Immunotargeting of cancer stem cells

Cancer stem cells (CSCs) represent a distinctive population of tumour cells that control tumour initiation, progression, and maintenance. Their influence is great enough to risk the statement that successful therapeutic strategy must target CSCs in order to eradicate the disease. Because cancer stem cells are highly resistant to chemo- and radiotherapy, new tools to fight against cancer have to be developed. Expression of antigens such as ALDH, CD44, EpCAM, or CD133, which distinguish CSCs from normal cells, together with CSC immunogenicity and relatively low toxicity of immunotherapies, makes immune targeting of CSCs a promising approach for cancer treatment. This review will present immunotherapeutic approaches using dendritic cells, T cells, pluripotent stem cells, and monoclonal antibodies to target and eliminate CSCs.

Key words: cancer stem cells, tumour immunotherapy, cancer, dendritic cells, adoptive cell transfer, monoclonal antibodies.

Eliza P. Kwiatkowska-Borowczyk[1,2], Agnieszka Gąbka-Buszek[1], Jakub Jankowski[1], Andrzej Mackiewicz[1,2]

[1]Department of Cancer Immunology, University of Medical Sciences, Poznan, Poland
[2]Diagnostic and Immunology Department, Greater Poland Cancer Centre, Poznan, Poland

Introduction

The mechanisms that control tumour initiation, growth, and dissemination by the immune system are still not fully understood. It is generally accepted that the host defence mechanisms may lose control of the cancer. There is also increasing evidence that at least some tumours can be eradicated by modulation/stimulation of the immune system by so-called immunotherapy. Moreover, various treatment modalities of cancer patients such as chemotherapy, radiotherapy, or surgery are accompanied by immunological reactions that coexist with the treatment, and "immunological support" is necessary for therapy efficacy. Since the discovery of cancer stem cells (CSCs), which form the core and drive the tumour growth, intensive research is being carried out to understand their biology and determine the means of their eradication. Due to their properties, CSCs have been proven be the force behind tumour initiation, progression, and maintenance. Moreover, they have transpired to be highly resistant to chemo- and radiotherapy that depends on CSC modified division patterns [1]. Their influence is great enough to risk the statement that a successful therapeutic strategy must target CSCs in order to eradicate the disease.

Current cancer immunotherapy strategies are based on vaccines, adoptive transfer of tumour-specific T-cells, and monoclonal antibodies targeting tumour cells or immunoregulatory molecules (immune check-points). Although the majority of clinical trials employing immunotherapy to treat cancer have been phase I, during the last 25 years the number of cancer immunotherapies in phase III clinical trials has exceeded 500. However, the only immunotherapeutic anticancer drugs that have received FDA approval are several monoclonal antibodies and sipuleucel-T (Provenge), the dendritic cell-based therapy for prostate cancer. It was recently reported that in certain settings CSCs might be immunogenic. Thus, they bear the potential to stimulate specific immune responses that could target and eliminate tumour CSCs in cancer patients.

Dendritic cell-based cancer vaccines

Dendritic cells (DC) are the most potent and versatile of antigen presenting cells, initiating and maintaining immune responses. To induce anticancer immunity, DCs presenting cancer antigens to T cells are required. Dendritic cells vaccines are pointed at boosting cancer-specific effector T cells to eradicate tumour cells, and at stimulating immunological memory to control the recurrence of cancer [2]. Although a number of clinical trials have been conducted, since the Food and Drug Administration's (FDA) approval of Provenge for prostate cancer treatment in 2010 no new DC-based vaccines have been granted marketing authorisation. That means that there is still potential to be discovered, possibly by retargeting dendritic cells at CSC antigens.

Research conducted since the discovery of CSC has revealed the potential of DC vaccination to target CSCs of several types of solid tumours in preclinical settings. In the GL261 mouse model of glioma, Pellegata *et al.* demonstrated that DCs pulsed with glioma CSCs antigens provided more efficient protection against GL261 tumours than DCs pulsed with adherent GL261 antigens [3]. Treatment with CSC-based vaccine induced higher lytic activity of splenocytes as well as robust tumour infiltration by CD8+ and CD4+ T cells. Dendritic cells-cancer stem cells (DC-CSC) vaccine cured 80% of "regular" GL261 tumours and 60% of CSC-initiated gliomas, whereas vaccination with regular vaccine cured none of the CSC-initiated tumours. In the rat 9L brain tumour model it was demonstrated that DC vaccination of with 9L CSCs significantly prolonged survival of gliosarcoma-bearing mice compared to vaccination with 9L cells [4]. The protective effect was correlated with higher interferon γ (IFN-γ) production by CD8+ T cells in rats treated with DC-9L CSCs vaccine.

It has been shown that stem cell-enriched populations can be isolated using aldehyde dehydrogenase (ALDH) as a marker [5, 6]. Using this technique Ning *et al.* purified CSCs from D5 murine melanoma and SCC7 squamous cell cancer and used it as a source of antigens to prime DCs [7]. The authors found that enriched CSCs were immunogenic and more effective than unsorted heterogeneous tumour cells in inducing immunity of the host to reject the challenge of tumour cells. In both melanoma and squamous cell cancer models, CSC-based vaccines led to significant inhibition of tumour growth. Cancer stem cells-vaccinated animals showed systemic humoral responses with high level of IgG, which recognised CSCs and were efficient in CSC lysis in the presence of a complement. Moreover, CTLs from CSC-vaccinated mice were capable of killing corresponding CSCs *in vitro*, which proves that CSCs can be destroyed by CSC vaccine-primed immune response.

Successful therapeutic strategy against sarcoma was described for the murine CMS-4 tumour model [8]. Dendritic cells loaded with the lysate of induced vascular progenitor (iVP) cells derived from induced pluripotent stem (iPS) cells protected 75% of CMS-4 fibrosarcoma-inoculated mice from the development of tumours for a seven-week period, and 50% of animals were still alive tumour-free six months after tumour implantation. The seven-week survival of the DC-CMS-4-immunised group was 50%, but all mice developed tumours after that time and died. In addition to slower growth, tumours that developed in mice vaccinated with DCs loaded with iVP cells revealed a limited vascular bed. Treatment elicited antitumor immunity targeting vascular and tumour cells demonstrated by CD8+ T lymphocytes cytotoxicity against endothelial cells and CMS-4 sarcoma.

Very recently the first report of a therapy targeting CSCs in solid tumour has been published. Seven patients that underwent surgery and chemo-radiotherapy were treated with immune therapy targeting autologous glioma stem cell (GSC) antigens [9]. Vaccination with DCs transfected with CSC-mRNA induced an immune response in all patients with no adverse autoimmune events or other side effects. *In vitro* studies of patient-derived T-cells revealed

proliferation in response to GSC-lysate, indicating successful mobilisation of the immune system. Compared to historical-matched controls, the vaccinated patients had significantly longer progression-free survival (median 694 days vs. 236 days, $p = 0.0018$). Five of the treated patients developed tumour recurrence, but later than most recurrences in the matched control group, in which all patients experienced progression. Currently, two other clinical trials are gathering participants for phase I studies to test the safety and effects of dendritic cell vaccines loaded with a lysate derived from an allogeneic glioblastoma stem-like cell line [10] or purified peptides from the CD133 CSC antigen [11] in newly diagnosed and recurrent glioblastoma.

Adoptive immunotherapy

Adoptive transfer of T cells bypasses the antigen presentation step of immune response by immediately delivering effector cells as a therapeutic agent. The leukocytes are isolated from the patient, manipulated *in vitro*, and transferred back to the affected individual. To broaden the efficacy, these T cells can be genetically modified to express antigen receptors with desired specificity by introducing tumour-specific TCRs or chimeric antigen receptors (CARs) [reviewed in 12, 13]. Moreover, the more accurate targeting of antigens may be crucial for successful therapy, and T-cell targeting CSCs could significantly enhance eradication of tumours.

There are reports showing that CSCs can be successfully targeted *in vitro* with both allogeneic as well as autologous T cells. In 2007 HLA-A2-restricted, naturally presented, CD8+ T-cell-defined tumour peptide of the CSC marker ALDH1 was identified for head and neck squamous cell carcinoma (HNSCC) [14]. In 2011 Visus *et al.* demonstrated that ALDH1A1$_{88-96}$ peptide-specific CD8+ T cells could recognise ALDH^{bright} cancer-initiating cells present in HLA-A2+ human head and neck, breast and pancreas carcinoma cell lines, xenografts, or surgically removed lesions *in vitro* [15]. Moreover, in the human tumour xenograft model adoptive transfer of ALDH1-specific CD8+ T cells inhibited growth of subcutaneously growing tumours and lung metastases. Liao *et al.* reported that putative CSCs generated from two HNSCC cell lines and cervical carcinoma cell line CaSki were very sensitive to MHC class I-restricted alloantigen-specific CD8+ CTL lysis after treatment with IFN-γ that up-regulated MHC I expression [16]. Also, the subset of ALDH^{high}-expressing CSCs presented greater sensitivity toward CD8+ CTL killing than the ALDH^{low} cells. Autologous tumour-infiltrating lymphocytes and autologous CTL clones derived from peripheral blood of patients were shown to specifically recognise and kill CSCs of malignant fibrous histiocytoma of the bone [17].

Ahmed *et al.* developed HER-2-specific T cells from glioblastoma multiforme (GBM) patients by genetic transfer of HER2-specific chimeric antigen receptor [18]. These HER2-specific T cells showed cytotoxicity against HER2-positive targets *in vitro* and secreted immunostimulatory Th1 cytokines. The HER2-positive T cells killed *in vitro* autologous CD133-positive GBM stem cells, expressing HER2. These cells are resistant to current standard

Table 1. Immunotherapeutic strategies that target cancer stem cells

Therapeutic agent	Targets	Disease	Status	References
Cancer vaccines				
Dendritic cells	CSCs	Glioblastoma	Preclinical (*in vitro*)	3, 4, 60
	CSCs/ALDH[+]	Melanoma, squamous cell cancer		7
	Vascular progenitor antigens	Sarcoma		8
	Survivin, hTERT	Glioblastoma	Clinical trial, phase I, II	9
	CSCs	Glioblastoma	Clinical trial, phase I	10
	CSCs/CD133^{+}	Glioblastoma		11
	CSCs	Melanoma	Clinical trial, phase II	61
	CSCs/HER2^{+}	Breast cancer		62
CTL	CSCs	Cervical cancer, head and neck cancer	Preclinical (*in vivo*)	16
	CSCs/ALDH^{+}	Malignant fibrous histiocytoma		17
	CSCs/CD44^{+}/CD24^{lo}	Breast cancer		63
	CEPP-55 peptide	Colon cancer		64
	CSCs/ALDH^{+}	Lung cancer		15
	CSCs/HER2^{+}	Glioblastoma multiforme		18
	DNAJB8 heat shock protein	Colorectal cancer		19
hiPSC/hESC	CSCs	Colon cancer	Preclinical (*in vivo*)	22
mESC		Lewis lung carcinoma		23
mESC with GM-CSF expressing fibroblasts		Lung cancer		24
hESC with MWCNTs		Colon cancer		58
PSCA cDNA mPSCA-VRP	CSCs-PSCA antigen	Prostate cancer	Preclinical (*in vivo*)	59
NK cells	CSCs/CD133^{+}	Melanoma	Preclinical (*in vitro*)	65
γδ T lymphocytes	CSCs	Ovarian cancer	Preclinical (*in vivo*)	66
Monoclonal antibodies				
H90	CD44	AML	Preclinical (*in vivo*)	67
P245		Breast cancer		28
H4C4		Pancreatic cancer		68
GV5		Cervix cancer, larynx cancer		30
RO5429083		HNSCC		31
RO5429083		Metastatic/locally advanced, CD44-expressing solid tumours	Clinical trial, phase I	33
RO5429083		AML		34
MT110 (Solitomab)	EpCAM	Ovarian cancer, colon cancer, pancreatic cancer	Preclinical (*in vivo*)	37, 38
		Lung cancer, gastric cancer, breast cancer, colorectal cancer, prostate cancer, ovarian cancer	Clinical trial, phase I	39, 76

Table 1. Cont.

Therapeutic agent	Targets	Disease	Status	References
Catumaxomab	EpCAM/anti-CD3/Fcγ	Ovarian cancer, pancreatic cancer, gastric cancer	Clinical trial, phase I-III	40, 41
		Epithelial carcinoma	Clinical trial, phase I	76
Demcizumab (OMP-21M18)	DLL4	Colon cancer, breast cancer, melanoma	Preclinical (*in vivo*)	51, 52
B6H12.2	CD47	Glioblastoma, breast cancer, bladder cancer, colon cancer	Preclinical (*in vivo*)	69
		Non-Hodgkin lymphoma, acute lymphoblastic leukaemia, multiple myeloma		70, 71
B6H12		Aggressive metastatic leiomyosarcoma		72
7G3	CD123	AML	Preclinical (*in vivo*)	73
CSL360			Clinical trial, phase I	74
AVE1642	IGF-IR	Myeloma, colon cancer	Preclinical (*in vivo*)	87
		Solid tumours	Clinical trial, phase I	77
Figitumumab (CP-751,871)		Colon cancer	Preclinical (*in vivo*)	78
		Lung cancer, solid tumours	Clinical trial, phase I-III	79–81
		Solid tumours, colorectal cancer, pancreatic cancer, lung cancer	Clinical trial, phase I	82–85
Bispecific antibody (BsAb)	EGFRvIIIț/CD133ț	Glioblastoma	Preclinical (*in vivo*)	86

therapies and may contribute to tumour recurrence in GBM. Adoptive transfer of HER2-specific T cells resulted in prolonged regression of autologous orthotropic GBM xenografts, confirming the potent antitumor activity of genetically modified T cells against HER2-positive tumours and their putative stem cells.

Very recently Morita *et al.* [19] found that DNAJB8 heat shock protein was preferentially expressed in CSCs derived from colorectal cancer (CRC) rather than in non-CSCs. They identified immunogenic DNAJB8 peptides and induced DNAJB8-specific cytotoxic T lymphocyte (CTL) response. A CTL clone specific for DNAJB8 peptide showed higher killing activity to CRC CSCs compared with non-CSCs *in vitro*. The antitumor effect of the DNAJB8-specific CTL clone was evaluated *in vivo* using a therapeutic CTL adoptive transfer model. The DNAJB8-CTL clone-transferred group showed a significant antitumor effect compared with that in the control group.

Pluripotent stem cells as cancer vaccines

The antigenic similarities between malignant and embryonic cells are reflected by the expression of oncofoetal antigens by both cancer-initiating and pluripotent cells. It was reported in 1906 that prior injection of mice with foetal tissues led to rejection of transplantable tumours [20]. Such tumour protection was observed later for chemically induced cancers of skin, liver, and gastrointestinal tracts [21]. It can be concluded that animals or humans immunised against embryonic antigens might be capable of recognising and destroying neoplastic cells, which has

been the premise for designing a novel immunotherapy approach. It has been shown that pluripotent ESCs induce modest delays in tumour growth in mouse models of transplantable colon and lung cancer [22, 23]. Protection against CT26 colon carcinoma, generated by vaccination with hESC line H9, correlated with expansion of tumour-responsive IFN-γ-producing cells and loss of CD11+Gr1+ MDSCs in spleen [22]. Furthermore, administration of ESCs in lung carcinoma-bearing mice induced potent antitumor effect and protected mice from tumour growth [23]. Yaddanapudi *et al.* reported that vaccination with ESC in combination with GM-CSF was effective in preventing implantable and carcinogen-induced lung tumours, without detectable toxicity or autoimmunity. The therapeutic efficacy of this vaccine was associated with tumour-specific primary and long-term memory CD8+ effector responses, infiltration of CD8+ T cells into the tumour, and reduced MDCSs in the spleen [24]. These findings provide a strong rationale for further developing this form of immunotherapy, although it has to be kept in mind that the use of human embryonic cells for therapy purposes is highly controversial.

Monoclonal antibodies

Monoclonal antibodies used in immunotherapies posses the ability to activate the immune system against tumour cells, inhibit cancer cell-intrinsic signalling pathways, bring toxins to close proximity of cancer cells, or interfere with the tumour-stroma interaction. Several antibodies have been approved by the FDA for the treatment of solid

and haematological tumours, e.g. rituximab (anti-CD20), cetuximab (anti-EGFR), trastuzumab (anti-HER2), bevacizumab (anti-VEGF-A), ipilimumab (anti-CTLA-4), and very recently pembrolizumab (anti-PD-1). The full potential of mAb is still to be exploited, and CSCs are currently increasingly important targets for antibody-based therapies.

CD44 is a transmembrane glycoprotein overexpressed in many tumour cells. It participates in the growth, survival, differentiation, and motility of cells, cell-adhesion, and the assembly of growth factors on the cell surface. Expression of CD44 is associated with metastasis, invasion, and aggressiveness of tumour growth [25]. CD44 is described as a marker of breast cancer stem cells and has been implicated as a CSC marker in bladder, gastric, prostate, pancreatic, ovarian, colorectal, hepatocellular, head, and neck squamous cell carcinomas [26]. Among CD44-based therapeutic strategies is the use of monoclonal antibodies. In vivo administration of anti-CD44 mAb to NOD-SCID mice transplanted with human acute myeloid leukemia (AML) led to efficient and selective eradication of AML leukaemic stem cells (LSC) [27]. Manipulation of CD44 function resulted in differentiation and inhibited proliferation, and engraftment and homing of CD34+CD38-LSCs from AML patients. Antibody-mediated CD44-targeting significantly reduced the growth of human breast cancer xenografts [28]. Moreover, mAb treatment during tumour remission induced by chemotherapy decreased tumour recurrence to 31% in mice injected with human triple-negative basal-like breast cancer cells. In mice with human pancreatic tumour xenografts anti-CD44 mAb reduced growth, metastasis, and post-radiation recurrence [29]. The antibody decreased the number of CSCs in both cultured pancreatic cancer cells and in xenograft tumours, as well as their tumourigenicity. Monoclonal antibody targeting CD44R1, an alternative splice variant of CD44 that is overexpressed in colon, bladder, lung, larynx and breast cancer, inhibited tumour growth of human cervix and larynx carcinoma xenografts [30]. In head and neck squamous cell carcinoma, treatment with anti-CD44 mAb displayed remarkable tumour growth inhibition, accompanied by the inhibition of constitutive EGFR phosphorylation on HNSCC cell line xenografts [31]. This phosphorylation has been associated with early relapse and poor prognosis in HNSCC patients [32]. Encouraging preclinical results have led anti-CD44 mAbs to clinical trials. RO5429083 has been tested alone and in combination with cytarabine in patients with AML or metastatic CD44-expressing malignant solid tumours [33, 34].

The epithelial cell adhesion molecule (EpCAM, CD326) is a transmembrane glycoprotein, overexpressed in most human carcinomas, which has been identified as an additional marker for cancer-initiating stem cells [35]. The EpCAM overexpression has been utilised in several EpCAM directed antibody-based preclinical studies and clinical trials [36]. Solitomab (MT110) is a single-chain bispecific T-cell engager (BiTE) antibody targeting EpCAM [37]. It prevented the outgrowth of SW480 human colon cancer xenografts and led to durable eradication of established tumours. Treatment with MT110 also resulted in remission of subcutaneous human ovarian cancer xenografts. Cioffi et al. reported elimination of tumour-initiating cells from

MT110 in vitro-treated primary human pancreatic cells [38]. In vivo studies using a mouse model of established primary human pancreatic cancer revealed disease stabilisation in response to MT110. Small remaining tumours were depleted of CSCs and contained mostly differentiated cancer cells. Since 2008 MT110 has been tested in dose escalation phase I clinical trials in patients with locally advanced, recurrent or metastatic lung, gastric, colorectal, breast, hormone-refractory prostate, and ovarian cancers. Anti-tumour response was evaluated in sixteen patients who received a dose of ≥ 24 μg/d [39]. In 38% of patients, disease stabilisation was observed with median duration of 155 days, and anti-tumour activity was demonstrated in a biopsy of liver metastases in one patient. Catumaxomab is a bispecific, trifunctional antibody that effectively binds to EpCAM antigen on tumour/CSC cells and to CD3 co-receptor on T lymphocytes. In addition, catumaxomab attaches to and activates FcγRI/IIa/III-expressing macrophages, DCs, and NK cells via its specific Fc region [40]. That makes catumaxomab a self-supporting system, without the need for additional immune system activation. Catumaxomab effectively eliminated CD133+/EpCAM+ CSCs from patients with severe ovarian, pancreatic, and gastric cancers [41]. Moreover, catumaxomab is in phase I-III of clinical trials for epithelial cancers. It is commercially available on the European Union market for therapy of malignant ascites caused by epithelial cancers [40].

CD133 (prominin-1) was first identified as a CD34+ haematopoietic stem cell marker [42]. Cancer stem cells expressing CD133 were subsequently reported in brain, colon, prostate, lung, ovarian, pancreatic, and hepatocellular carcinomas [43]. In order to eradicate CD133high cancer cells, Huang et al. generated a bispecific antibody (BsAb) against CD3 and CD133 [44]. They combined antibody therapy with cellular immunotherapy by arming cytokine-induced killer (CIK) cells with the novel BsAb to create BsAb-CIKs. The BsAb-CIK cells killed CD133high pancreatic and hepatic cancer cells in vitro more efficiently than the parental CIK or CIK cells bound with anti-CD3 (CD3-CIK) without CD133 targeting and produced significantly higher amounts of IFN-γ. In nude mice, the BsAb-CIK cells significantly inhibited CD133high tumour growth by selectively targeting this cell population.

Notch signalling modulates differentiation, survival, proliferation, and apoptosis of CSCs [45, 46]. Aberrant Notch signalling is responsible for promotion of tumorigenesis, multiresistance, and self-renewal of CSCs [46]. Delta-like ligand (DLL) is one of the canonical ligands in Notch signalling [47]. Overexpression of DLL in tumour cells led to activation of Notch signalling and cancer progression, while blockade of DLL resulted in poor tumour perfusion, hypoxia, and tumour shrinking [48–50]. Blockade of DLL with monoclonal anti-human antibody in human colon tumour xenograft produced a decrease in tumour growth that correlated with a 50% decrease in the CSC population [51]. Beviglia et al. reported a significant inhibition of both primary tumour and metastases in the lungs, liver, intestine, and brain in a xenograft mice human melanoma model after treatment with anti-DLL4 mAb [52]. Yen et al. found that treatment with anti-DLL4

antibody decreased CSC frequency in residual tumours after conventional chemotherapy in patient-derived breast cancer and pancreatic cancer xenografts [53]. That led to growth inhibition of resistant tumours and re-sensitised them to the chemotherapeutic agent. With a rationale from pre-clinical studies to target CSCs through interference with Notch pathway anti-DLL4 mAb went to clinical trials. Demcizumab (OMP-21M18) is currently being tested in combination with chemotherapy in several phase I/Ib trials, in advanced or metastatic solid tumours [54–56]. In February 2014 the safety and tolerability phase I study of REGN421 (SAR153192), a fully humanised mAb against DLL4, in patients with advanced solid malignancies, was completed. Although full results have not been published yet, preliminary data show an acceptable safety profile of REGN421 and anti-tumour activity, which include 2 partial responses and 16 patients with stable disease [55].

Conclusions

As shown above, numerous *in vitro* and *in vivo* studies or clinical trials indicate that immune targeting of CSCs presents a promising approach for cancer treatment. The major advantages of most immunotherapeutic strategies are low or acceptable toxicity and the ability to target defined molecules or cell populations. On the other hand, immunotherapy is more effective in some types of cancer and often needs to be accompanied by traditional treatment strategies such as chemotherapy. Nevertheless, it is becoming a fascinating tool in the fight against cancer, and its further development in the near future is guaranteed.

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Address for correspondence

Eliza P. Kwiatkowska-Borowczyk
Department of Cancer Immunology
University of Medical Sciences
Garbary 15
60-866 Poznan, Poland
e-mail: eliza.kwiatkowska@wco.pl