

Review

Search for “Weapons of Mass Destruction” for Cancer - Immuno/ Gene Therapy Comes of Age

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The complexity of a cancer, such as cell heterogeneity, and the existence of hypoxia, stromal cells and stem cells has so far prevented successful development and treatment of patients suffering from the later stages of cancers. At present, the use of conventional therapies, such as chemo/radio therapy is limited, and only therapies that are focused on utilizing the patient's immune response to combat against the disease appear to be the most reliable and promising. Two decades ago, cytokines were discovered to be able to activate the immune systems and mount an anti-tumour response. Then, dendritic cells were hailed as the most significant regulators of immunity and are employed in a variety of cancer management schemes. This review introduces current development in the field, focusing on combination of the components of the rapidly growing fields of immunotherapy and gene transfer/therapy, providing useful and significant detailed information for readers of cellular and molecular immunology. *Cellular & Molecular Immunology*. 2005;2(5):351-357.

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Introduction

Immuno/gene therapy is one of the very few options that can provide hopes and promises for the treatment of cancer. Taking malignant melanoma as an example, the current conventional therapeutic strategies include chemotherapy, radiation therapy, combination of chemo and radiation therapy, immunotherapy and more recently, gene therapy. In chemotherapy, dacarazine (DTIC) is the most active single agent for melanoma treatment, with an expected 40-50% response rate (1). This agent is ineffectual in cerebral metastases, as it does not cross the blood-brain barrier. The lipid-soluble agents, nitrosoureas, which have the ability to cross this barrier, showed a considerably lower response rate, and in combination with DTIC showed increased toxicity to

patients (2). Although the majority of the combination chemotherapy trials have not shown superiority to single agent DTIC treatment, the combination of DTIC, carmustine, cisplatin and tamoxifen first devised by Del Prete et al. (3), has produced encouraging response rates of approximately 50% (4, 5). The cost to the patients' quality of life after treatments is severely affected. The doses of radiation received by patients are comparable or higher than those experienced by atomic bomb survivors (6). Infertility is a common side effect of current cancer therapies, which causes possible risk to germ cell damage.

Immuno/gene therapy may be a more suitable approach to treating cancer. Cancer immuno/gene therapy specifies treatments that instigate antitumour responses primarily through the host immune-defence mechanisms. Cancer immuno/gene therapy can be divided into: 1) Passive specific immunotherapy - specific antibody or antibody conjugates, or immune cells such as cytotoxic T lymphocytes (CTLs), tumour-infiltrating lymphocytes (TILs) or modified TILs (adoptive specific immunotherapy) are transferred into the patient (7); 2) Passive non-specific immunotherapy - transfer of lymphokine-activated killer (LAK) cells (adoptive non-specific immunotherapy); 3) Active non-specific immuno/gene therapy - induction of an immune response with microorganisms (e.g., *Bacillus Calmette-Guerin* (BCG), *Corynebacterium parvum*), microbial components (e.g., endotoxins) or immunomodulators (e.g., cytokines), or genetically modified microorganisms; 4) Active specific immuno/gene therapy - immunisation with anti-idiotypes or tumour-based vaccines (8) or gene transfer/gene modified immune cells or vaccines (9).

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Cancer immunotherapy with cytokines and tumour-based vaccines

The first observed case of cancer immunotherapy was probably in the early 1890's when injection of the bacterium *Streptococcus pyogenes* into the tumour site induced its regression (10). Later it was found that the active component of the injected "toxin" and the active mediator of tumour regression were the endotoxin and the cytokine tumour necrosis factor (TNF), respectively (11). Other bacterial agents, such as BCG and *Corynebacterium parvum* have been used in large clinical trials with some demonstrated benefit to the patients (12). A little over two decades ago, cytokines were discovered to be able to activate an allogeneic T-cell response that was previously inert against a tumour cell line (13). Inevitably, a great deal of enthusiasm for tumour immunotherapy was generated and a large amount of research into cytokine therapy eventuated from the findings.

A study showed that administration of interleukin-2 (IL-2) alone or with LAK could reduce tumour volume (14). One of the roles of IL-2 (T-cell growth factor) *in vivo* is to stimulate activated T-lymphocyte proliferation and *in vitro* it can transform LAK cells (lymphocytes from the spleen or blood) into non-specific cytotoxic cells (15). Although, high doses of IL-2 are correlated with tumour regression, its clinical usage is limited as systemic IL-2 leads to capillary shock syndrome (16). Several clinical trials have been based on systemic administration of IL-2 (17, 18), however the delivery caused accumulation of the molecule in the vasculature instead of the tumour site (18). Cytokines are transient and short-range molecules for signalling between cells, and therefore in order to avoid the potential side effects from systemic administration, they should be delivered at the tumour site to induce a local inflammatory response. This can be achieved by *in vitro* modification of tumour cell lines to produce one or more types of cytokines *in situ*. For example, a vaccine based on irradiated B16-F10 murine melanoma cells transfected with another cytokine, granulocyte macrophage colony stimulating factor (GM-CSF) was demonstrated to induce long-term immunity to the tumour (19). Syngeneic mice immunised with GM-CSF modified tumour cells were shown to provide better protection against subsequent challenge with parental B16-F10 cells than with other cytokines in this particular study. In another experiment, the *in vivo* release from transduced B16 cells of modified IL-1 α , a potent cytokine produced mainly by activated monocytes and macrophages, showed significant tumour reduction compared with mock-transduced cells (20). The presence of IL-1 β appeared to enhance the tumour infiltration of macrophages, CD4⁺ and dendritic cells, and upregulated adhesion molecules, ICAM-1. This could be the result of IL-1 instigating the increase of several other cytokines (e.g., GM-CSF, IL-4 and TNF α) that are known to have an antitumour response (19).

Experiments discussed above are examples of many recent cancer immunotherapeutic strategies that use tumour cells modified (either by transfection or transduction) with

genes encoding immune response-enhancing molecules. Tumour cells are usually irradiated prior to their use so that they no longer constitute a malignant threat. Our own results showed that live tumour cell-based vaccines however, seem to be more potent and give better immunological memory (21). This could be due to higher number of antigens and longer period in which the antigens are present *in vivo*. Unfortunately, vaccines based on live tumour cells raise substantial safety concerns. The idea of gene therapy by transferring therapeutic genes directly into the tumour site may overcome the safety issues, but because of the lack of vector systems that are capable of delivering adequate amounts of transgene *in situ* for a long period of time, this method of gene therapy of cancer is not clinically viable at the present though many clinical trials are going on around the world.

Cancer therapy with antibodies

The concept of using antisera to treat diseases is not novel. The discovery that sera from individual affected with diphtheria toxin could cure diphtheria from another over a century ago was possibly the first application of passive immunotherapy. Given more than enough time to develop, there are disappointingly few successes from antibody therapy of malignant diseases.

The use of anti-idiotypic antibodies (Ab₂) as vaccines has been successful in generating an immune response against microbial antigens, however it has proved to be less successful in melanoma clinical trials, resulting in only moderate partial responses from patients (22). The antigen-binding site of Ab₂, which physically resembles that of the original tumour antigen, is used to "fool" the immune response. It is believed that because this artificial antigen is presented in the context of a foreign protein (Ab₂s are usually generated from nonhuman origins, e.g., murine monoclonal antibodies), it will overcome the tolerance effects that sometimes occur with native tumour antigens (23).

Another application of antibody technology in cancer therapy is the use of immunotoxins. Immunotoxins are complexes of specific antibodies linked to a toxin molecule. They are intended to be used to target certain cell surface oncoproteins or TAAs and kill the target cells directly, or they block the function of the target intracellular tumour associated proteins. Immunogenicity against the toxin protein, inefficient accessibility of immunotoxins to cancer cells and side effects are current problems with this approach. Chen S and Marasco WA developed a novel approach in which a highly immunogenic toxin protein was replaced by an expression plasmid to enable intracellular synthesis of the toxin in the target cell (24). The presence of the plasmid DNA is nonimmunogenic or only weakly immunogenic (25), unlike that of most toxins. Neutralizing antibodies against the toxins can be produced, thereby limit the possibility of repeated administration in patients (26, 27). The success of this approach is still likely to be hindered by insufficient delivery of the DNA/immunotoxins, even with repeated

administration, to the tumour cells to elicit significant tumour regression. Also, naked DNA is rapidly degraded by endogenous endonucleases before the gene(s) is/are expressed (28).

To generate a potent immune response to effectively induce tumour regression, it may be necessary to present the immune response with multiple antigenic peptides (with differing MHC binding requirements) from parent proteins. It is unlikely that immunisation with single types of peptides, with or without adjuvant, will be sufficient to evoke diverse and potent immune responses.

Dendritic cells and cancer therapy

Dendritic cells

Though the tumour cells themselves express the target tumour-associated antigens (TAAs), they are generally not capable of initiating an immune response. This is because they do not present these antigens in the context of costimulatory signals (e.g., B7) necessary for T cell activation. In the absence of appropriate signals, interaction of the T cell receptor itself usually leads to ignorance, anergy or apoptosis of the antigen-specific T cell. To produce effective and long-term immunity against tumour cells the functions of antigen presenting cells (APCs), T-helper cells and cytotoxic T cells are required. Dendritic cells (DCs) are the most potent of all APCs that are capable of stimulating a naïve T lymphocyte. DCs are found in high numbers at the cellular interstitium and near barriers to the environment (skin, mucosa). DCs represent discrete leukocyte populations of APCs that have a great capacity for inducing a primary immune response (29). These cells are mobile and have notable cytoplasmic extension/retraction capability. They are able to migrate through tissues and passage to the T-lymphocyte dependent areas of lymph node. DCs express major histocompatibility complex (MHC) molecules, particularly, MHC class II loci products, in high density. Short-lived migratory DCs can be processed by recipient DCs in the lymph nodes, distributing their peptide efficiently to other DCs to form MHC II-peptide complexes (30). DCs are extremely proficient at forming these complexes from cellular fragments released by phagocytosed cells, with efficiency up to 10 thousand times greater than exposure to the synthetic peptide epitope (31).

A small portion of DC precursors (and mature recirculating forms) circulates in the blood and migrates from the bloodstream into the tissues. These interstitial DCs have extensive cellular processes and act as guards looking out for foreign antigens and/or inflammatory stimuli. The DCs that circulate in peripheral blood will bind to the P- and E-selectins expressed by capillary endothelial cells, and thus are poised to exit from the blood at inflammatory sites, allowing expeditious accumulation of DCs (32). Under cytokine stimulation by factors such as granulocyte/macrophage-colony stimulating factor (GM-CSF) or tumour necrosis factor- α (TNF- α), DCs mature and may interact with T-cells at the tumour site or may migrate to the regional

lymph nodes. Reciprocal T lymphocyte feedback provides antigen specific control of DC antigen presentation (33). DC/T-cell interaction may protect T cells from activation-induced cell death (34). Recent data suggested that DCs might also have a direct role in regulating B cell maturation. Dubois et al. showed direct stimulation of activated B cells to proliferate and produce high amounts of IgM and IgG by purified Langerhans/DCs (35).

Only several relatively DC-specific antigens have been identified as the isolation of pure DCs for immunisation had been difficult. CD11c, widely used as a DC marker, is an integrin adhesion molecule, although it has also been described in a wide variety of human leukocytes including monocytes and macrophages (36). Recently, high-purity preparations of low density DCs have been made possible due to discoveries of monoclonal antibodies against CD83 and CMRF-44. Now reasonably large numbers of DCs can be generated, from either murine or human sources, by treatment with GM-CSF alone or in combination with other growth factors such as IL-4 (30). A review by Derick Hart provides comprehensive coverage of DC antigens as well as the biology of DCs (37).

Antigen presentation by DC

Although MHC class I molecules almost exclusively form complexes with peptides derived from cytosolic proteins (38), APCs appear to be an exception. DCs take up exogenous antigens by macropinocytosis and release them into the cytosol for degradation by intracellular proteases and peptidases (39). These peptides bind to transporter (TAP: transporter associated with antigen processing) gene products and are delivered to be incorporated into MHC class I molecules for presentation. Also, exogenous peptides present in high concentration externally may exchange with peptides on mature MHC class I molecules. The receptors for the Fc portion of immunoglobulin (Ig) G, Fc γ R, have been shown to influence murine DCs in the internalisation of antigen-IgG complexes, promote efficient MHC class I-restricted presentation, and induce DC maturation (40).

Early DCs can delay antigen presentation by regulating MHC class II transport and compartmentalisation to prevent "premature" presentation to T cells in the periphery. DCs accumulate antigen in the periphery and efficient display of antigen (delivery to lymphoid organs) occurs 24-48 hours after antigen internalisation (41). DCs require various signals to prime T cell responses. The first signal is provided by the antigens which are processed into peptides and complexed with MHC molecules. Either inflammatory cytokines or bacterial components (e.g., LPS) then provide cell activation signals to induce the expression of MHC and T cell costimulatory molecules on the DC surface. This results in subsequent T cell priming *via* DC migration from peripheral tissues to secondary lymphoid organs.

Generation and isolation of mouse DCs for in vitro modification

Not until recently, the rarity of DCs *in situ*, laborious time consuming isolation processes, and subsequent low yield

restricted extensive studies on these unique cells. Generally, about $1 \times 10^{5-6}$ DCs can be generated from one mouse, either from its spleen, thymus, blood or bone marrow (31). Lutz et al. recently described an advanced culture method for generating up to 3×10^8 DCs from mouse bone marrow at 90-95% purity (42). This was achieved simply by prolonging the culture period to 12 days, reducing the GM-CSF dose and initially plating the bone marrow cells out at low density.

Applications for DCs in cancer therapy are increasing rapidly, as efficient isolation and preparation of both human and murine DCs are now possible. Also, early experiments suggested that DC injections into human patients are safe (37). *Ex vivo* cancer therapy based on DCs can be conducted by inducing expression of TAAs on dendritic cells. There are several methods used to achieve this: 1) Pulsing of *ex vivo* generated DCs using a tumour-specific antigenic peptide or tumour cell lysate; 2) Fusion of DCs and tumour cells (43) providing direct access of DCs to antigens; 3) Pulsing of DCs with tumour-derived mRNA; 4) Transfection or viral transduction of genes encoding specific TAAs to DCs. *Ex vivo* modified DCs have been shown to migrate to the nearest lymph node to initiate T-cell activation after administration (44).

The methods most commonly used currently are peptide pulsing and gene transfer. The first method involves the delivery of tumour-specific antigens (in the form of peptides) into *ex vivo*-generated DCs. The second method involves transferring tumour-associated antigen coding genes into DCs. The aim of these two strategies is to induce a CTL response by getting DCs to present a particular tumour antigen(s). The first method has already been shown to work in animal models to protect against tumour challenge by eliciting T-cell immunity (43, 45, 46). The use of the whole tumour-associated antigen for vaccination strategies (transduction into DCs) should promote induction of a T-cell response against multiple epitopes of the protein.

Pulsing of DCs with tumour-associated peptides and cell lysates

Proteins or peptides are the most common forms of antigens used in immunotherapy. DCs pulsed with protein or peptide mixtures isolated from tumour cells have been shown to be an exceptionally effective vaccine against tumours in mouse models (31, 46). Celluzzi CM et al. showed that MHC class I-presented peptide antigen pulsed onto DCs induced protective immunity against a challenge from tumour containing antigen (43). This action was antigen specific and was obliterated by depletion of CD8⁺ T cells, demonstrating CD8-mediated antitumour immunity instigated by peptide-pulsed DCs. Immunity from DC-based vaccines was confirmed to be primarily cell mediated in another experiment where the transfer of spleen cells, but not the transfer of sera, from immunised mice protected irradiated naïve mice against subsequent tumour challenge (46). The experiment also showed abrogation of the therapeutic effects of DC vaccine in the absence of CD4⁺ or CD8⁺ T cells.

A specific range of peptide/MHC densities on APCs has to be achieved with peptide pulsing since an antigen dose

above this range has induced apoptotic cell death in a high avidity CD8⁺ CTL line and may lead to tolerance (47). The peptide approach provides a limited base of CTL epitopes. It is more desirable to provide a complete protein antigen rather than haplotype-specific epitopes, given the high MHC haplotypic diversity of the human population. Also, with this strategy, no B cell or helper T cell epitopes are provided for the generation of the full immune complement of both antibody and cellular responses. There is evidence that human peripheral blood DCs degrade synthetic class I peptides at their cell surface within minutes after loading (48). This is the result of the activity of ectoenzymes whose degradation performance interestingly increases in the presence of GM-CSF/IL-4 (48), the cytokines widely used *in vitro* to induce DC activation and proliferation. Peptide-MHC class II complexes, on the other hand, are stable and have much longer half-lives (> 100 h) (49). The MHC restriction and short-term presentation may be bypassed by genetic transduction of DCs. The degraded product of the tumour antigen transgene in DCs can then be "custom-made", to fit the patient's own HLA molecules. This method will also allow constitutive expression of the proteins which will prolong the presentation of the antigens. Additionally, multiple and unidentified epitopes encoded by the entire tumour-associated gene may enhance T cell activation.

TAA gene transfer to DCs

Physical methods such as lipofection, electroporation, or Ca₃(PO₄)₂ precipitation were found to be ineffective procedures to introduce foreign genes into DCs as they all yield a low level of transgene expression (50). Many gene transfer vectors based on adenoviruses (AdV) are available for clinical use. These vectors do not integrate into the host chromosome, and therefore, unlike retroviral vectors, have negligible oncogenic potential. Their disadvantages include the development of inflammation and the transient expression of the recombinant genes. These effects are believed to be the results of an induced host immune response, specifically the CD8⁺ CTLs acting against the cells containing the transgene's product and, currently unidentified, adenovirus antigens (51). Song W and co-workers showed that AdV vectors encoding β-gal as a model antigen could effectively transfer and express the transgene in DCs (52). Administration of these β-gal modified DCs *in vivo* elicited an antigen-specific CTL response, protecting syngeneic mice against tumour challenge, suppressing pre-existing tumours and prolonging the survival rate. Transferring genes into DCs by conjugating them with an UV-irradiated AdV has recently been shown to be a consistent and efficient gene delivery method without concomitant expression of AdV gene products (53). However, high viral titre and precise AdV UV irradiation dosage are required in order to achieve efficient gene transfer and to completely block adenoviral genomic expression.

Another viral vector that has been successfully used to genetically modify DCs is the MLV-based retroviral vector. This vector has some advantages over the AdV-based delivery of antigenic transgene. As a retroviral vector stably

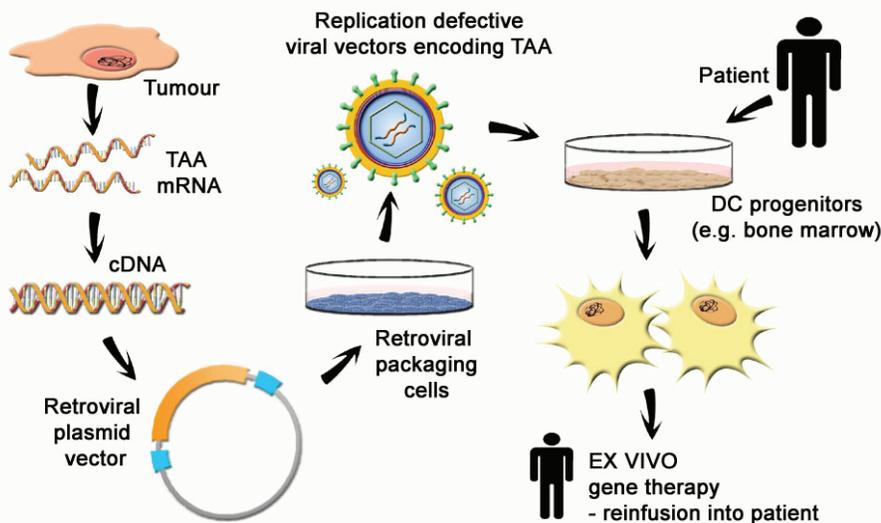


Figure 1. A simplified sequence of steps showing one of the methods to which DCs can be modified to express a TAA. Tumour-antigen encoding cDNA is isolated from tumour and cloned into a retroviral vector. The replication incompetent virus particles are generated from a packaging cell line and used to transduce DC progenitors which are then matured, in the presence of certain cytokines (i.e., GM-CSF and IL-4), into DCs. These DCs can be used as a vaccine to treat tumour.

integrates into the genome of the target cell, the product may be expressed indefinitely and the multiple CTL epitopes generated from cytoplasmic degradation of the product may be continuously available for presentation by the cell's MHC molecules. Long-term expression of MHC-peptide complexes may be essential for an effective immune response given recent reports on the relatively short half-life of MHC class I-peptide complexes on DCs (49). The infection process of retroviruses appears to cause no harm to the host cell and there is a lack of a significant immune response from the patient against retrovirus vectors, even at high dose (54). Like any vector with integrative ability, there is a theoretical risk of insertional mutagenesis and oncogene activation. However, the development of cancer from replication-incompetent retrovirus vectors has never been reported (55). Unfortunately, as the MLV-based vectors are not capable of transducing fully differentiated cells (56) such as DCs, only the proliferating DC progenitors isolated from bone marrow or cord blood may be transduced before they are cultured to matured DCs. It was shown that retrovirally transduced DCs retained the normal physical and functional characteristics of DCs (57, 58). Integration of replication incompetent virus resulted in no alteration of the DCs' antigen presenting and T-cell stimulating functions. Figure 1 shows the steps involved to retrovirally transduce DCs with a tumour antigen.

Apart from this laborious process required to transduce DCs, it appears that the transcription of transgenes delivered by MLV-based vectors often shuts off after the transduced cells return to a non-proliferating state (59). The reason for this is not well understood but the lack of expression from a retroviral vector after transduction has been associated with the methylation of proviral DNA *in vivo* (60). As lentiviruses do not require cells to be actively dividing at the time of transduction, lentivirus-based vectors may not suffer from the same transcription silencing mechanism (59). We had shown that lentiviral vectors have the potential to directly and effectively transduce fully matured DCs with high efficacy

providing a new area of research to appropriate feed DCs with tumor antigen and generate sufficient DCs for tumour therapy (9). Since most lentiviral vectors were based on a human pathogen, there were always worries about its use in human. In order to apply lentiviral gene transfer technology to the clinical settings, our laboratory has developed a bovine lentiviral vector system and in the process to test its use in immuno/gene therapy of later stage solid tumours (61, 62).

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