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REVIEW

Genetic Modification of T Cells Improves the Effectiveness of Adoptive Tumor Immunotherapy

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Abstract Appropriate combinations of immunotherapy and gene therapy promise to be more effective in the treatment of cancer patients than either of these therapeutic approaches alone. One such treatment is based on the application of patients' cytotoxic T cells, which can be activated, expanded, and genetically engineered to recognize particular tumor-associated antigens (TAAs). Because T cells recognizing TAAs might become unresponsive in the process of tumor development as a result of tumor evasion strategies, immunogenic viral antigens or alloantigens could be used for the expansion of cytotoxic T cells and then redirected through genetic engineering. This therapeutic approach has already demonstrated promising results in melanoma patients and could be used in the treatment of many other tumors. The graft-versus-leukemia, or more generally graft-versus-tumor, reaction based on the application of a donor lymphocyte infusion can also be ameliorated through the incorporation of suicide genes into donor lymphocytes. Such lymphocytes could be safely and more extensively used in tumor patients because they could be eliminated should a severe graft-versus-host reaction develop.

Keywords Adoptive tumor immunotherapy · Gene therapy · Graft-versus-tumor

Abbreviations

CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CML	Chronic myelogenous leukemia
DLI	Donor lymphocyte infusion
GVH	Graft-versus-host
GVHD	Graft-versus-host disease
GVL	Graft-versus-leukemia
GVT	Graft-versus-tumor
HSV-tk	*Herpes simplex* thymidine kinase
IL	Interleukin
LMO-2	LIM domain-only
MHC	Major histocompatibility complex
NK	Natural killer
TAA	Tumor-associated antigen
TCR	T-cell receptor
TIL	Tumor-infiltrating lymphocyte

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Introduction

Gene therapy can be defined as the introduction of genetic material into cells with the aim to achieve a therapeutic effect. Beginning with the first human gene therapy trial in 1989 (Rosenberg et al. 1990), over 1,400 clinical trials have been completed or are ongoing in the treatment of many diseases, but so far with limited success. In some gene therapy trials, serious side-effects have been observed and four patients undergoing gene therapy have already died (Cavazzana-Calvo and Fischer 2007; Frank et al. 2009; Raper et al. 2003; Stein et al. 2010), although the death of one of them has not been related directly to the gene therapy (European Society of Gene Therapy 2006; Wadman 2007). One of the remaining three patients who died as a consequence of gene therapy was an 18-year-old boy treated for ornithine transcarbamylase deficiency. He died from an inflammatory reaction to antigens encoded by adenovirus vector, leading to disseminated intravascular coagulation

and multiple organ failure (Raper et al. 2003). In the two remaining patients, murine Moloney leukemia virus was used as a gene therapy vector. One of them was suffering from X-linked severe combined immunodeficiency, which is caused by mutation of the gene encoding the gamma common (γc) cytokine receptor chain shared by receptors for interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15, and IL-21 (Cavazzana-Calvo and Fischer 2007). His death followed retroviral vector insertion close to proto-oncogene *LMO-2*, resulting in the activation of *LMO-2* by retroviral enhancer sequences and the development of T lymphoblastic leukemia (Thrasher 2008). Another patient suffered from X-linked chronic granulomatous disease and died 27 months after gene therapy. His death, originally supposed to be unrelated to the gene therapy (European Society of Gene Therapy 2006), turned out to be caused by retrovirus-mediated insertional activation of *EVI1* and *EVI1-MDS* proto-oncogenes causing myelodysplasia (Dunbar and Larochelle 2010; Stein et al. 2010). At least six other deaths in patients participating in gene therapy trials have probably not been reported to the National Institutes of Health, contrary to the official guidelines (No authors 1999).

The majority of clinical gene therapy trials target neoplastic diseases. Despite encouraging and sometimes exciting results of pre-clinical studies, clinical gene therapy for cancer has not yet lived up to its expectations. Likewise, clinical tumor immunotherapy has shown little success so far (Rosenberg et al. 2004), except for favorable results of intravesical administration of bacillus Calmette Guérin vaccine in the treatment of superficial bladder carcinoma (Razack 2007) and the successful application of monoclonal antibodies, particularly in the therapy of certain types of lymphomas (Cheson and Leonard 2008) and several other types of tumors (Weiner et al. 2009). Unfortunately, very encouraging results of idiotype vaccination in patients with follicular lymphoma (Inogès et al. 2006) have not been confirmed in a phase III trial (Freedman et al. 2009) and the early enthusiasm surrounding the effectiveness of various vaccines in patients with prostate cancer has recently waned (Drake 2009). Some combinations of immunotherapy and gene therapy seem to be more promising. Gene immunotherapy for cancer can use different approaches (Table 1). The most effective of these seem to include adoptive therapy with gene-modified T cells and suicide gene therapy to allow safe application of the graft-versus-leukemia (GVL) and graft-versus-tumor (GVT) reactions without the risk of life-threatening graft-versus-host disease (GVHD).

Table 1 Different approaches to gene immunotherapy for cancer

Vaccination with tumor cells transfected with genes encoding cytokines which stimulate the development of antitumor immunity
Vaccination with tumor cells transfected with genes encoding MHC and/or costimulatory molecules
Vaccination with genetically modified dendritic cells (various genes)
Vaccination with recombinant viruses encoding TAAs
Vaccination with genetically manipulated TAA demonstrating improved immunogenicity
Adoptive immunotherapy with T cells transfected with genes encoding cytokines which demonstrate antitumor effects
Adoptive immunotherapy with T cells with modified antigen receptors
Various combined therapeutic modalities

Table 2 Mechanisms of tumor immune escape

Loss of tumor antigens
Diminished or abolished expression of MHC class I molecules in tumor cells
Incorrect processing or presentation of tumor antigens
Down-regulation of CD3ζ chain
Lack of costimulation
Suppression by T regulatory cells and/or myeloid suppressor cells and tumor-associated macrophages
Suppression by factors such as IL-10 and TGF-β released from tumor-infiltrating cells, including T regulatory cells and tumor cells
Production of indoleamine 2,3-dioxygenase by a subset of dendritic cells and/or tumor cells
Release of molecules such as MICA, MICB, and HLA class I molecules disturbing the activity of NK cells and cytotoxic T cells, respectively
Counterattack by tumor cells using FasL or PD-L1
Induction of immune tolerance

Application of T Cells with Modified Antigen Receptors

Adoptive tumor immunotherapies using lymphokine-activated killers (Sankhla et al. 1996) or tumor-infiltrating lymphocytes (TILs) (Khammari et al. 2007) have not given very encouraging clinical results although recent observations of the clinical application of TILs seem to be much more promising (Dudley et al. 2008). Among the main obstacles to the application of lymphocytes isolated from a tumor patient for effective tumor therapy are tumor evasion strategies (Table 2). Probably the most important of these are the immunosuppressive effects elicited by several types of suppressor and regulatory cells and soluble immunosuppressive factors (Bronte and Mocellin 2009) as well as the induction of immunological tolerance to tumor-associated antigens (TAAs) (Willimsky and Blankenstein 2005), hampering the development of an effective immune response. Tumors usually develop from single tumor cells. Therefore the immune system of a tumor patient becomes exposed to very low, but gradually increasing concentrations of TAAs for prolonged periods of time, thereby facilitating the development of immune tolerance and the

loss of or functional inactivation of TAA-reactive T cells. The above hypothesis is supported by the phenomenon of "sneaking through", whereby a small number of tumor cells injected into an experimental animal are not rejected and form a tumor, similarly to the injection of a large-size inoculum as opposed to the rejection of a medium number of tumor cells (Gatenby et al. 1981; Mengersen et al. 1975). This should occur even if the TAAs are rare tumor-specific antigens which can be expected to evoke an immune response. Most TAAs are present at least in some normal tissues and self tolerance to them develops naturally during lymphocyte maturation. An important evasion strategy also results from the loss of expression of major histocompatibility complex (MHC) class I molecules by tumor cells, precluding their recognition by $CD8^+$ T cells (Marincola et al. 1994). At least some of these escape mechanisms can now be counteracted as a result of genetic manipulation of either the tumor cells (del Campo et al. 2009) or the patient's T cells (see below).

As the preparation of tumor-reactive lymphocytes may not always be successful for the above-mentioned reasons, patient's T cells could be stimulated with potent immunogens such as viral antigens (Heemskerk et al. 2004), although allogeneic major histocompatibility antigens are also appropriate candidates (Kershaw et al. 2002b). The specificity of ex vivo activated and expanded T cells can then be redirected following transduction with genes for α and β chains isolated from tumor-reactive T cells found in some rare patients (Morgan et al. 2006). The application of redirected dual-specificity T cells recognizing both TAAs and viruses causing persistent infections in man, such as cytomegalovirus or Epstein–Barr virus, has an additional important advantage. Such T cells will be constantly activated, enabling them to survive for prolonged periods of time in virus-infected individuals (Pule et al. 2008) provided they are not rendered anergic or tolerant during chronic viral infection (Virgin et al. 2009).

Another development in the genetic refinement of tumor-reactive T cells is the construction of so-called chimeric antigen receptors (CARs). CARs consist of two parts. They recognize antigen with single-chain Fv fragments formed with variable parts of light and heavy chains of tumor-specific antibodies linked to an intracellular signaling domain usually consisting of the cytoplasmic region of CD3ζ chain (Sadelain et al. 2009). The additional advantage of CARs compared to classical T-cell receptors (TCRs) is their ability to recognize and induce a response to TAAs which are not MHC-restricted. T cells transfected with CARs could thus be used in the therapy of tumors which have lost the expression of MHC molecules or have deficiencies in MHC class I antigen processing or presentation. To increase the proliferative potential and tumor-eradicating capacity of such T cells, CARs have been constructed which additionally contain a CD28 signaling domain transmitting a costimulatory signal (Emtage et al. 2008; Haynes et al. 2002; Wang et al. 2009; Westwood et al. 2009). Signal-transmitting domains of other costimulatory molecules, including 4-1BB (CD137), OX40 (CD134), 2B4 (CD244), and inducible costimulator, have also been used, resulting in improved antitumor activity (Altvater et al. 2009a; Carpenito et al. 2009; Finney et al. 2004; Imai et al. 2004; Milone et al. 2009; Sadelain et al. 2009). T cells for adoptive tumor therapy with triple-fusion CARs containing signaling domains from three different costimulatory molecules have already been constructed (Wilkie et al. 2008). Strengthened antitumor effects have been observed in tumor-bearing mice using T cells with CARs recognizing TAAs and containing intracellular signaling domains of CD3ζ chain, CD28, and CD137 (Tammana et al. 2010). However, strengthening activation signals through the inclusion of costimulatory molecules should be approached with caution as this could result in severe toxic effects, similar to those which were recently observed following application of agonistic anti-CD28 monoclonal antibodies (Suntharalingam et al. 2006).

Before adoptive transfer of genetically modified T cells, patients could undergo lympho-depleting preconditioning with chemotherapy or a combination of chemotherapy and X-irradiation (Dudley et al. 2008; Rosenberg and Dudley 2004; Muranski et al. 2006), which results in the creation of a lymphopenic environment, depletion of regulatory T cells, increased levels of IL-7 and IL-15 as a consequence of diminished use of these cytokines by depleted lymphocytes, and improved survival and efficacy of transferred T cells (Dudley et al. 2008). More space and more growth and differentiation factors are now available for administered T cells.

Genetically modified T cells recognizing Mart-1 or both Mart-1 and gp100 antigens have already been successfully used in patients with metastatic melanoma, with 2 out of 17 treated patients experiencing complete regression of metastatic melanoma lesions in one study (Morgan et al. 2006) and with up to 30% of patients showing objective tumor regression in another trial (Johnson et al. 2009). Genetic modification of T cells to recognize many other TAAs using the TCR (Clay et al. 1999; Cohen et al. 2005; Engels et al. 2005; Hughes et al 2005; Morgan et al 2003; Tsuji et al 2005; Zhao et al. 2005) or CARs (Carpenito et al. 2009; Emtage et al. 2008; Kochenderfer et al. 2009; Maher and Wilkie 2009; Tammana et al. 2010; Till et al. 2008; Westwood et al. 2009; Wilkie et al. 2008; Yoon et al. 2009) is now feasible and clinical trials with genetically engineered T cells have been conducted (Gonzalez et al. 2004; Kershaw et al. 2006; Park et al. 2007; Pule et al. 2008; Sadelain et al 2009; Till et al. 2008). For a review, see Sadelain et al. (2009).

Probably both $CD8^+$ and $CD4^+$ TAA-specific T cells should be used in combination to obtain an optimal therapeutic effect (Kuball et al. 2005), as such cells will include both cytotoxic T cells as well as T helper cells. Although $CD8^+$ T cells are preferentially used in adoptive tumor immunotherapy, $CD4^+$ T cells recognizing particular TAAs have already shown antitumor effects in clinical studies (Hunder et al. 2008). As cytotoxic T cells have a limited life-span, memory T cells with redirected specificity, especially central memory cells, should also be included because their ability to survive for prolonged periods of time and expand and differentiate into effector T cells will guarantee extended therapeutic effects (Berger et al. 2008). Simultaneous application of lymphocytes recognizing different TAAs of a given tumor should further increase the efficacy of this therapeutic modality as tumor evasion strategies include the loss of particular TAAs. As some tumor cells are known to produce chemokines, the effectiveness of adoptive immunotherapy could be further improved by transducing lymphocytes with particular chemokine receptor genes (Di Stasi et al. 2009; Kershaw et al. 2002a).

One of the problems encountered during the construction of T cells with modified TCRs is mispairing of the introduced TCR α and β chains, which instead of assembling with each other, cross-pair with endogenous TCR chains (Kuball et al. 2007). Additionally, endogenous, introduced, and mispaired TCR α and β chains compete for binding CD3 molecules to form a TCR–CD3 complex (Heemskerk et al. 2007). This can diminish or abolish the ability of transfected T cells to recognize the appropriate tumor antigen, but it also constitutes a risk of creating autoreactive T cells. There are some promising strategies to abolish or at least reduce cross-pairing. One of them is to introduce additional cysteines into the constant domains of introduced TCR α and β chains, strengthening their pairing (Kuball et al. 2007). Another strategy is specific silencing of endogenous TCR chains. This has been achieved using small interfering RNA constructs specifically down-regulating the expression of endogenous TCR chains (Okamoto et al. 2009). It has also been demonstrated that the avidity of re-targeted T cells can be increased by removal of particular *N*-glycosylation sites from the introduced TCR α and β chains (Kuball et al. 2009).

Redirecting the specificity of effector cells for adoptive tumor immunotherapy has concentrated on $\alpha\beta$ T cells although both $\gamma\delta$ T cells (Rischer et al. 2004) and natural killer (NK) cells have also been successfully transfected to express CARs containing CD3ζ and other signaling molecules counteracting the activity of inhibitory receptors and recognizing TAAs (Altvater et al. 2009b; Imai et al. 2005; Kruschinski et al. 2008). As effective expansion of human NK cells on a large scale is now feasible (Fujisaki et al. 2009), their being equipped with CARs could facilitate their clinical application in adoptive tumor immunotherapy.

Infusion of Donor Lymphocytes Expressing Suicide Genes

Genetic engineering of T cells can also improve the effectiveness of adoptive tumor immunotherapy in a less direct way. In the treatment of hematological malignancies with transplantation of hematopoietic cells, lower relapse rates have been observed in patients obtaining allogeneic versus syngeneic or autologous cells (Anderson et al. 1993; Bruno et al. 2007; Jones et al. 1991; Martinelli et al. 2000) and in those developing GVHD (Anderson et al. 1993; Toze et al 2005; Weiden et al. 1979) and higher relapse rates in those obtaining T cell-depleted hematopoietic cells (Horowitz et al. 1990; Marmont et al. 1991). All these results support GVL or more generally GVT effects of allogeneic transplants. The GVL effect has been very successfully exploited in donor lymphocyte infusions (DLIs) for the treatment of relapse of chronic myelogenous leukemia (CML) following transplantation of allogeneic hematopoietic cells, resulting in a high rate of durable remissions (Bacigalupo et al. 1997; Bar et al. 1993; Collins et al. 1997; Dazzi et al. 2000; Kolb et al. 1990; Porter et al. 1994). DLI has also been applied, but usually with lower response rates, in chronic lymphocytic leukemia (Ritgen et al. 2004; Russell et al. 2005), acute myelogenous leukemia (Collins et al. 1997; Kolb et al. 1995), acute lymphoblastic leukemia (Collins et al. 1997, 2000), multiple myeloma (Lokhorst et al. 1997, 2004), non-Hodgkin's lymphoma (Mandigers et al. 1998), renal cell carcinoma (Takahashi et al. 2008), and breast carcinoma (Bishop et al. 2004). One of the reasons that relatively slowly progressing CML is a disease well suited for treatment with DLIs is the fact that their therapeutic effects sometimes need several months to occur (Loren and Porter 2008). Another proposed reason for the high efficacy of DLI in CML could be the ability of leukemic cells to behave as antigen-presenting cells (Chen et al. 2000). Unfortunately, infusion of donor lymphocytes is threatened by the risk of morbidity and mortality of GVHD. On the other hand, effective separation of GVL from GVH effects remains a yet unresolved problem because target antigens which are responsible for the DLI effect, namely minor histocompatibility antigens and most TAAs, are present not only on tumor cells, but also on hematopoietic and some other normal recipient cells (Bleakley and Riddell 2004; Feng et al. 2008).

As the magnitude of GVHD accompanying DLI is difficult to predict, suicide genes can be transferred into

donor lymphocytes before their infusion into the recipient (Bondanza et al. 2005; Bonini et al. 1997). The gene encoding thymidine kinase from *Herpes simple* virus (HSV-tk) is usually used for this purpose (Bonini et al. 2007; Ciceri et al. 2009). Infused donor lymphocytes direct themselves against minor histocompatibility antigens present on normal and tumor cells and against TAAs exerting GVT activity, but when GVHD develops, producing serious toxicity, patients can be given ganciclovir, which is converted into cytotoxic metabolites by HSV-tk in genetically manipulated lymphocytes controlling the disease. An additional advantage of using DLI is improved immune reconstitution of the tumor patient. It is worth mentioning that suicide genes were originally introduced into tumor cells in cancer gene therapy causing direct and indirect antitumor effects. However, although encouraging results of this therapeutic modality were obtained in experimental animal models, clinical trials so far have not demonstrated its efficacy (Fillat et al. 2003). What limits the application of HSV-tk-modified T cells is the immunogenicity of the HSV-tk gene product, leading to their premature elimination (Berger et al. 2006).

Concluding Remarks

Gene therapy has not fulfilled its early promises in cancer patients so far. However, its combination with immunotherapy could result in an improved effectiveness of tumor treatment. One example of such a combination is the redirection and amplification of cytotoxic T cells of the tumor patient to use them in effective adoptive immunotherapy. Another is equipping donor lymphocytes with suicide genes for their application in the GVT reaction with the possibility to induce their death in case of serious GVHD complications. Infusion of genetically modified patients' T cells recognizing TAAs have already demonstrated effectiveness in patients with metastatic melanoma, but there is a growing possibility to construct T cells recognizing many different tumor antigens and to broaden the application of this therapeutic modality. One could argue that engineering the T cells of the tumor patient with TCRs recognizing specific tumor antigens should replace donor lymphocyte transplantation because such T cells could kill tumor cells without the risk of GVHD induction. However, infused allogeneic lymphocytes probably recognize an array of different molecules on tumor cells, including tumor-associated, tumor-specific, and minor histocompatibility antigens, many of them remaining largely unknown. This versatility could make them an effective weapon against tumor cells and decrease the efficiency of tumor evasion strategies, but unfortunately at the price of GVHD risk.

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