Cancer Immunol Immunother (2002) 51: 235–247
DOI 10.1007/s00262-002-0276-4

REVIEW

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The role of CD8$^+$ T cells in immune responses to colorectal cancer

Received: 1 November 2001 / Accepted: 24 January 2002 / Published online: 20 April 2002

Abstract Cytotoxic T lymphocytes (CTL) are essential effectors of the cell-mediated immune response. The ability of CTL to specifically recognise and lyse malignant cells expressing the relevant surface antigens under optimal in vitro conditions justifies attempts to boost their number and activity through various forms of immunotherapy. Considering the high prevalence of colorectal cancer and poor survival rates for patients with advanced-stage disease, the development of new protocols based on CTL stimulation represents a genuine and promising treatment option. Significant advances in recombinant DNA technology and molecular biology have led to the identification of a number of tumour-associated antigens (TAA). These have served as vaccine constituents and/or stimuli for obtaining CTL used for adoptive immunotherapy after in vitro stimulation and expansion. The present review describes the properties and functions of CTL as effectors of the immune response against tumours, and summarises the known TAA recognised by CTL and the current status of CTL-related immunotherapeutic interventions in colorectal cancer patients.

Keywords Colorectal cancer · Cytotoxic T lymphocyte · Immunity

Introduction

The past four decades have been marked by efforts to understand the role of immune surveillance mechanisms in the prevention of cancer, and to elucidate the failure of the immune system as regards malignant transformation and cancer progression.

The immune response against cancer cells is complex, involving the interaction of many different cell types and cell products, and it is not possible to consider cell-mediated and antibody-mediated responses in isolation. However, it is well recognised that cytotoxic T lymphocytes (CTL) constitute one of the most important effector mechanisms of anti-tumour immunity [38, 88]. CTL are able to perform tumour-specific recognition via their clonal T cell receptors (TCR) generated via a somatic recombination mechanism. Activated CTL can mediate specific destruction of tumour cells by the release of lytic components and direct cell–cell interaction. In parallel with the characterisation of the functions and mechanisms of CTL, the identification of antigens inducing T cell responses has progressed at an accelerated pace [87].

In general, CTL are CD8$^+$ and are therefore class I major histocompatibility complex (MHC)-restricted, even though in some instances CD4$^+$ class II MHC-restricted T cells have also been shown to perform cytolytic activities [10]. CD4$^+$ T cells play an important role in generating and sustaining protective immunity against malignant cells, mainly through the production of cytokines that act on the CTL to increase their cytolytic capacity; their presence, however, is not essential for lytic activity [27]. The remainder of this review will focus on CD8$^+$ CTL.

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Cytotoxic T lymphocytes and cancer immune surveillance

CTL are generated by immune activation of naive CTL precursors (CTLp), which appears to require at least two sequential signals [11].

Signal 1 is provided by the specific interaction of the TCR on the membrane of the CTL with the antigen on the surface of the antigen-presenting cells (APC). CD8$^+$

T cells recognise short peptide antigens (8–10 amino acids) presented by class I MHC molecules; CD8 serves as a co-receptor, augmenting the TCR–MHC–peptide interaction without influencing its specificity [62]. Endogenous protein antigens are cleaved into peptides by a multifunctional cytoplasmic protease complex called the proteasome. Resulting peptides are than transported into the rough endoplasmic reticulum (RER) by the transporter associated with antigen processing (TAP) system, which is a membrane-spanning heterodimer consisting of two proteins: TAP1 and TAP2. Here, the peptide associates to and stabilises the α-chain and $ß_2$-microglobulin components of the MHC molecule into a stable MHC–peptide complex that can now exit the RER and proceed to the cell surface via the Golgi apparatus.

Signal 2 is a co-stimulatory signal that is not antigen-specific and is mediated by the interaction between several pairs of ligand–receptor molecules. The best-characterised interaction is between the CD28 receptor on the surface of the CTL and the CD80 (B7.1) ligand on the membrane of certain specialised APC [114]. The engagement of CD28 by CD80 promotes the survival and clonal expansion of antigen-specific T cells by stimulating the expression of anti-apoptotic proteins of the Bcl family [8] and the production of interleukin-2 (IL-2) [40, 65]. Recent data suggest that while CD28 co-stimulation is required for T-cell proliferation, it is not essential for receptor-mediated target cell lysis [45]. Since all mature somatic cells express class I MHC/peptide complexes but lack expression of the CD80 molecule, the co-stimulatory signal represents an effective mechanism to prevent triggering of CTL activity against normal tissue components [109].

Once activated, CTL are highly cytolytic against tumour cells bearing the appropriate antigenic determinant. Perforin and enzymatic proteases (granzymes) are released into a confined junctional space between the two cells, causing cell death by disruption of the cell membrane and activation of the apoptotic pathway, respectively [3]. An alternative cytotoxic mechanism is the Fas–FasL interaction [52], where Fas and FasL are members of the tumour necrosis factor (TNF)/nerve growth factor (NGF) receptor superfamily that plays an essential role in apoptotic cell death. While the Fas receptor (45 kDa) is ubiquitously expressed on a variety of normal and tumour cells, its ligand, FasL (40 kDa), is present only on activated T cells, natural killer (NK) cells, Sertoli cells of the testis, and the retinal and corneal epithelium of the eye [95]. Ligation of Fas on target cells with membrane-bound FasL on CTL elicits a death-inducing caspase cascade that causes genomic DNA fragmentation and target cell apoptosis [49].

However, tumour cells can also acquire FasL expression and use it to deliver a death signal to activated Fas^+ T cells, offering a potential method for evading immunologic recognition [82, 99]. The Fas pathway is not only responsible for killing tumour infiltrating lymphocytes (TIL), but can also facilitate local tumour invasion by inducing apoptotic cell death in normal hepatocytes at the metastatic tumour margin [120]. This "tumour counterattack hypothesis" has recently been challenged by Favre-Felix et al. [30], who found that all of the seven colon cancer cell lines studied lacked FasL on their membrane, and were incapable of killing Fas-positive T cells. The authors propose a suicidal or fratricidal apoptotic mechanism by activated CTL co-expressing Fas and FasL [5] to explain TIL death associated with immune tolerance to cancer [24, 121]. Further studies are needed before the significance and various aspects of the Fas–FasL interaction between CTL and tumour cells can be fully determined.

Tumour cells may escape CTL detection by several other mechanisms. A partial or total loss of MHC class I expression on the cell surface has been well documented in the case of colorectal cancers [14, 35]. Downregulation of MHC class I expression may be secondary to mutations involving $ß_2$-microglobulin [17], the TAP complex [53, 94] or the LMP-2 and LMP-7 subunits of the proteasome [21].

Colorectal cancers have also been shown to induce defects in the expression and function of the TCR-associated ζ-chain [19, 116]. Matsuda et al. have found that decreased expression of TCR-related signalling pathways by TIL, and to a lesser extent, by peripheral blood lymphocytes (PBL) in colorectal cancer patients is correlated with disease stage [66]. In addition, a significant proportion of cancer cells and APC that infiltrate colorectal stroma fail to express the CD80 co-stimulatory molecule [16], contributing to the low immunogenicity of colorectal tumours.

MHC class I-associated antigenic epitopes in colorectal cancer

Oncogenic proteins are involved in the malignant transformation and maintenance of the transformed phenotype, and represent potential targets for CTL. Developments in molecular cloning and biochemical techniques have led to the identification of several TAA from colorectal cancers. Although many tumour-associated gene products are now known, corresponding T cell epitopes have been described for relatively few of them (Table 1). The majority are nonapeptides that bind specifically to HLA–A2, explained by the high prevalence of this MHC class I molecule in Caucasian populations.

Carcinoembryonic antigen

The carcinoembryonic antigen (CEA) is expressed in up to 85% of colorectal cancers [69] and therefore represents a potentially promising source of epitopes for targeting by CTL. Tsang et al. have succeeded in inducing a T cell response against CAP-1, a HLA–A2-restricted epitope of CEA, in three $HLA–A2^+$ patients

Table 1. MHC class I antigenic epitopes in colorectal cancer

TAA	Residue	Antigenic peptide sequence	HLA	Reference
CEA	571–579	YLSGANLNL	A0201	[86, 107]
CEA	Heteroclitic	YLSGADLNL	A0201	[123]
CEA	694–702	GVLVGALI	A0201	[92]
CEA	652–660	TYACFVSNL	A2402	[58]
CEA	268–277	QYSWFVNGTF	A24	[81]
CEA	652–660	TYACFVSNL	A24	[81]
CEA	61–69	HLFGYSWYK	A0301, A1101, A3101, A3301, A6801	[55]
Ep-CAM	263–271	GLKAGVIAV	A0201	[86]
Ep-CAM	184–192	ILYENNVIT	A0201	[105]
Ep-CAM	184–193	ILYENNVITI	A0201	[105]
Ep-CAM	Heteroclitic	YQLDPKFIV	A0201	[105]
Ep-CAM	Heteroclitic	ILYENNVIV	A0201	[105]
Ep-CAM	Heteroclitic	ILYENNVITV	A0201	[105]
her-2/neu	654–662	IISAVVGIL	A0201	[13, 85]
her-2/neu	8–16	RWGLLLALL	A24	[100]
her-2/neu	369–377	KIFGSLAFL	A2	[31, 122]
her-2/neu	754–762	VLRENTSPK	A0301, A1101, A3101, A3301	[55]
WT p53	149–157	STPPPGTRV	A0201	[101]
WT p53	264–272	LLGRNSFEV	A0201	[101]
WT p53	161–169	AIYKQSQHM	A24	[108]
hTERT	540–548	ILAKFLHWL	A0201	[70, 112]
hTERT	865–873	RLVDDFLLV	A0201	[70]
FBP	191–199	EIWTHSTKV	A2	[84]
SART1	690–698	EYRGFTQDF	A24	[57, 91]
SART1	736–744	KGSGKMKTE	A26	[91, 96]
SART3	302–310	LLQAEAPRL	A0201, A0207	[50]
SART3	309–317	RLAEYQAYI	A0201, A0207	[50]
p21 ras	1–25	MTEYKLVVVGAGAVGKSALTIQLIQ	B12(44)	[34]
MUC1	141–148	APDTRPAP	A2	[2]
MUC1	130–138	STAPPAHGV	A2	[2]
MUC1	12–20	LLLLTVLTV	A2	[12]
MUC1	950–958	STAPPHVNV	A2	[12]
MUC1	9–17	STAPPAHGV	A11	[25]
MUC2	476–484	LLNQLQVNL	A2	[7]
MUC2	654–662	MLWGWREHV	A2	[7]

with advanced colorectal cancer after vaccination with a recombinant CEA vaccinia virus [107]. PBL from these patients were used to establish a $CD8^+/CD4^+$ T cell line (V24T), which was capable of lysing the $CEA^+/HLA\text{-}A2^+$ SW403 colon carcinoma cell line in an HLA-restricted fashion. This was the first study demonstrating that a specific CTL response against CEA epitopes could be generated in colorectal cancer, as well as the MHC class I restricted nature of T-cell mediated lysis of human cells expressing an endogenously processed peptide.

In an attempt to increase the immunogenicity of self-antigens overexpressed by tumour cells, Zaremba et al. have substituted single amino acids of the CAP-1 peptide based on their predicted interaction with TCR. Of the four peptides tested, the agonist epitope of CAP-1, designated CAP-1–6D, was able to induce $CD8^+$ CTL that were at least two orders of magnitude more efficient at lysing human carcinoma cell lines expressing CEA in the context of HLA–A2 when compared with T cells sensitized by CAP-1 [123].

Kawashima et al. have isolated a CEA nonapeptide (^{61}HLFGYSWYK^{69}) that bound to five alleles of the HLA–A3 super-type family, and used it to induce specific CTL in vitro using peripheral blood mononuclear cells (PBMC) and peptide-pulsed autologous dendritic cells (DC) obtained from healthy $HLA\text{–}A1^+/A3^+$ donors [55]. $CD8^+$ CTL lysed the peptide-pulsed Epstein–Barr virus (EBV)-transformed human B cell line EHM and the $A3^+/CEA^+$ colon tumour cell line SW403 in a standard ^{51}Cr release assay. The authors have also described an HLA–A3 specific epitope of her-2/neu (^{754}VLRENTSPK^{762}) that is able to produce CTL active against the colorectal cancer cell line SW403 under identical experimental conditions.

Kim et al. have stimulated anti-CEA CTL by culturing PBMC from an HLA–A2402 healthy donor with the CEA peptide ^{652}TYACFVSNL^{660} [58]. Resultant CTL were able to kill $HLA\text{–}A2402^+$/CEA-expressing tumour cell lines and peptide-pulsed HLA–A2402 target cells, but not tumour cells lacking CEA. The lytic activity was blocked by anti-CD8 and anti-MHC class I monoclonal antibodies (mAb), confirming the central role of $CD8^+$ T lymphocytes in cell lysis. The technique described by Kim et al. provides a simple and pertinent option for the identification of other TAA epitopes without the need to obtain TIL or tumour-associated lymphocytes (TAL), which is an extremely difficult task.

Two other HLA–A24-restricted CEA epitopes, ^{268}QYSWFVNGTF^{277} and ^{652}TYACFVSNL^{660}, have recently been described by Nukaya et al. [81]; here

peptide-pulsed DC from a healthy HLA–$A24^+$ volunteer induced CTL in vitro that lysed colorectal cell lines expressing CEA in an antigen-specific and class I MHC-restricted fashion.

A novel T-cell independent approach for the identification of specific TAA epitopes has recently been described by Schirle et al. [92]. Using high performance liquid chromatography–mass spectrometry (HPLC–MS), the authors identified an HLA–A2-restricted CEA peptide (^{694}GVLVGVALI^{702}) in the colon carcinoma cell line SW1116 and a Duke's stage C colon tumour. To test the immunogenicity of this epitope, HLA–A2 monochain-transgenic H-$2D^b/\beta_2$-microglobulin double-knockout mice were immunised with the peptide, and yielded CTL that recognised $CEA_{694–702}$ loaded onto T2 cells and lysed the SW1116 colon carcinoma line. These CTL also stained positive with HLA–A2 tetramers folded with $CEA_{694–702}$. This was the first time that a tumour-associated MHC class I ligand was isolated from solid tumour tissue, and represents a promising avenue for the rapid screening of TAA peptides presented by any HLA molecule with a known sequence.

Epithelial cell adhesion molecule

Epithelial cell adhesion molecule (Ep-CAM; 17–1A, GA733) is a transmembrane glycoprotein with an important role in cell adhesion, that has been found to be over-expressed in over 90% of colorectal cancers and other epithelial tumours [37]. Ras et al. used spleen cells obtained from HLA–A0201 K^b transgenic mice previously immunised with a HLA–A0201-specific Ep-CAM-derived nonapeptide (^{263}GLKAGVIAV^{271}) in a standard ^{51}Cr release assay to assess this epitope's immunogenic potential [86]. Although isolated mice splenocytes were able to lyse Jurkat HLA–A0201 K^b target cells pulsed with peptide, no studies to date have confirmed the immunogenicity of this epitope in a human system. Similar results were obtained by this group for an HLA–A0201-restricted epitope of CEA (^{572}YLSGANLNL ^{579}), confirming the earlier results of Tsang et al. [107].

Recently, Trojan et al. have demonstrated that human CTL can be readily generated in vitro against two native and three heteroclitic Ep-CAM-derived peptides using PBMC obtained from three HLA–$A2^+$ healthy volunteers. Resultant CTL lysed the human colon cancer line SW480 in a HLA-restricted fashion, with CTL specific to the heteroclitic peptides demonstrating increased lytic efficiency compared with CTL raised against the native epitopes [105].

Her-2/neu

Her-2/neu (C-erbB2, p185) is a 185-kDa transmembrane glycoprotein with tyrosine kinase activity, broadly amplified and overexpressed in more than 80% of colorectal carcinomas, especially in the advanced stages [54]. The E75 nonapeptide has been identified by Fisk et al. as a HLA–A2-restricted her-2/neu epitope recognised by TIL isolated from HLA–$A2^+$ ovarian cancer patients [31]. Zaks et al. have used the E75 epitope to immunise patients with advanced colorectal breast and ovarian carcinomas, and have successfully isolated peptide-specific CTL in three out of four patients, none of whom exhibited CTL activity against E75 prior to immunisation. All patients were HLA–$A2^+$, and the her-2/neu antigen was over-expressed by all tumours. Despite being capable of lysing E75-pulsed target cells, CTL were unable to react either with a panel of her-$2/neu^+$/HLA–$A24^+$ tumour cell lines, or with HLA–$A24^+$ cells transfected with a recombinant vaccinia virus encoding her-2/neu [122], raising doubts about it being a naturally processed epitope.

Brossart et al. pulsed autologous DC generated from the PBMC of a healthy HLA–$A2^+$ donor with the her-2/neu-derived peptides E75 and GP2 [85], and used them as APC for CTL priming. High CTL activity toward peptide-pulsed targets was obtained after two weekly restimulations. Furthermore, these CTL lysed, in an MHC- and antigen-restricted fashion, a panel of colon, breast and renal cancer cell lines expressing her-2/neu [13].

Recently, Tanaka et al. have identified a novel HLA–A24-restricted her-2/neu epitope HE1 capable of inducing specific CTL from healthy donor $CD8^+$ T cells when presented in vitro by DC. CTL showed cytolytic activity not only against HE1-pulsed target cells, but also against HLA–$A24^+$ colorectal tumour cell lines expressing her-2/neu [122]. Together, these results suggest that epitopes derived from the her-2/neu protein might constitute attractive candidates for broadly applicable vaccines, and may prove useful for adoptive immunotherapies in colorectal cancers.

P53

Inactivation of the p53 tumour suppressor gene through mutations or deletions occurs in many different types of tumours, including colorectal cancer. The modified p53 protein is markedly overexpressed by cancer cells, representing a potential target for CTL. Due to the diversity of mutations that can arise in p53, only peptides representative of the wild type (WT) can be studied as potential immunogens. Considering that the WT p53 molecule is also expressed at low levels by the normal thymus, spleen and lymphohaematopoietic cells, T cells are likely to be tolerant of antigens derived from this molecule. Using transgenic mice that express the HLA–A0201 molecule, Theobald et al. have identified two peptide sequences (^{149}STPPPGTRV^{157} and ^{264}LLGRNSFEV^{272}) from the human WT p53 molecule that bind A0201, and serve as endogenously processed target epitopes for CTL recognition [101]. CTL lines specific for both peptides were able to recognise and lyse

a broad range of p53-expressing and A0201$^+$ tumour cells, including the SW480 colorectal cancer cell line. Other studies have also found the two peptides capable of stimulating a specific and HLA–A0201-restricted CTL response in PMBC expanded in vitro [48, 79, 80]. The array of WT p53 epitopes has recently been expanded by Umano et al., who discovered a HLA–A24-specific nonapeptide that when pulsed on DC was capable of inducing a CTL line active in vitro against the HT29 colorectal cell line [108].

Nevertheless, high avidity CTL specific for naturally processed WT p53 peptides have been found to be functionally tolerant [102]; this would need to be overcome if such epitopes were to be used in human immunotherapy studies. Unfortunately, no efficient methods to circumvent such functional tolerance have been proposed so far.

Telomerase

Telomerase is a ribonucleoprotein that mediates RNA-dependent elongation of telomeric DNA, preventing cells from ageing and allowing them to divide continuously. Telomerase activity has been observed in over 85% of solid tumours [59], and in up to 97% of colorectal cancers [28]. Vonderheide et al. have identified a HLA–A0201 epitope of the reverse transcriptase (hTERT) unit of telomerase, designated I540, and proved it to be capable of generating specific CTL in vitro. CD8$^+$ T cells from the peripheral blood of HLA–A2$^+$ healthy donors were primed and then re-stimulated weekly with autologous DC pulsed with I540. More than 80% of cultured cells expressed the CD3$^+$/CD8$^+$ phenotype after three cycles of re-stimulation. CTL from five out of seven donors were able to kill the U2OS, HLA–A0201$^+$ osteosarcoma cell line transfected with an amphotrophic virus bearing the full hTERT sequence. The HLA-restricted nature of the cytotoxic activity was again proven by blocking with an anti-MHC class I mAb, but not with a control mAb. Furthermore, CTL were able to lyse a panel of cancer cells of diverse histological origin expressing telomerase activity: ovarian, malignant melanoma, renal, multiple myeloma and lung cancer [112]. Minev et al. found that CTL raised against I540 and another HLA–A0201 epitope named p865 in prostate cancer patients were able to lyse not only the LnCap prostate cell line and target cells pulsed with the two peptides, but also HLA–A2$^+$ colon, breast and lung cell lines expressing telomerase activity [70]. In the case of the colorectal cell line SW480, cytolytic T cell activity was observed only with the p865-, and not for the I540-specific CTL. In order to test the capacity of I540 and p865 to induce CTL in vivo, the authors immunised HLA–A0201 monochain transgenic mice with these two peptides and successfully induced a specific CTL response against both of them, without the development of any autoimmune reactions. Due to the almost ubiquitous expression of telomerase by colorectal tumours and its high prevalence in all solid tumours, I540 and p865 warrant further investigation for inclusion in future immunotherapy trials.

Folate binding protein

Folate binding protein (FBP) is a membrane-associated glycoprotein identified in more than 90% of ovarian and endometrial carcinomas, but also present in 20% to 50% of colorectal cancers [115]. Peoples et al. have demonstrated that one HLA–A2-associated FBP epitope, designated E39, is capable of inducing CTL in vitro, which can lyse not only breast and ovarian cancer cells, but also the SW480 colorectal cell line [84]. So far however, no studies exploring the possibility of inducing CTL specific for FBP epitopes have been undertaken, and thus its immunogenic potential in colorectal cancers remains unproven.

Ras

Mutated *ras* proto-oncogenes, which play an important role in signal transduction, have been found in 40% to 60% of colorectal cancers [9]. Fossum et al. have described the isolation of human CD8$^+$ T cell clones from a colon cancer patient harbouring a K-*ras*, codon 13, point mutation (Gly–>Asp) that lysed a colon carcinoma cell line expressing the corresponding mutation in vitro [34]. The same group had previously identified both CD4$^+$ and CD8$^+$ T-cell clones reactive against a 25-mer, 13Gly–>Asp mutated K-*ras* peptide in a colon cancer patient, but the corresponding mutation could not be found in the cancer cells [33]. Although one possible explanation is that in this patient a specific T cell response resulted in the eradication of tumour cells harbouring the 13Gly–>Asp mutation, more evidence is needed before this peptide's immunogenic potential can be fully determined.

Squamous tumour-rejecting antigen

The squamous tumour-rejecting antigen 1 (SART1) gene is expressed in 39% of colorectal adenocarcinomas [91]. One HLA–A24-specific SART1 peptide, ^{690}EYRGF-TQDF^{698}, was recognized by HLA–A24-restricted and tumour-specific CTL established from an oesophageal cancer patient. After a third stimulation in vitro, this peptide induced CTL active against SART-1$^+$ tumour cells in PBMC of all HLA–A24 homozygous and the majority of HLA-A24 heterozygous cancer patients and healthy donors tested [57]. Shichijo et al. obtained similar results with a HLA–A26-restricted SART1 nonapeptide, ^{736}KGSGKMKTE^{744} [96].

Ito et al. [50] have established an HLA–A0201-restricted and tumour-specific CTL line from the TIL of a patient with colon cancer, and tested it for

interferon-gamma (IFN-γ) production after stimulation with T2 cells loaded with two HLA–A0201-specific SART3 peptides: ^{302}LLQAEAPRL^{310} and ^{309}RLAEYQAYI^{317}. Both peptides were capable of inducing IFN-γ secretion by CTL in a dose-dependent manner. The two peptides were subsequently tested for their ability to induce specific CTL in vitro in two HLA–A2^{+} patients with colorectal cancer. Both patients (one HLA–A0201 and the other HLA–A0207) produced significant numbers of CD8^{+} T cells capable of secreting IFN-γ and lysing HLA–A2^{+} cells lines, but not HLA–A2^{-} cells. The same test yielded negative results for three healthy HLA–A2^{-} subjects. In another study, two HLA–A2402-specific SART3 peptides, ^{109}VYDYNCHVDL^{118} and ^{315}AYIDFEMKI^{323}, induced CTL in vitro from the PBMC of four patients with epithelial cancers that lysed HLA–A24^{+} tumour cells in a Cr^{51}-release assay [119]; however, none of the patients studied had a colorectal tumour. Although SART peptides have been proved to be immunogenic in vitro, further studies on the biological function of the SART antigens and the non-response observed in healthy volunteers are needed before their potential in stimulating CTL immunity in colorectal cancers can be fully assessed.

Mucins

Mucins are glycoproteins that are strongly over-expressed in carcinomas of the colon, breast, ovary and pancreas. Four MUC-1 [2, 12] and two MUC-2 [7] HLA–A2-restricted peptides, and one MUC-1 peptide specific for HLA–A11 [25] have been identified, but none of them has yet been proven to induce CTL reactive with colorectal tumour cells either in vivo or in vitro.

Melanoma antigens

Mori et al. [72] and Hasegawa et al. [42] have shown that different antigenic peptides of the melamona antigen (MAGE) group of genes are expressed in 30% to 90% of colorectal cancers. Even though no reports have yet appeared on CTL active against colorectal tumours bearing MAGE antigens, the relatively high prevalence of MAGE expression could justify the use of these peptides for further study in colorectal cancer patients.

Although tumour-reactive T cells against various TAA have been expanded from both TIL and PBL, in most cases this has involved a period of in vitro stimulation and culture, resulting in both quantitative and qualitative changes. A recent ex vivo study by Nagorsen et al. has identified the presence of CTL directed against HLA–A2-associated epitopes of CEA, Ep-CAM and her-2/neu in approximately one-third of colorectal cancer patients [75]. These CTL were capable of secreting IFN-γ and upregulating CD69, but their cytolytic ability was not tested against tumour cells. A positive CTL response was only observed in advanced stages of the disease (Duke's stages C and D), prompting the hypothesis that the spread of tumour cells into the periphery, especially the lymph nodes, is a prerequisite for the induction of CTL against TAA. An alternative explanation is that in cases of limited disease, CTL are restricted to the primary tumour as TIL, whereas disseminated cases can also elicit a CTL response detectable in the periphery. Ongoing studies will elucidate if CTL against other common self-antigens can be detected in colorectal cancer patients, and establish whether they are indeed capable of lysing tumour cells.

Immunotherapeutic interventions aimed at stimulating CTL activity in colorectal cancer patients

The induction of tumour-specific T cell responses that are effective in eradicating disseminated tumours and in mounting a persistent tumour-protective immunity remains a major goal of tumour immunotherapy. So far, development of T cell therapy for the treatment of colorectal cancer has been hindered by the relative lack of identifiable antigenic epitopes recognised by CTL and by difficulties in circumventing tumour escape mechanisms; and more so, as the majority of studies are still in phase I–II. As more antigenic determinants become available, the number of such studies and their rate of progress to large-scale clinical trials would be expected to increase significantly.

Active immunotherapy

Active immunotherapy employs full-length or known epitopes of TAA administered systemically either alone or combined with other immunogens, with the aim of stimulating the patient's own CTL immune response.

Early attempts to stimulate CTL immunity included the following: bacillus Calmette–Guérin (BCG), levamisole, cytokines, autologous tumour cells, tumour cell extracts and antigen mixtures from cell culture supernatant fluids alone or in combination. Although some of these trials [41, 47, 110] have shown occasional tumour regression and improved patient survival, the cellular immune responses have only been measured in terms of delayed sensitivity skin tests. Sobol et al. have administered subcutaneously (s.c.) a mixture of irradiated tumour cells and IL-2-transfected fibroblasts in ten patients with colorectal cancer, but, despite a five-fold increase in the frequency of tumour-specific CTL in two out of six evaluable patients, all the patients included in the study eventually developed progressive disease [97]. Interestingly, patients without detectable CTLp before treatment failed to show any increase in specific CTL frequency after therapy with the tumour cell/IL-2 combination. The lack of a quantifiable clinical response compounded with the problems of using tumour cells as

antigen, i.e. the difficulty in obtaining sufficient cells for immunisation and potential for transmitting putative contaminating tumour viruses, have made this approach less attractive and prompted the use of other techniques.

Active specific immunotherapy attempts to stimulate the immune system to target a particular antigen administered in various forms and combinations as a vaccine (Table 2).

The simplest strategy to stimulate the host's immune response against tumours is the s.c. injection of peptides derived from relevant TAA, often accompanied by an immunoadjuvant. Peptide immunisation offers the possibility of specifically directing the immune response against tumour-expressed epitopes, avoiding the potential induction of autoreactivity towards sequences present in the remainder of the protein. Synthetic ras oncogene peptides administered in combination with recombinant granulocyte–macrophage colony stimulating factor (GM–CSF) as adjuvant have successfully generated an immune response in phase II trials in colorectal cancer patients [36]. More recently, vaccination with two SART3 peptides induced a significant increase in the cellular immune response to colon cancer cells in seven out of 11 and seven out of the ten patients tested, respectively [71]. Many similar studies with different peptides and cytokines are ongoing (Table 2).

Over the past decade, increasing attention has been focused on DC as vehicles for antigen delivery in active immunotherapy trials. DC are considered to be the most potent APC, and can now be readily generated in vitro from autologous PBMC [6, 73, 90]. PBMC-derived DC can be loaded with single or multiple antigens, and administered systemically to cancer patients with little or no side-effects and a resultant increase in specific T cell anti-tumour immunity [18]. Nair et al. have successfully induced CTL against CEA after in vitro stimulation of PBMC from colorectal cancer patients with autologous DC pulsed with CAP-1 (in HLA–$A2^+$ patients) or transfected with CEA mRNA (in HLA–$A2^-$ subjects). CTL were capable of lysing target cells pulsed with CEA in a standard europium release assay in 16/17 patients included in the study; similar levels of CTL activity were seen whatever method of "immunisation" was used [77]. Alters et al. obtained similar results in breast and pancreatic carcinoma patients and normal donors [1]. A recent study by Fong et al. reported an objective response in two out of 12 patients with advanced colorectal cancer vaccinated with an altered peptide (YLSGADLNL) of CEA loaded onto DC expanded by immunisation with the Flt3 ligand, a haematopoietic growth factor [32]. The clinical response showed a statistically significant correlation with the percentage of $CD8^+$ cells that stained positive for the corresponding peptide–HLA–A2 tetramer. Many other DC-based trials are now underway and it is hoped that they will confirm the positive results observed in melanoma [78] and prostate cancer [74, 104].

Table 2. Types of recombinant colorectal cancer vaccines

Synthetic peptides with adjuvant pulsed with DC
Heat-shock proteins
Insertion of tumour-associated gene into a vector
Vaccinia virus
Avipox virus
BCG
Polynucleotides
Anti-idiotype antibodies

A major criticism of peptide vaccination is the limited number of peptide epitopes to which the immune system can be exposed. Heat-shock protein (HSP) preparations contain a broad array of natural tumour peptides tightly bound to HSP molecules and theoretically have the advantage of being able to generate an immune response against a multitude of TAA. Although several studies have shown that injection of HSP preparations from a given tumour into syngeneic rats or mice renders them resistant to the particular tumour [83, 98, 113], there are no reports that prove the effectiveness of this approach on human colorectal tumours.

Zhu et al. have succeeded in generating CEA-specific CTL in seven out of eight HLA–$A2^+$ patients vaccinated with a replication-defective avipox vector (ALVAC) containing the CEA gene. Post-vaccination, PBMC were incubated with the CAP-1 peptide and IL-2, and the resultant CTL expressed lytic activity against the HLA–$A2^+$/CEA-expressing colorectal cell lines, but not a HLA–$A2^-$/CEA^+ line [124]. This demonstrated that ALVAC–CEA could be used to vaccinate cancer patients, and elicit CTL specific for the CAP-1 epitope, which are capable of lysing human tumour cells expressing this antigen.

Similarly, von Mehren et al. have used the ALVAC–CEA/B7.1 vector to vaccinate patients with advanced or metastatic CEA-expressing adenocarcinomas, the vast majority of which were of colorectal origin. Twelve out of 15 HLA–$A2^+$ patients that could be analysed demonstrated a statistically significant increase in CEA-specific T-cell precursor frequency after vaccination in an ELISPOT assay [111]. The interpretation of these results is complicated by the fact that most patients also received prior immunosuppressive treatment. An inverse correlation between the number of prior chemotherapy regimens and the CTL response to the dual-gene vaccine was observed, suggesting an immunomodulatory effect. Furthermore, no objective clinical responses to the vaccine could be demonstrated, and the majority of patients showed a rise in their serum CEA values. However, 27% of all evaluable patients experienced stabilisation of the disease after four immunisations; this group included all patients with an initial decrease in the serum CEA. Further studies that include patients with no or minimal residual disease, and those which have not undergone any other adjuvant therapy are needed before the efficacy of this vaccine can be fully evaluated.

The observation that intramuscular injection of plasmid DNA preparations can result in myocyte gene expression and induce immune responses to encoded immunogens has encouraged attempts for its use in

cancer immunotherapy. Conry et al. have constructed a plasmid encoding the full-length cDNA for human CEA, and have demonstrated that this can function as a polynucleotide vaccine to elicit a CEA-specific cellular and humoral immune response in mice [20]. The observed immune response protected mice against tumour challenge with syngeneic CEA-transduced colon carcinoma cells. However, to our knowledge no reports have yet been published on the efficacy of this approach or of that concerning any other intramuscular vaccines in colorectal cancer patients.

Based on Jerne's network concept [51], anti-idiotypic antibodies (ab2) can be used as the "internal image of the antigen" recognised by a defined therapeutic antibody (ab1). In addition to inducing an anti-anti-idiotypic antibody (ab3) humoral response, ab2 also stimulates the activation of T cells recognising the nominal TAA [68]. However, tumour cells not expressing the antigen that the anti-idiotypic antibody mimics may escape lysis, thereby reducing the efficacy of the vaccine [67]. Fagerberg et al. have observed tumour regression in five out of 24 patients with metastatic colorectal carcinoma treated with mAb 17–1A, which recognises the GA733–2 (Ep-CAM) epitope [29]. Four out of the five responders demonstrated T cells recognising GA733–2, while all non-responding patients lacked such cells. Similarly, Buckley et al. [15] observed tumour killing by activated $CD8^+$ T cells and NK cells in 75% of patients with advanced colorectal cancer immunised with the human anti-idiotypic antibody 105AD7 that mimics the 791Tgp72 (CD55) antigen, present in 70% to 80% of colorectal tumours [4]. In a separate study, Durrant et al. immunised six patients with rectal cancer preoperatively with 105AD7, and observed significant killing of autologous tumour cells by cryopreserved lymphocytes or lymph node cells of three patients at one to two weeks post- immunisation, but not with pre-treatment biopsies [26]. These results indicate that immunisation with anti-idiotypic antibodies has the potential to enhance cytotoxic activity in colorectal cancer patients by specific and non-specific effector mechanisms, but any potential clinical benefit has yet to be confirmed by larger randomised trials.

Adoptive immunotherapy

Adoptive immunotherapy employs in vitro generated allogenic CTL against selected TAA, which are then administered to a tumour-bearing host where they mediate tumour regression. Both PBMC and TIL can be used as a source of cytolytic cells, although there are reports suggesting that expanded TIL are the more efficient effectors [56]. In many cases, CTL are re-administered with an adjuvant that potentiates their activity, most commonly IL-2 [93]. IFN-α [64] and IFN-γ [22], as well as interleukin-6 (IL-6) [22, 106] have all been found to enhance TAA expression by cancer cells, and therefore could be included as adjuvants to facilitate target recognition by CTL [43].

Xiang et al. have demonstrated that a recombinant humanised anti-EpCAM antibody-IL-2 fusion protein (huKS1/4-IL-2) administered systemically was capable of inducing a MHC class I-restricted $CD8^+$ T-cell-mediated eradication of established pulmonary and hepatic metastases in syngeneic BALB/c mice [117]. In a subsequent study [118], the authors found that $CD4^+$ and $CD8^+$ lymphocytes obtained from the spleens of mice that previously rejected pulmonary metastases bearing the Ep-CAM antigen were also effective in protecting naive BALB/c scid/scid mice challenged with Ep-CAM^+ tumour cells. The central role of $CD8^+$ T cells in tumour rejection was confirmed by the fact that only mice injected with $CD8^+$ cells from BALB/c mice previously cured of metastatic disease by treatment with huKS1/4-IL-2 showed a complete absence of macroscopic pulmonary metastases; $CD4^+$ cells obtained from the same animals were far less efficient at preventing pulmonary metastases in naïve BALB/c scid/scid mice. Transferred $CD8^+$ cells were effective in rejecting tumour challenges up to five months after the initial treatment, thereby demonstrating the long-lived nature of this immune response. The results provide positive support for TAA–cytokine fusion proteins as vaccination strategies, possibly in conjunction with adoptive therapy of antigen-specific $CD8^+$ cells.

Whilst direct in vitro induction of $CD8^+$ T cells against particulate antigens [39] and known antigenic epitopes of soluble antigens [76] has been successfully achieved, the same response against whole soluble proteins like CEA, which are known to preferentially stimulate a T helper response [89], was thought to be more difficult. Kim et al. [58] have proved this possible by generating CEA-specific CTL from fresh PBMC in a healthy donor after several cycles of in vitro stimulation with autologous PBMC loaded with CEA-bound latex beads. The resultant cell population contained 72.9% $CD8^+$ cells that were able to lyse CEA-positive cancer cell lines and target cells loaded with CEA. The same effector cells also lysed targets loaded with a HLA–A2402-restricted CEA peptide, demonstrating that the exogenous antigen on the latex beads was processed and presented by MHC class I molecules after delivery to the cells. This method could be widely applicable to generate autologous CTL capable of responding polyclonally to various TAA epitopes, which might then be used for adoptive immunotherapy.

Bispecific monoclonal antibodies (bi-mAb), which have one of the two Fv fragments recognising an ubiquitous T cell antigen (most commonly CD3) and the other targeting a defined TAA, bypass the need for TCR/MHC class I–peptide complex interaction for T cell activation, and which represent a useful alternative for targeting CTL to antigens for which specific HLA epitopes have not yet been isolated. Hombach et al. have generated a bi-mAb OKT3/NSI19–9 by somatic fusion of two hybridoma lines secreting antibodies against CA19–9 and CD3, respectively. This bi-mAb in combination with a co-stimulatory anti-CD28 mAb activated

resting peripheral T cells, which could lyse CA19–9$^+$ tumour cells in vitro [46]. However, systemic administration of bi-mAb in cancer patients produced severe side-effects (fever, rigors and dyspnoea), thought to be due to the non-specific activation of T cells and cytokine release by contaminating intact antibody after incomplete proteolytic digestion when using the hybrid-hybridoma technology [60, 103]. Recombinant bispecific antibodies created by joining two single-chain Fv fragments are devoid of any Fc portion and/or anti-CD3 homodimers, thus reducing the potential for side-effects due to non-specific T cell stimulation. A single-chain bispecific antibody for CD3 and the Ep-CAM antigen was found to induce both CD8$^+$ and CD4$^+$ cytotoxic T cells active against various tumour cell lines that express Ep-CAM [61, 63], but has not yet been employed in clinical trials.

A new strategy, which combines the advantages of antibody-based antigen recognition and cell-mediated cytolysis, is to graft T cells with a chimeric antibody that binds to a TAA and mediates cellular activation. Darcy et al. have cloned a scFv anti-CEA chimeric receptor gene into a retroviral vector and used it to transduce enriched naive T cells from BALB/c mice. The mouse T cells expressing scFv anti-CEA demonstrated the ability to bind to CEA-expressing target cells in a rosette assay and exerted cell lysis on a CEA$^+$ colon carcinoma cell line in a Cr^{51}-release assay. Furthermore, transduced T cells adoptively transferred into scid mice with established s.c. colon carcinoma completely rejected tumour growth in two out of five mice, and partially inhibited tumour growth in the remaining three [23]. The main advantage of this approach is the non-MHC-restricted nature of tumour cell lysis performed by the modified CTL, but the efficacy of tumour cell targeting by immune-receptor-grafted effector cells is limited by a potential host-versus-graft response against xenogenic parts of the receptor and the number of peripheral CTL that can be successfully grafted with the recombinant receptor. To partially address this, Hombach et al. have constructed an entirely humanised TCR composed of an antibody-derived extracellular domain for CEA binding and a CD3 ζ-chain-derived signalling domain for cellular activation and inserted it into a retroviral vector [44]. The construct was then packed into the GALV- and A-MuLV-pseudotyped viruses, and used to infect human peripheral blood T cells to generate anti-CEA T cells. The number of anti-CEA receptor-expressing T cells was further increased by magnetic activated cell sorting. The ζ-chain receptor induced an effective and MHC-independent immune response of grafted T cells that were capable of lysing CEA$^+$ tumour cells in vitro at effector:target ratios as low as 10:1. These initial results suggest that the adoptive transfer of gene-engineered T cells has potential as a widely applicable immunotherapeutic strategy, particularly for tumours such as colorectal cancers, for which few specific CTL clones have been isolated.

Conclusions

Characterisation of the mechanisms for cancer cell recognition and lysis by CTL, and of the processes involved in tumour cell escape is essential for further development of immunotherapeutic strategies. Although CTLp against colorectal cancer antigens can be detected in the peripheral blood and peri-tumoral inflammatory infiltrate, their frequency is extremely low and they probably have little anti-tumour effect. Current efforts are aimed at boosting specific CTL numbers and activity in the hope that this may overcome the pre-existing immunologic tolerance and lead to tumour rejection and prevention of recurrence.

Stimulation of the CTL response against TAA is a promising and safe adjuvant therapeutic option. The large number of patients with a small tumour burden following surgery makes colorectal cancer an attractive candidate for adjuvant immunotherapy trials. The advent of reliable and sensitive systems such as the ELISPOT assay and multimer analysis that can quantify the amplitude of CTL specific to various TAA offer reliable tools for monitoring and correlating the effect of CTL expansion with tumour response. As new antigenic epitopes continue to be defined and more effective antigen delivery systems become available, the role of CTL-based immunotherapy in colorectal cancer is expected to become more important.

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