevacizumab is an antivascular endothelial growth factor monoclonal antibody that has an indirect effect by inhibiting vascularization. These

BOX II. TNM Staging System.

Tumor	T1	tumor invades submucosa
	T2	tumor invades muscularis propria
	T3	tumor invades through the muscularis propria into the subserosa, or into the pericolic or perirectal tissues
	T4	tumor directly invades other organs or structures, and/or perforates
Node	No	no regional lymph node metastasis
	N1	metastasis in 1 to 3 regional lymph nodes
	N2	metastasis in 4 or more regional lymph nodes
Metastasis	Mo	no distant metastasis
	M1	distant metastasis present
Stage groupings	Stage I	T1 No Mo; T2 No Mo Cancer has begun to spread, but is still in the inner lining
	Stage II	T3 No Mo; T4 No Mo Cancer has spread to other organs near the colon or rectum. It has not reached the lymph nodes
	Stage III	any T, N1-2, Mo Cancer has spread to lymph nodes, but has not been carried to distant parts of the body
	Stage IV	any T, any N, M1 Cancer has been carried through the lymph node system to distant parts of the body. This is known as metastasis. The most likely organs to experience metastasis from colorectal cancer are the lungs and liver.

agents are combined with chemotherapy and further improve the clinical outcome for patients with metastatic colorectal cancer [19]. With these current treatment strategies, higher response rates have been achieved, but these patients still have a poor prognosis, with an overall survival of 20 months [20]. Other therapies that are more selective for tumor tissue are needed and cellular immunotherapy specifically targeting colorectal cancer is a potential alternative.

Carcinoembryonic antigen

In colorectal cancer patients spontaneous systemic T-cell immunity against several tumor associated antigens (TAAs) has been described [21 and references therein]. One of the first described TAAs that has also been intensively studied as a target for immunotherapy of colorectal cancer is carcinoembryonic antigen (CEA). CEA was first described in 1965 when it was isolated from a colon carcinoma specimen [22] and the gene encoding human CEA was cloned in 1987 [23]. CEA is a 180,000-200,000 kD protein that was initially considered to be an oncofetal glycoprotein. At the present time, CEA should be viewed as a normal epithelial molecule with retained expression in tumors. It consists of an Ig variable region-like amino-terminal domain followed by six Ig constant region-like domains and it is anchored to the cell membrane via a glycosylphosphatidylinositol (GPI) moiety (Fig. 1). In vitro studies have demonstrated that CEA acts as a cell adhesion molecule when expressed on the tumor cell surface [24,25]. It has also been demonstrated that the N-domain is directly involved in the cell adhesion phenomena [26 and references therein]. However, the relevance for these findings for the in vivo situation is not clear. CEA expression on normal adult tissues is detectable in colon, stomach, tongue, oesophagus, cervix, sweat glands and prostate (Table I). The highest CEA production

Figure 1. Model of a CEA molecule. It consists of one IgV-like N-domain and six IgC-like domains (A and B). The GPI-linkage to the cell membrane is shown by an arrowhead. Glycosylation sites are shown as lollipops. CEA has been named in the CD system as CD66e.

in healthy individuals takes place in the colon. There, it is released from the apical surface of mature columnar cells into the gut lumen and disappears with the faeces (50-70 mg/day). Therefore only low levels of CEA are detectable in the blood of healthy people (<2.5 ng/ml). Serum levels of CEA are also often used as a diagnostic marker because it is expressed at high levels in positive tumors. It has been shown that in colorectal cancer 80% of the patients show elevated levels in the serum prior to evidence of clinical recurrence. In 40-73% of patients with breast cancer CEA elevations may be found. Also patients with bronchogenic lung cancer, small cell carcinoma of the lung, pancreatic and gastric malignancies or epithelial neoplasms of the female reproductive tract can show elevated serum levels of CEA that may correlate with stage of disease [27]. In colon can-

TABLE 1. Expression or concentration of CEA in tissue respectively faeces, colonic tissue or serum of mice transgenic for CEA, compared to humans. Data are collected from the literature.

	Adult human tissue	CEA-tg (W. Zimmerman)	CEA-tg (J. Primus)
Colon/rectum	+	+	+
Tongue	+	+	–
Oesophagus	+	+	–
Stomach	+	+	+
Small intestine	–	+	+/–
Trachea/lung	–	+	–
Cervix	+	ND	–
Sweat glands	+	ND	ND
Prostate	+	ND	ND
Faeces (ng/mg of total protein)	13 800 ± 12 400	40 000 ± 14 000	11.7 ± 4.0
Colonic tissue (ng/mg of total protein)	108 ± 38	1500	25.9 ± 7.5
Serum	<2.5	20 ± 8	<2.5

cer, the tumor cells have lost their polarity and CEA is distributed around the cell surface. Through draining lymph nodes and blood vessels it can then end up in the blood. However, serum levels may also rise in some non-malignant conditions (such as chronic cirrhosis, pulmonary emphysema and heavy smoking). Therefore, serum levels are not always a very reliable factor. CEA has primarily been studied as a target for immunotherapy against cancers of epithelial origin, in particular colorectal cancer. Notably, the presence of CEA on epithelial cells and in serum might hamper the induction of specific immune responses by the induction of self-tolerance. On the other hand, when the induction of potent CEA-specific immune responses would succeed, CEA expressing epithelial cells may be a target for these T cells, which might lead to severe auto-immunity. Side effects that are not hazardous for the patient might be acceptable when therapy is effective, but autoimmune responses might also be very dangerous when vital tissues, like colon or stomach, are targeted.

CEA-specific immunity in humans

Specific immunotherapy alone or in combination with other drugs is now worldwide under investigation to prevent or treat colorectal cancer. Many strategies of immunotherapy targeting CEA have been tested in colorectal cancer patients. For example, vaccination with canarypox virus expressing human CEA has been shown to increase CEA-specific T-cell precursors and antibody production [28-30]. Increased frequencies of CEA-specific IFN-γ producing cells were also described after vaccination with dendritic cells [31-33] or after combined chemoimmunotherapy [34]. Analysis of the CEA-specific T-cell response in humans has also resulted in the identification of several cytotoxic T cell and T-helper epitopes [35-37]. However, despite these findings and improvements, these vaccines still only result in low levels of circulating immune cells.

Table II. Results of clinical studies in patients with metastatic colon cancer.

Vaccine type	Vaccine	Study Phase	Patients responding	Reference
Dendritic cells	Autologous DCs loaded with CEA peptide	I	2/12	Fong et al. (2001)
	Autologous DCs loaded with CEA peptide	I/II	0/9	Babatz et al. (2006)
	Autologous DC's modified with rF-CEA-TRICOM	I	0/14	Morse et al. (2005)
Virus	Vaccinia-CEA	I	0/20	Conry et al. (1999)
	Vaccinia-CEA/ALVAC-CEA	I	0/18	Marshall et al. (2000)
	Vaccinia-CEA-B7.1	I	0/18	Horig et al. (2000)
	ALVAC-CEA	I	0/15	Marshall et al. (1999)
	ALVAC-CEA-B7.1	I	0/39	Von Mehren et al. (2000)
Chemotherapy / peptide	Standard chemotherapy + CEA CAP-1 peptide	I/II	5/17	Weihrauch et al. (2005)

Pox virus vaccines have been reported to increase circulating antigen-reactive T cells from fewer than 1 in 200,000 to about 1 in 40,000 [38,39]. In addition, conclusions about clinical responses are mostly based on surrogate or subjective endpoints like lymphocyte infiltration or tumor necrosis, instead of objective cancer regressions [40]. So, many of these studies describe the induction of CEA-specific immune responses, but striking clinical effects of CEA-specific immunity have not been reported until now (Table II). In accordance with the lack of objective cancer regression, the responding patients in these clinical trials did also not show any signs of auto-immunity in CEA-expressing tissues. Vaccination targeting other cancers like melanomas did also not result in effective anti-tumor immunity. Even after the induction of high numbers of tumor-Ag reactive T cells in patients with melanoma by peptide vaccinations, no significant decrease on the incidence of recurrent tumors was achieved [41]. Melanoma specific vaccinations comprising peptide-pulsed dendritic cells, autologous tumor cells or synthetic peptides have also been described to induce antigen-specific autoimmune reactions (vitiligo), but again no striking clinical responses were observed [42,43]. However, non-specific therapy in which patients with metastatic melanoma were treated with anti-CTLA-4 caused substantial tumor regression [16,44]. Intriguingly, tumor regression was correlated with the induction of autoimmune pathology [15]. 25% of the patients developed grade 3-4 autoimmune toxicity (including mostly colitis and dermatitis) and 36% of these patients showed evidence of tumor regression. These data indicate that the induction of effective anti-tumor immunity by immunotherapy can cause severe autoimmune pathology. This immune reaction can be antigen-specific when T cells damage healthy tissue expressing the same target antigen or non-specific as tissues are targeted by T cells specific for other (self-)antigens.

CEA-specific immunity in mice

In normal mice CEA is a non-self/foreign antigen and no CEA homologue could be identified in mice. Because this would not be comparable to the human setting, several transgenic mouse models expressing human CEA have been developed. Two of these

models used the complete CEA gene, including the flanking regulatory elements, to generate CEA-tg mice with tissue-specific CEA expression that closely resembles that seen in humans [45,46]. In the mice generated in the group of W. Zimmerman, CEA was found in oesophagus, stomach, small intestine, cecum, colon and trachea [45] (Table I). In mice prepared in the group of J. Primus, strong cytoplasmic staining was only found in cecum and colon whereas small intestine villi had only a few positive cells [46] (Table I). Most studies have been performed in the first model with relatively high CEA levels compared to humans. With this mouse strain immune tolerance can be studied and CEA serum levels are more comparable with levels in late-stage cancer patients. Initial studies have shown that immunization of CEA-tg mice with whole CEA protein resulted in T- and B-cell responses that were strongly reduced as compared to vaccination of CEA negative littermates [47]. However, repeated CEA-specific vaccination of CEA-tg mice using recombinant poxviruses, fusion proteins or DNA has been shown to induce CEA-specific immunity and to delay and in some cases prevent the outgrowth of CEA-positive tumors [48 and references therein]. Unfortunately, analyses of the immune responses in these reports were not performed in sufficient detail. CEA-specific immunity only contributed partially to the anti-tumor efficacy, while most likely innate immune responses and T cells targeting other antigens expressed by the tumor were mostly responsible for the observed anti-tumor effect. These studies were all performed with transplantable CEA-expressing tumors that grow out to large tumors within 4-6 weeks. In addition, the subcutaneous location of the tumor is not comparable with the normal situation in which the tumor is located in the colon and has often metastasised to the liver. Therefore this model does not provide the most physiological conditions to critically evaluate cancer vaccines. Other mouse models have been developed now, in which tumors arise spontaneously in the intestine due to a mutation in the Apc tumor suppressor gene. Germline mutations of the Apc gene itself are responsible for familial adenomatous polyposis (FAP), an inherited autosomal dominant condition leading to the development of multiple adenomas in the colorectum [49,50]. The Apc gene is also found to be mutated in the majority of human sporadic colorectal tumors regardless of their degree in malignancy. A consequence of Apc gene mutation is β-catenin accumulation in the cytoplasm. In normal cells the breakdown of β-catenin is regulated by the Wingless/Wnt pathway. However, mutations in Apc prevent complex formation with Apc and β-catenin, and therefore β-catenin levels rise in the cytoplasm. β-catenin associates with transcription factor Tcf4 and induces constitutive activation of c-myc, cyclin D1 and c-jun [51]. The disruption of the Wnt/β-catenin pathway is thus a major event in most colon cancers. As in humans, different mutations lead to different phenotypes. For instance, $Apc^{+/min}$ mice develop 30-50 adenomas within 4-5 months with a high density of tumors in the second half of the jejunum [52]. Whereas $APC^{+/1638N}$ mice only develop 4-8 tumors within 8-10 months and these are mainly located in the upper GI tract with a characteristic clustering at the transition from stomach to the small intestine [53]. These Apc mice are promising models of human colorectal cancer. However, a major drawback is that the tumors occur predominantly in the small intestine, not the colon. Crossing Apc mice with CEA-tg mice resulted in the development of intestinal adenomas with strong CEA expression, as well as CEA expression in the normal GI tract. It has been re-

ported that CEA-specific vaccination of the $Apc^{+/Min}$/CEA-tg mice resulted in the induction of CEA-specific immune responses and in a reduction of the number of intestinal tumors [54,55]. However, the CEA-specific effect was very low and other vaccine components, like non-specific stimuli as IL-2 and/or GM-CSF and/or cyclooxygenase-2 inhibitor, had a much greater impact on tumor development. These data argue that the limited CEA-specific T-cell repertoire can suffice when these mice receive a strong non-specific stimulus. The need for non-specific stimuli has also been described for the induction of effective CTLs against murine melanocyte/melanoma antigen gp100. Adoptive transfer of gp100 specific T cells in combination with both antigen-specific vaccination and system administration of IL-2 was necessary for clearance of B16 melanoma [56]. Despite the high CEA expression levels in the intestine and other epithelia of the CEA-tg mice in all mentioned models, in none of these reports efficient anti-tumor immunity was accompanied by the induction of auto-immunity. This paradox might be explained by the use of non-specific stimuli that may have effect on CEA-specific cells but will also activate T cells with different specificities and cells from the innate immune system.

Conclusion

All data together from clinical trials and mouse models are still not conclusive about whether CEA is a good target for immunotherapy of cancer. Therefore, I performed a detailed analysis of anti-tumor immunity against tumor-associated antigen CEA. To achieve effective anti-tumor efficacy it is important to identify immune mechanisms available for targeting CEA-positive tumors. I assessed the specificity and the character of the CEA-specific immune response by determining the specificity and magnitude of CEA-specific T- and B-cell responses in immunized wild-type mice. Because CEA is a human self-antigen, and no homologue for CEA is found in normal mice, the responses found in wild-type mice were compared with CEA-tg mice that have a similar expression pattern of CEA as humans. CEA-tg mice showed a severely limited CD4 T-cell repertoire compared to wild-type mice. Next, the question was raised whether this tolerance was induced in the periphery or in the thymus. My data show that CEA expression in thymic epithelial cells results in the tolerization of the T-cell repertoire against this antigen. All these issues together are extremely useful for the design of the vaccination strategy. Different immunization protocols have been tested to activate the available endogenous repertoire, but none of these were effective. To determine whether central tolerance could be circumvented, the T-cell repertoire in CEA-tg mice was reconstituted by adoptive transfer of CEA-specific T cells from wild-type mice. These data indicated that, in addition to central tolerance, also peripheral tolerance limited the CEA-specific T-cell repertoire in CEA-tg mice. Suppression of peripheral regulatory mechanisms could lead to better anti-tumor efficacy, but might also increase the risk for autoimmune reactions. Intriguingly, most modalities we tested, in which tolerance was overcome, showed anti-tumor efficacy that was accompanied by severe autoimmune pathology. I critically evaluated the different vaccination schemes by studying the specificity of the immune responses and the possible implementation in human cancer patients.

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