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REVIEW

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New developments in dendritic cell–based vaccinations: RNA translated into clinics

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Abstract Dendritic cells (DCs) are the most powerful antigen-presenting cells that induce and maintain primary immune responses in vitro and in vivo. The development of protocols for the ex vivo generation of DCs provided a rationale for designing and developing DC-based vaccination studies for the treatment of infectious and malignant diseases. Recently, it was shown that DCs transfected with ribonucleic acid (RNA) coding for a tumour-associated antigen or whole tumour RNA are able to induce potent antigen and tumour-specific T-cell responses directed against multiple epitopes. The first RNA-transfected-DC-based clinical studies have shown that this form of vaccination is feasible and safe. In some cases, clinical responses were observed, but the preliminary data require further extensive investigations that should address the technical and biological problems of manipulating human DCs, as well as the development of standardised protocols and definitions of clinical settings.

Keywords CTL · Dendritic cells · Immunotherapy · RNA transfection

Introduction

Dendritic cells (DCs) are the most powerful professional antigen-presenting cells (APCs), with the ability to initiate and maintain primary immune responses. Recently, several procedures to generate large number of DCs from circulating precursors, including peripheral blood monocytes and $CD34^+$ stem cells, have been developed for clinical use to treat patients with infections and malignant diseases. Hence, numerous attempts to optimise delivery of tumour antigens to DCs, as well as routes and schedules of administration to cancer patients, are currently being analysed in clinical trials [18, 25, 52, 66, 83]. DCs can be pulsed with synthetic peptides derived from a known tumour-associated antigen (TAA) or with full-length proteins as antigen, thus allowing the induction of immune responses against different epitopes [4, 17, 50, 60, 62, 64, 77]. Other approaches utilising whole tumours as a source of antigen have been developed using DCs loaded with tumour lysates or dying tumour cells or fused with tumour cells [19, 38, 44, 69, 70].

Recently, it has been demonstrated that transfection of DCs with specific or whole cell ribonucleic acid (RNA) is more effective than transfection with DNA-vector constructs [8, 56]. A multitude of studies have impressively demonstrated the power of this approach to induce antigen-specific cytotoxic T lymphocytes (CTLs) in vitro and in vivo. The first RNA transfected DC based clinical studies have indicated that this form of vaccination is feasible and safe; furthermore, in some cases, clinical responses were observed, even in patients heavily pretreated with standard chemotherapy and/or radiotherapy approaches. These preliminary data, although encouraging, require further extensive investigations, that should address the technical and biological problems of manipulating human DCs, as well as the development of standardised protocols and definitions of clinical settings that could benefit from immunotherapeutic approaches.

DCs as therapeutic cancer vaccines

Human DCs can be generated in vitro from $CD34^+$ bone marrow and peripheral blood progenitor cells after culture with different cytokine combinations including tumour necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), Flt3 ligand, CD40 ligand (CD40L), stem cell factor (SCF), or

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transforming growth factor (TGF)-β. Alternatively, DCs can be generated from $CD14^+$ peripheral blood-adhering monocytes. When cultured for 2 to 7 days in the presence of GM-CSF and interleukin (IL)-4, these cells differentiate into immature DCs making it possible to gain access to large numbers of DCs for vaccine production [6, 12, 15, 16, 22, 26, 68]. Immature DCs are very efficient in antigen capture but express relatively low levels of major histocompatibility complex (MHC) class I and II and costimulatory molecules. Furthermore, application of immature DCs can result in the induction of tolerance or regulatory T cells (T_{reg}) to the antigens used. In contrast, mature DCs switch from an antigen-capturing to an highly efficient antigen-presenting and T-cell–stimulating mode mediated by the expression of MHC I, costimulatory molecules, and secretion of cytokines and chemokines involved in T-cell activation [43, 79].

The efficacy of antigen delivery into DCs is crucial for the induction of effective T-cell–mediated immune responses. The application of antigenic peptides is limited to use in patients who express a defined, specific human leukocyte–associated antigen (HLA) haplotype and requires the determination of the T-cell epitopes and TAAs [50]. Furthermore, using HLA class I–restricted peptides ignores the important role of HLA class II–restricted T-helper cells in initiating and maintaining an effective immune response.

Another approach is to use full-length proteins as antigen, thus allowing the induction of immune responses against different epitopes that could potentially be restricted by multiple HLA alleles [10, 42]. In addition, methods using whole tumours as a source of antigen have been developed using DCs loaded with tumour lysates, dying tumour cells (apoptotic bodies, necrotic cells) or fused with tumour cells [1, 4, 17, 19, 38, 44, 60, 62, 64, 69, 70, 77].

An alternative strategy is gene-based delivery of specific TAAs into DCs that does not require prior knowledge of the patient's HLA type or the relevant T-cell peptide epitope. DCs can be transduced with recombinant viruses such as retroviral or adenoviral vectors or transfected with DNA coding for a specific tumour antigen [11, 21, 45].

Various viral and nonviral transfection methods are available for introducing DNA into mammalian cells, depending on the purpose of the experiment. In particular, retroviruses are suitable, since their life cycle involves the stable integration of viral DNA into the genome of infected cells making them an important tool for a broad range of applications including gene therapy [5, 65, 71]. Nonviral transfection methods include direct microinjection of DNA into the cell nucleus, formation of complexes between nucleic acid and cationic liposomes that fuse with the plasma membrane (lipofection) [27, 75], and exposure of cells to a brief electric pulse that transiently permeabilises the cell membrane (electroporation) [2, 20]. However, plasmid DNA transfer into DCs is not very efficient, and the use of viral vectors requires more complex and time-consuming manipulations associated with concerns regarding safety. Moreover, DCs infected by viral vectors have been reported to have an impaired capacity to stimulate T lymphocytes [48, 65, 71].

RNA as an agent for DC transfection

It was recently shown that DCs transfected with RNA coding for a TAA or even whole tumour messenger RNA (mRNA) are able to induce potent antigen and tumour-specific T-cell responses directed against multiple epitopes [8, 31, 39, 54, 56]. The latter technique does not require the definition of the TAA or HLA haplotype of the patients and has the potential for broad clinical application. Such a polyvalent vaccine may reduce the probability of a clonal tumour escape and elicit CTL responses directed against naturally processed and presented immunodominant tumour antigens. Additional targeting of HLA class II–restricted epitopes may further amplify and prolong the induced T-cell responses. In contrast to other whole-tumour vaccine approaches using tumour cell lysates, fusions of DCs with tumours or dead cells that are limited by the requirement of large amounts of tumour samples, RNA transfected DCs could be applied even in patients with small tumours.

The first functional data using RNA-loaded DCs were presented by Boczkowski et al. [8]. These pioneering experiments demonstrated that DCs pulsed with total or $poly(A)^+$ mRNA from ovalbumin (OVA)-expressing tumour cells, as well as in vitro transcribed OVA RNA, were as effective as DCs pulsed with OVA peptide in stimulating CTL responses. Mice vaccinated with DCs pulsed with OVA RNA were protected against a lethal challenge with OVA-expressing tumour cells. Induction of OVA-specific CTLs was inhibited when the $poly(A)^+$ RNA was treated with specific antisense oligodeoxynucleotides or RNase H, demonstrating that the observed effects indeed were mediated by OVA mRNA.

Among other things, RNA represents the templates for protein synthesis in the cell. In particular, a class of RNA molecules called mRNA are the information-carrying intermediates in protein synthesis. The 3′ end of most eukaryotic mRNA is defined not by termination of transcription, but by cleavage of the primary transcript and addition of a poly(A)-tail, a processing reaction called polyadenylation. Over the past few years, attempts have been made to define the role of the 3′-poly(A)-tail in translation by injecting adenylated and deadenylated mRNA into *Xenopus laevis* oocytes. The results of these studies have generally shown that mRNA lacking a 3′-poly(A) sequence translates less protein, but the deadenylated mRNA was also reported to be less stable than adenylated mRNA [23, 24, 28, 49]. A second important characteristic of eukaryotic mRNA is the modification of the 5′ end of pre-mRNA soon after its synthesis, by the addition of

a structure called a 7-methylguanosine 'cap'. This 5′-cap aligns eukaryotic mRNAs on the ribosome during translation. Proper capping of RNA promotes correct initiation of protein synthesis, as well as stability and processing of mRNA in vivo. Uncapped RNA is rapidly degraded by cellular RNases after microinjection or transfection into cells. Capped RNA is also typically translated more efficiently in reticulocyte lysate and wheat germ in vitro translation systems [23, 47]. According to these findings the use of intact native or in vitro transcribed biological active mRNA with the eukaryotic features poly(A)-tail and 'cap' is essential for successful RNA transfection of eukaryotic cells. RNA is commonly regarded as a very sensitive molecule exposed to ubiquitous ribonucleases and therefore difficult to handle in the laboratory. Nevertheless, the investigation of Eli Gilboa's team has resolved all doubts and demonstrated that RNA transfection of DCs is a potent alternative to DNA transfection and viral transduction of primary cells with promising applications in immunotherapy.

RNA-delivery into DCs

We and others have analysed the efficiency of RNA transfection into immature monocyte–derived DCs applying different delivery strategies including electroporation, lipofection and transferrin receptor (CD71)–based endocytosis. To evaluate the sensitivity of these different approaches, in vitro RNA transcripts from vectors coding for the enhanced green fluorescence protein (EGFP) or TAA were utilised. Strobel et al. [72] showed the use of liposomes to be superior to electroporation. However, Van Tendeloo et al. [81] and others found that electroporation was more potent compared with lipofection or CD71-based endocytosis [31]. Transfection rates of up to 90% have been described in the current literature for RNA delivery into DCs by electroporation. Recently, Ueno et al. [80] reported that they obtained up to 90% efficiencies by electroporation of human DCs generated in vitro from haematopoietic $CD34^+$ progenitor cells with in vitro transcribed GFP-RNA. Most remarkably they detected high GFP fluorescence for at least 14 days.

A summary of the different RNA transfection methods and efficiencies is given in the review by Ponsaerts et al. [63].

However, it seems that such high levels of antigen delivery are not required to elicit an efficient T-cell response. In our experiments, even the use of the transferrin receptor system for RNA uptake, which possesses a very low transduction efficacy, resulted in the induction of tumour-specific CTLs against the renal cell carcinoma (RCC) tumour cell line A498, thus suggesting that even low levels of antigen delivery, as judged by reporter gene expression, may lead to induction of antigen-specific CTL responses [31].

In vitro induction of human antigen-specific CTLs by RNA-transfected DCs

Since the first report by Nair et al. [56] on the induction of specific human CTLs by carcinoembryonic antigen (CEA) mRNA-transfected DCs, the effectiveness of this approach has been demonstrated in many in vitro studies using RNA coding for different antigens. In their study, Nair et al. [56] demonstrated that RNA encoding a chimeric CEA/LAMP-1 (lysosome-associated membrane protein-1 [32]) lysosomal targeting signal additionally enhanced the induction of CEA-specific $CD4^+$ T cells, thus providing a potential strategy to induce T-helper cells that may be necessary to generate and/or maintain an effective $CD8^+$ CTL response in vivo. To make the RNA transfection also available for small numbers of tumour cells, Boczkowski et al. [9] developed in 2000 a PCR-based procedure to amplify biologically active total RNA pools. This technique allowed the introduction of RNA into DCs isolated from human tumour cells that were microdissected from frozen tissue sections and subsequently amplified to stimulate primary CTL responses in vitro. The efficiency of this procedure was further confirmed in mice that were treated with DCs transfected with amplified melanoma RNA and thereupon developed CTL responses and protective immunity in a postsurgical metastasis setting. Heiser et al. [35–37] investigated the efficacy of autologous DCs transfected with mRNA encoding PSA to stimulate CTLs against prostate-specific antigens (PSA) in vitro. The authors showed that PSA mRNA-transfected DCs indeed were capable of eliciting primary CTL responses against prostate-specific antigens in vitro [35]. Thornburg et al. [76] evaluated the use of DCs transfected with RNA encoding the human papillomavirus (HPV) E6 and E7 oncoproteins for the development of cervical cancer immunotherapies. These authors demonstrated that DCs transfected with RNA stimulated effective antigen-specific CTL responses in vitro. Nair et al. [57] proved in other experiments that human telomerase reverse transcriptase (hTERT) [84] RNA-transfected human DCs induced hTERT-specific CTLs in vitro that lysed human tumour cells, including autologous tumour targets from patients with renal and prostate cancer. Weissman et al. [82] observed in vitro that a single stimulation of T cells by immature DCs transfected with mRNA coding for human immunodeficiency virus (HIV) capsid proteins (*gag*) resulted in primary $CD4^+$ and $CD8^+$ T-cell immune responses at frequencies of antigen-specific cells ranging from 5–12.5%. These data were similar to primary immune responses observed in vivo in murine models. Furthermore, they induced DC maturation by the *gag* mRNA transfection itself as monitored by the expression of the maturation marker CD83. The authors note that the induction of both $CD8^+$ and $CD4^+$ T cells and induction of maturation is observed only with RNA loading of DCs and not with other transfection techniques.

Strobel et al. [72] transfected DCs with influenza matrix protein 1 (M1) RNA and were able to stimulate autologous peripheral M1-specific memory CTLs, as well as M1-specific CTL clones. Heiser et al. [36] demonstrated that autologous DCs transfected with RNA amplified from microdissected tumour cells are capable of stimulating CTLs against a broad set of yet-unidentified prostate-specific antigens. In a further study, Su et al. [73, 74] sought to determine whether human DCs transfected with mRNA encoding a chimeric hTERT/LAMP-1 protein, carrying the endosomal/lysosomal sorting signal of the LAMP-1, are capable of stimulating hTERT-specific $CD8^+$ and $CD4^+$ T-cell responses in vitro. They demonstrated that processing of hTERT/LAMP-1 transcripts leads to enhanced stimulation of hTERT-specific $CD4^+$ T cells and does not negatively affect intracellular generation and subsequent presentation of MHC class I epitopes, hence, generating a CTL response [73]. Milazzo et al. [51] transfected monocyte-derived DCs with total RNA from the myeloma cell lines LP-1 and U266 by electroporation and investigated the potential of these DCs to induce myeloma-specific CTLs. They determined that RNA-transfected DCs induce CTLs that lyse the LP-1 and U266 myeloma cells in an antigen-specific and MHC class I–restricted manner. Interestingly, LP-1–specific CTLs exhibited no specificity for the idiotype, indicating that idiotype-derived epitopes do not represent immunodominant antigenic peptides. Consistent with studies demonstrating Mucin 1, a transmembrane mucin glycoprotein (MUC1), as a myeloma-associated antigen [78], they detected MUC1-directed cytotoxic activity of the CTLs induced with U266-derived RNA. As corresponding epitopes, Milazzo et al. [51] tested the MUC1-derived peptides M1.1 and M1.2 [14] and found a strikingly fine specificity for M1.2, assuming a possible immunodominance of this peptide. Nencioni et al. [59] presented data showing that DCs transfected with RNA from colorectal cancer cells present HLA class I–restricted antigenic epitopes to induce a primary antitumour CTL response in vitro. Müller et al. [54] analysed the induction of HLA class I– and II–restricted T-cell responses against MCF-7 breast cancer cells. Using this approach we were able to elicit CD4- and CD8-mediated antitumour responses. The CTLs specifically lysed MCF-7 cells and DCs electroporated with MCF-7 RNA, but spared control cell lines. Interestingly, these polyclonal CTLs selectively recognised two epitopes derived from the MUC1 and HER-2/neu tumour antigens [13, 54]. In another study, Müller et al. [55] could demonstrate that DCs generated from monocytes of patients with B-chronic lymphocytic leukaemia (CLL) induce leukaemia-specific cytotoxic and proliferative T-cell responses upon transfection with total RNA isolated from autologous leukaemic B lymphocytes. Kobayashi et al. [46] demonstrated that tumour mRNA-loaded DCs can elicit a specific $CD8^+$ CTL response against autologous tumour cells in patients with malignant glioma. CTLs from three patients expressed strong cytotoxic activity against autologous glioma cells, did not lyse autologous lymphoblasts or EBV-transformed cell lines and were variably cytotoxic against the NK-sensitive cell line K562.

All these studies demonstrate that using in vitro transcribed RNA coding for a defined TAA or whole tumour derived RNA is a powerful approach to evoke antigen-specific CD8- and CD4-positive T cells efficient at recognising tumour cells.

Vaccination studies in humans using RNA-transfected DCs

The in vitro studies provided promising data and the rationale for the development of DC-based vaccines and suggested the transfer from the laboratory to the clinic. The principle behind all these studies is to isolate peripheral blood mononuclear cells (PBMCs) or $CD34^+$ progenitor cells from the patients' peripheral blood to generate DCs in vitro, to transfect them with RNA-encoding antigens and to inject these genetically modified DCs back into the patients with the intention of inducing antigen-specific CTLs in vivo that specifically attack tumour cells. The RNA can be synthesised in vitro from plasmid vectors encoding defined tumour-specific antigens (TSAs) or TAAs. Alternatively, mRNA can be isolated directly from tumour cells thus harbouring the complete information of the cells.

The first vaccination study using RNA-transfected DCs was published by Heiser et al. [37]. This phase I clinical trial was designed to evaluate this strategy for safety, feasibility and efficacy to induce T-cell responses in vivo. Patients with metastatic prostate cancer stages D1–D3 were treated with PSA RNA-transfected DCs. Prostate cancer is one of the few tumour systems for which a highly specific marker, namely the self-protein PSA, is available for monitoring disease progression. DCs with typical immature phenotype were generated from plastic adherent monocytes by the addition of IL-4 and GM-CSF and transfected with in vitro–transcribed PSA RNA. Transfection was carried out as a passive pulsing by simple coincubation of RNA with the DCs. The genetically modified DCs were administered intravenously using escalating doses of 1×10^7 to 5×10^7 DCs at intervals of 2 weeks. Additionally, 10^7 cells were administered intradermally at each vaccination. Vaccination was well tolerated, and no major toxicity during the vaccination period was observed in any of the patients treated. To determine whether vaccination with PSA RNA-transfected DCs is capable of stimulating T-cell responses in the treated patients, the authors first analysed and compared the numbers of PSA-specific T cells directly from pretherapy and posttherapy PBMC samples using an IFN-γ ELISpot assay [3]: all patients analysed had PSA-reactive INF-γ–secreting T cells after vaccination, whereas in the pretherapy samples virtually no INF-γ–secreting cells were detectable. To further analyse the effector function of the in vivo–generated PSA-specific T cells and their ability to lyse

PSA-expressing cells, a standard chromium-release assay with PSA RNA-transfected DCs as target cells was performed. In all nine patients tested there was a significant increase of PSA-specific killing of target cells mediated by MHC class I–restricted $CD8^+$ T cells compared with the samples prior to vaccination. To validate the vaccine-induced eradication of tumour cells, levels of PSA transcripts in the peripheral blood of patients were analysed by real-time PCR. The data of three patients suggested that three vaccination cycles with PSA RNA-transfected DCs were associated with the transient elimination of tumour cells from the peripheral bloodstream of some prostate cancer patients.

In the same year a second report from the Duke University Medical Center, Durham, was published [58]. This time only one patient with metastatic adenocarcinoma in the subcutaneous tissue and pulmonary nodule was treated with RNA-transfected DCs. In contrast to the first study where pure specific in vitro–generated PSA mRNA was used, total RNA isolated from the autologous tumour cells of the patient was applied. The goal of this approach was to elicit T-cell responses against multiple antigens, instead of targeting a single defined antigen. The RNA was introduced into immature autologous precursor-derived DCs by lipofection. The patient received an intravenous infusion of 3×10^7 DCs loaded with autologous total tumour RNA followed by intradermal injection of 1×10^6 autologous total tumour RNA-loaded DCs intradermally every 4 weeks for four immunisations. Vaccination was well tolerated, and no major toxicity during the vaccination period was observed, but there was no obvious clinical response since the patient had ongoing progression of disease. CTLs were evaluated for their ability to specifically lyse antigen-expressing target cells in vitro. PBMCs used for this cytotoxicity assay were obtained before any immunisation, after immunisation with CEA RNA-transfected DCs (in the context of a another clinical trial 6 months before immunisation with total tumour RNA-transfected DCs), and after immunisation with total tumour RNA-transfected DCs. Total tumour RNA-transfected DCs and total PBMC RNA-transfected DCs were utilised as targets in the assays. Both the PBMCs obtained post CEA RNA immunisation and those obtained post whole tumour RNA immunisation induced tumour-specific CTLs. Remarkably, the lytic activity of the PBMCs obtained after immunisation with DCs transfected with total tumour RNA was higher than the activity of the PBMCs obtained after immunisation with DCs transfected with pure in vitro–generated CEA RNA. The authors speculate that the tumour cells express proteins in addition to CEA that serve as tumour antigens. Therefore total tumour RNA-transfected DCs could elicit responses to multiple other yet-unidentified tumour antigens, whereas DCs transfected with pure CEA-transcript stimulate only CEA-specific responses.

A potential drawback concerning the whole tumour RNA approach is the risk of autoimmunity [29]. This point should be irrelevant when targeting TSAs that represent neoantigens arising from somatic mutations in normal gene products and are specific for the tumour cells (e.g. the *BCR-ABL* fusion protein). These tumour antigens are the ideal targets for tumour vaccination. However, the majority of tumour antigens that have been identidfied to date are TAAs that are not unique to the tumour cells and are expressed also on normal cells. And even more risky than the use of TAAs as targets could be the approach of using whole tumour RNA for DC transfection. That means that the whole mRNA content of a cell is delivered into DCs, and self-peptides potentially are presented to T cells, thus eliciting immune responses to normal antigens. To address this problem, Nair et al. [56–58] generated B-lymphoplastoid cells from the PBMCs of a normal individual. These cells were used as a source of total tumour RNA for DC transfection. DCs transfected with RNA extracted from the B-lymphoplastoid cells and RNA from normal B cells served as target cells in a cytotoxicity assay. In this experimental setting the CTLs induced by the DCs transfected with the B-lymphoblastoid RNA did not lyse DCs transfected with normal B-cell RNA. The authors therefore conclude that the antigen(s) recognised by the CTLs induced by DCs transfected with tumour RNA are not present in significant amounts in the normal B cells from which the B-lymphoblastoid line was derived. Thus, in these experiments, autoimmunity was not induced. These findings are in accordance with our own data: we were able to show that DCs generated from monocytes of patients with B-CLL induce leukaemia-specific cytotoxic and proliferative T-cell responses on transfection with total RNA isolated from autologous leukaemic B lymphocytes. Standard ^{51}Cr-release assays showed specific MHC class I–restricted cytotoxic activity against the autologous leukaemic B cells and DCs transfected with CLL-RNA, whereas nonmalignant B cells were spared [55].

Another phase I study was performed in patients with advanced CEA-expressing malignancies that was followed by a phase II study in patients with resected hepatic metastases of colon cancer. The authors used immature DCs transfected with pure mRNA coding for CEA [53]. The immunisations were well tolerated, and there were no toxicities observed. Of 24 evaluable patients in the phase I trial, there was 1 complete response, evaluated by tumour marker monitoring, 2 minor responses, 3 with stable disease, and 18 with progressive disease. In the phase II study, 9 of 13 patients have relapsed at a median of 122 days. Manifestations of immunological responses were proven in biopsies of DC injection sites and peripheral blood of a subset of patients.

RCC is highly resistant to systemic chemotherapy, and no agent should be considered standard in the treatment of metastatic disease. However, the spontaneous regression of RCC in some cases suggests that these tumour cells are susceptible to immunologic mechanisms [30]. Clinical trials were performed utilising DCs loaded with lysate of cultured autologous or

allogeneic permanent RCC tumour cells as a vaccine [40]. In this setting, 2 of 27 patients showed complete response, 1 patient had an objective partial response, 7 patients had stable disease, and the remaining 17 patients had progressive disease. Su et al. [74] tested an alternative approach using RCC RNA-transfected DCs. In a phase I clinical trial, 10 patients with metastatic RCC received immature cryopreserved and reconstituted DCs which have been transfected with total RNA isolated from renal tumour tissue. Unfortunately, the authors were unable to reliably assess potential vaccine-induced clinical responses because the majority of patients (8 of 10) received secondary therapies generally applied in the form of IL-2 administration. Nevertheless, the study produced evidence that the application of RCC RNA-transfected DCs is not only safe and feasible but also capable of stimulating the expansion of tumour-specific, polyclonal T cells in the treated patients. The vaccine-induced T-cell responses were directed toward several different antigens including hTERT, RCC-associated antigen G250, and oncofetal antigen (OFA), but not against normal cellular antigens expressed by autologous normal renal tissue. Additionally, the authors showed that the vaccine-induced tumour-specific CTLs are functionally intact: in cytolytic assays CTLs stimulated from posttherapy PBMCs were more potent in recognising and lysing RCC RNA-transfected DC targets than were CTLs that were stimulated from pretherapy PBMC samples.

General conclusions

The preclinical in vitro studies for the induction of antigen-specific CTLs by DCs transfected either with defined TAAs or even with whole tumour RNA demonstrated the power and feasibility of this approach.

The investigators were able to elicit tumour-specific CTLs against various antigens such as the CEA, PSA antigens, papillomavirus oncoproteins, human telomerase reverse transcriptase, Mucin 1, HER-2/neu and melanoma antigen. The types of cancer faced by these technique were cervical cancer, prostate cancer, myeloma, colorectal cancer, breast cancer, melanoma, glioma, CLL, adenocarcinoma and RCC.

In all reported clinical trials the vaccinations were well tolerated, and no major toxicity was seen in any of the patients treated. These observations should legitimate treatment of patients with an early stage of disease.

An important and interesting finding of some studies was the induction of both $CD4^+$ and $CD8^+$ mediated T-cell responses. The current concept is that the kind of immune response induced depends on how the antigens are taken up and presented by the DCs. Endogenous cytosolic protein antigens from pathogens replicating inside the APC, such as a virus, are cleaved into peptides by the multicatalytic proteasome which are then transported via transporter associated with antigen presentation (TAP) into the endoplasmatic reticulum (ER) cellular compartment. In the ER, peptides assemble with MHC class I molecules. These complexes are then transported to the cell surface for presentation to $CD8^+$ CTLs. Antigens from extracellular pathogens are usually processed differently from endogenous antigens. Exogenous antigens are engulfed by APCs, internalised into the endocytic pathway and degraded in phagosomes, membrane-bound cellular compartments. MHC class II heterodimers, which are assembled in the ER, are targeted to the endocytic compartment where they are loaded with peptides and are then transported to the cell surface for presentation to a subset of $CD4^+$ helper T lymphocytes [33, 34, 41, 67]. However, these two pathways of antigen presentation are not strictly separated. Professional APCs including DCs can break through these routes and present exogenous antigens on MHC class I molecules, a phenomenon called cross-presentation [7, 10]. In the corresponding studies, the reverse occurred: peptides from the endogenous pathway were presented on MHC class II molecules and $CD4^+$ helper T cells were induced. Nair et al. [56] engineered a chimeric RNA encoding an antigen fused to a lysosomal targeting signal. With that strategy they were able to induce additionally antigen-specific $CD4^+$ T cells. Weissman et al. [82] observed induction of $CD8^+$ and $CD4^+$ T cells by *gag*-RNA transfection of DCs, because some of the *gag* protein was secreted and endocytosed into the exogenous pathway. Müller et al. [54, 55] were able to induce breast cancer–specific $CD8^+$ and $CD4^+$ cells by transfecting DCs with total tumour RNA. However, the molecular mechanisms by which peptides processed from endogenous proteins enter the MHC II presentation pathway are still elusive. Recently, autophagy was identified as a mechanism responsible for the presentation of a cytosolic antigen on MHC II molecules [61]. In this concept, the presentation on MHC II is independent from the machinery active within the endogenous pathway, the proteasome and TAP, but is dependent on the endosomal/lysosomal compartment in which the cytoplasmatic processing and turnover of proteins by autophagy merge in the exogenous pathway. Beside this antigen-processing pathway, secretion of some peptides and subsequent re-uptake as observed by Weissman et al. [82] is an alternative route by which cytoplasmatic peptides gain access to the endosomal/lysosomal compartment.

Clinical studies that have addressed the efficacy of RNA-transfected DC-based vaccine therapy have revealed promising data; nevertheless, many problems remain to be resolved including the design of broadly applicable clinical protocols, the optimal source of DCs for clinical applications (monocyte-derived, $CD34^+$ PBMCs etc.), the most efficient way of loading DCs with antigens, and the best route of DC administration. More importantly, all of these procedures need standardisation to allow comparison of clinical outcomes in further in vivo studies. Future clinical trials should also focus on patients with minimal residual disease or on the

adjuvant setting, as this group might be the one that could benefit most from DC-based cancer immunotherapies.

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