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REVIEW ARTICLE

Gene Therapy Applications to Cancer Treatment

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Over the past ten years significant advances have been made in the fields of gene therapy and tumour immunology, such that there now exists a considerable body of evidence validating the proof in the principle of gene therapy based cancer vaccines. While clinical benefit has so far been marginal, data from preclinical and early clinical trials of gene therapy combined with standard therapies are strongly suggestive of additional benefit. Many reasons have been proposed to explain the paucity of clinical responses to single agent vaccination strategies including the poor antigenicity of tumour cells and the development of tolerance through down-regulation of MHC, costimulatory, signal transduction, and other molecules essential for the generation of strong immune responses. In addition, there is now evidence from animal models that the growing tumour may actively inhibit the host immune response. Removal of the primary tumour prior to T cell transfer from the spleen of cancer bearing animals, led to effective tumour cell line specific immunity in the recipient mouse suggesting that there is an ongoing tumour-host interaction. This model also illustrates the potential difficulties of clinical vaccine trials in patients with advanced stage disease.

INTRODUCTION

In spite of the slow clinical progress, efforts to develop specific nontoxic cancer therapies are increasing exponentially [1, 2, 3, 4, 5, 6, 7], with the result that over 500 gene therapy trials have been listed with the FDA to date [8]. A number of strategies are currently being pursued in cancer treatment, aiming to either

(i) enhance immunological rejection of the tumour by the host,

(ii) decrease tumour cell proliferation and increase cell cycle control by restoring functions such as p53 and RB,

(iii) specifically poison tumour cells according to a 2-step design; incorporation of an enzyme followed by administration of a prodrug to be specifically activated in tumour cells harbouring the enzyme, or

(iv) specifically lyse tumour cells defective in the p53 or RB pathways using oncolytic viruses which are able to invade the "defective" tumour cells.

VECTORS (TABLE 1)

Genetic material is optimally transported into host cells by naturally evolved vectors such as viruses or bacteria. Efforts are ongoing to improve on natures' designs with increasingly sophisticated vector systems aimed at allowing prolonged transgene expression at high titre in the desired cell type whilst remaining nontoxic to the host [9]. Ideally, vectors should also carry a low risk of recombination with wild-type pathogens. Currently, the most promising approaches are based on replication-competent agents that allow efficient tumour penetration. Exciting results are anticipated with poxviruses [10, 11] and with selectively replicating/targeted adenoviruses [12, 13, 14, 15, 16], although pre-clinical models suggest that significant response rates will only be achieved by combination with standard therapies.

Poxviruses

Vaccinia virus (VV)-based strategies have been brought to clinical fruition by a number of different sources [17, 18, 19]. The large potential size (25 kb) of the gene insert, the absence of viral integration into the host cellular genome, and the excellent immune stimulation induced by this virus all combine to make it an attractive candidate for immune based therapy in cancer. Vaccinia virus infects all cells, however the host immune response to the vector does not abrogate the tumour immune response even following repeated injections. The availability of attenuated virus (*tk*- modified *vaccinia ankara*) [10] allows the use of vaccinia in immuno-delicate cancer patients and there is evidence that this vector enhances immunological rejection of the tumour.

In preclinical studies, use of a diversified immunization scheme employing a recombinant vaccinia virus followed by recombinant avian pox virus was shown to be superior to the use of either vector alone in eliciting

Table 1. Gene therapy vectors.

Vector	Preexisting immunity	Proliferation needed	Genome integration	Pathogenicity	Viral persistence	Specificity	Limitations (Viral titres and safety)
Adenovirus	+	–	No	+ + +	No	CAR receptors	+
AAV	+	–	?	No	Yes		
Retrovirus	–	Yes	Yes	No	Yes		+
Lentivirus	–	–	Yes	No ?	Yes	CD4 +	+
Poxvirus	+/–	–	No	No	No		
Bacterial vvectors, eg, salmonella	?	–	No	Antibiotics	No	Inflammation	?
Liposomes	–	–	No	–	–		–
Naked DNA	–	–	?	No	No		

CEA-specific T-cell responses. Multiple boosts of ALVAC-CEA following rV-CEA priming further potentiated the antitumour effect and CEA specific T-cell response [20]. Using tetrameric-MHC complexes *ex vivo* as well as lytic assays, Estcourt et al [21] were able to show that "prime-boost" immunization with DNA vaccines and recombinant poxvirus vectors generates high frequencies of cytotoxic T lymphocytes (CTL) that recognize target cells expressing very low levels of the specific antigen. These cells persisted for at least 6 months [21]. Harrington et al [22] quantified the T-cell responses to both the viral vector and the insert following infection of mice with VV expressing a CTL epitope (NP118–126) from lymphocytic choriomeningitis virus and demonstrated potent and long-lasting CD8 and CD4 T-cell responses to the vector peaking at approximately 1 week. These numbers decreased to approximately 5×10^5 CD8 T cells (approximately 5% frequency) and approximately 10^5 CD4 T cells (approximately 0.5% frequency), respectively, by day 30, at which levels they were stably maintained for over 300 days. The CD8 T-cell response to the foreign gene (NP118-126 epitope) was correlated with the response to the vector during all three phases (expansion, contraction, and memory) of the T-cell response [22].

Clinical results are still limited to marginal benefit but the proof of concept is established. Responses to an intradermally administered live vaccinia virus HPV 16 and 18 E6/E7 gene construct (TA-HPV, Cantab Pharmaceuticals) were seen in 1/3 of the evaluable patients with advanced cervical cancer, in 3/12 CIN III volunteers, and in 4/29 patients with early invasive cervical cancer [19]. A HLA-A*O201 restricted CD8 T cell response has also been recorded in the single HLA-A*O201 patient whose tumour was shown to be HPV16 positive. Vaccination in breast cancer patients using a poxvirus vector, MUC1, and IL-2 was well tolerated [23] and did exhibit evidence of some clinical activity (unpublished results, 2002). Common toxicities included a local skin reaction at the site of the vaccine, usually of 4–5 days' duration, and mild flu-like symptoms of 1–2 days' duration. Cellular immune response did not correlate with clinical response. The presence of a strong immunogenic vector appears to be important, since vaccination in the absence of a viral vector (MUC1-KLH conjugate plus QS-21) while immunogenic (high IgM and IgG antibody titers against synthetic MUC1), did not result in a cellular immune response in breast cancer patients [24].

Adenoviral vectors and adeno associated vectors. [8, 12, 25, 26]

Adenoviral vectors also have a large transgene capacity, a high level of expression, and can infect a large variety of cell types, however limitations are the absence of adenoviral receptor expression in certain cell types and the strong preexisting immunity, which limits transgene expression. In this regard, a direct relationship between low susceptibility of tumours to adenovirus injections and the absence of CAR (Coxsackie adenovirus receptor) expression on tumour cells has been demonstrated.

Ongoing preclinical emphasis is on designing improved, better targeted, and infectivity-enhanced adenoviral vectors. Since CAR deficiency in tumours clearly limits current adenovirus-based therapies, the tropism has been altered through genetic modification of the adenovirus capsid by mutating critical residues in the fibre knob [1] such that tumour cells can be infected via CAR independent mechanisms [27]. Double mutant AdV additionally lacking the integrin-binding penton base RGD motif were shown to efficiently target epidermal growth factor receptor or epithelial cell adhesion molecules, depending on the choice of the bispecific linker, resulting in a relative glioma/normal brain transduction ratio of 60 times that achieved with native AdV. Adenovirus-mediated IFN-γR gene transfer was shown to be effective

in augmenting the biological activity of IFN-γ, a strategy which should be useful in studying other applications of cytokine receptor-based gene therapy for cancer [28]. Regarding the transfer of p53, Ad5CMV-p53-infected cells underwent apoptosis, and cell growth was greatly suppressed. Ad5CMV-p53 treatment significantly reduced the volumes of established subcutaneous tumors in vivo [29]. In another model using stably transfected mammary carcinoma cells, a dominant negative (DN) mutant of EGFR, (EGFR-CD533) could act as a potent inhibitor of EGFR (epithelial growth factor receptor) and its cytoprotective signaling after exposure to ionizing radiation. In a genetic approach, using replication-incompetent adenovirus-mediated transfer of EGFR-CD533, the vector was able to enhance the radiosensitivity in vitro of representative cell lines [30]. Adenovirus-mediated expression of dominant negative-estrogen receptor-induced apoptosis in breast cancer cells and regression of tumors in nude mice [31]. In a different approach, the antisense RNA transcript of the E6 and E7 genes of human papillomavirus (HPV) 16 were transfected into cervical cancer cells harbouring HPV 16, via a recombinant adenoviral vector, Ad5CMV-HPV 16 AS. Expression of these genes suppressed greatly the growth of the Ad5CMV-HPV 16 anti-sense infected cells [32]. A rapid induction of cytotoxic T-cell response against cervical cancer cells by human papillomavirus type 16 E6 antigen gene delivery into human dendritic cells was also demonstrated using an adeno-associated virus vector [33].

Clinical results

The majority of patients who have been treated with adenovirus vectors received them with the aim of replacing defective genes, in particular p53, however, thus far clinical efficacy has been limited [34]. Testing by PCR for adenovirus shedding in body fluids of NSCLC patients injected intratumorally with adenoviral vectors at doses of 10^7–10^9 plaque forming units, revealed detectable viral genome for up to 90 days after injection. Screening of the clinical staff proved consistently negative and did not provoke a rise in antivirus antibody titres. (Escudier B, Institut Gustave Roussy, personal communication, NDDO meeting, Valencia, 2001, oral presentation.) Novel strategies that exploit our knowledge of the function and regulation of p53 are being actively investigated [35, 36, 37]. Intravesical instillation of Adenovirus p53 (SCH 5850) combined with a transduction-enhancing agent is safe, feasible, and biologically active in patients with bladder cancer [38]. Direct bronchoscopic injection of Adp53 into endobronchial NSCLC is safe and with acceptable levels of toxicity. Initial clinical results demonstrating relief of airway obstruction warrant further clinical investigation [39].

Conditionally replicative adenovirus vectors with oncolytic potential [14, 15, 16, 40, 41, 42, 43]

While overall approximately 50% of tumour cells are defective in the p53 pathway, it is estimated that one hundred percent of tumour cells present one of several defects in the Rb pathway, the most prevalent being p16 mutations, cyclin D amplification, HPV E7 overexpression, or a defective Rb expression itself.

Preclinical studies

The cumulated deletions of two E1B-gene fragments (E1B 19K and E1B 55K) in Adl 118, engineered by Ramon and Cajal [42] resulted in clear cytopathic effects in most human cancer cell lines. Intravenous injection of this conditionally replicative adenovirus, in an adjuvant situation after excision of the primary tumour, reduced metastatic disease and could eventually be seen as a strategy to prevent tumour metastasis in high risk breast carcinomas. These results were improved on with concomitant use of chemotherapy. Another potent adenovirus, (ONYX 411, carrying an E1A mutation in the Rb binding domain) was significantly superior to ONYX 015 in all models. The E1A gene of ONYX 411 is not complexed by Rb (if Rb is still expressed) allowing the virus to replicate even in the presence of Rb. Tumour cells have high levels of free E2F and therefore genes that have E2F responsive elements (E1A, TS, TK, dhfr, E2F itself etc.) should be more highly expressed in tumour cells. High E2F levels in tumour cells will also drive viral E1A expression allowing effective tumour cell kill by the virus. Similar oncolytic adenoviruses with selectivity for Rb pathways but without the CR2 mutation are also under development. Another strategy is to utilize tumour selective promoters to control early viral gene expression. Insertion of the E3 region enhances selectivity in tumour cell killing. E3 is composed of a series of genes involved in evasion of immune cell control, decrease in host cell MHC, Fas, and TNF expression and gives a consistent better tumour cell to normal cell kill-ratio. The efficacy of these new vectors has been shown in xenograft models following intratumour injection. Another recombinant adenovirus vector in which p53-dependent expression of a fusion protein (E2F-Rb) selectively attenuated viral replication in normal cells, was further modified by insertion of the viral late promoter (MLP) in the E3 region with the aim of driving overexpression of Ad5-E3 11.6K protein, thereby increasing cytotoxicity in tumour cells, while decreasing cytotoxicity in normal cells. Selective targeting could be achieved by Ad5-Delta 24RGD, an adenovirus selectively replication-competent in cells defective in the Rb/p16 pathway, such as ovarian cancer cells. The fiber of Ad5-Delta 24RGD contains an integrin binding RGD-4C motif, allowing Coxsackie adenovirus receptor-independent infection of cancer cells [44].

Clinical results

Over 230 cancer patients have been treated to date with the dl-1520 (ONYX-015 [15]) a replication-selective adenovirus. Kirn recently confirmed excellent tolerance using various injection routes, and documented reproducible evidence of viral replication. Tumour regression

was seen following treatment with single agent therapy in H&N cancer patients (15–20%) but not in other tumours. An early clinical trial of intraperitoneal delivery, efficacious in nude mouse-human ovarian carcinomatosis xenografts, showed no major toxicity without clinical response [16].

Other vector systems

Reovirus is an ubiquitous and relatively benign virus which may infect cells of the upper respiratory and GI tracts of humans, but is usually asymptomatic. Based on the finding that cells become highly susceptible to reovirus upon transformation with oncogenes in the Ras signalling pathway, administration of reovirus in cancer bearing animals confirmed a specific antitumour activity which could be enhanced by combination with chemotherapy and immune suppressive drugs. In vivo studies of reovirus therapy revealed that viral administration caused tumour regression in an MDA-MB-435S mammary fat pad model in severe combined immunodeficient mice [45].

Evidence of antitumour activity of the G207 herpesvirus vector in a phase I study of malignant glioma was shown by MRI (magnetic resonance imaging). This vector was also shown to be nerve-sparing [46]. Preclinical evaluation showed increased efficacy when administered in association with either radiotherapy, Cisplatin, or cytokines such as IL-12, GM-CSF, or the costimulatory molecule B7.1 [47, 48, 49].

VNP 2009, an attenuated and genetically modified strain of *Salmonella typhimurium* showed tropism for tumour cells as well as antitumour activity in dogs with melanoma, rhabdo-myosarcoma or fibro-sarcoma 50. Shiga toxin B subunit has become a powerful tool to study retrograde transport between the plasma membrane and the endoplasmic reticulum and may be used for tumour antigen insertion and presentation by antigen presenting cells [50]. Retroviral vectors are often favoured for GPAT (gene prodrug-activated therapy), their advantages being their simple genome, the availability of AZT, and their mode of transmission which prevents epidemic outbreak. So far, tumour eradication has been obtained in vivo only when replicative, but not defective, vector systems were used to transfer a suicide gene 51. Both retroviral and lentiviral vectors were shown to be able to efficiently transduce cycling hepatocarcinoma cell lines in vitro. Following cell cycle arrest, transduction efficacy remained the same for lentiviral vectors while it decreased by 80% for retroviral vectors. The CMV promoter allowed a stronger transgene expression than the PGK promoter, but expression rapidly decreased with time due to promoter silencing [51]. Liver failure which occurred following TK expression in nontumour cells, emphasized the need to target the expression of the *tk* gene to tumor cells using a hepatoma-specific promoter such as AFP promoter.

RECOMBINANT STRATEGIES OF INTEREST

Tumour antigens

Many clinical trials in cancer are designed to enhance immune responsiveness of the host against the so-called tumour antigens. The advantage of using viral strategies to transfer tumour antigens is the potential to deliver the full length genetic information of a protein allowing it to be processed in accordance with the patients MHC type. Tumour antigens fall into three main categories.

The first are those coded by viral genomes [54, 55]. In principle, these are attractive targets for immunotherapeutic attack [56, 57, 58], since the cells capable of responding to these antigens should not have been removed from the repertoire by central tolerance-inducing mechanisms. The immune response to these exogenously coded antigens should be vigorous; therefore interference by other factors (such as peripheral tolerance or escape mechanisms) is theoretically minimal. The success of therapy directed at EBV in transplant patients and HPV in cervical cancer patients suggest that under ideal circumstances, this type of response can indeed be effective [33, 59].

The second category of antigens are self antigens altered by genetic changes and rendered more visible by overexpression. Most, if not all, tumours accumulate multiple mutations during the process of malignant transformation and provide treatment targets. Another type of altered self-antigen is exemplified by MUC1, where the altered pattern is caused by genetic changes affecting glycosylation. Just how distinct these neo-epitopes of MUC1 are, however, is called into question by evidence that most serologically detected epitopes on tumour mucins are equally seen in the lactating breast. In practice, there is a little firm evidence for the development of high frequencies of MUC1-reactive T cells in tumour bearing patients or even in those immunized with MUC1 [60]. Nevertheless, the overexpression of MUC1 by tumour cells and evidence of the generation of MUC1-specific T cells in response to vaccination [61, 62] suggest that this may be a good tumour antigen. Clinical activity has been seen with poxviral vectors carrying MUC1 (unpublished results, 2002). Poxvirus-based vaccines can reproducibly generate T-cell responses to tumours expressing CEA or PSA [63]. Disease stabilization has been seen in up to 37% of patients treated with these vaccines [64]. A phase III trial of ALVAC CEA B7.1 in colon cancer is under discussion [65]. Many clinical trials are ongoing in the prostate cancer field, the antigenic proteins to be expressed and presented to the immune system being PSA or PSMA [18] as well as MUC1. Selecting an appropriate therapeutic gene and vector system to carry the gene driven by a tissue specific promoter such as the PSA promoter (PSAP) in prostate cancer may be important [66, 67, 68]. Trials with complex designs, alternating vectors (prime-boost) [20, 21, 22, 69], and associating immune modulating agents with classical therapies are ongoing.

The remaining category of tumour antigens, originally described by Boon and colleagues, are unaltered self-antigens [70, 71] with an expression profile limited to specific tissues at certain times in development.

Immune modulatory agents

IL-12. In his introductory session at the NDDO meeting in Valencia, Woo [72] focused on preclinical models using various combinations of immuno-modulatory gene therapy for cancer. Following intrahepatic implantation of colon or breast carcinoma cells in syngeneic Balb/c mice, intratumour treatment with a recombinant adenovirus expressing murine IL-12 was followed by expression of very high IL-12 (25000 pg/ml) and Interferon gamma (6000 pg/ml) titres at the tumour site as well as tumour rejection and long term survival. This IL-12-dependent antitumour activity was shown to be mediated by NK cells, despite the fact that these tumours were MHC class-I-positive [73]. The NK antitumour response could be complemented through ligation of the 4-1BB receptor by an agonistic monoclonal antibody leading to long-term tumour-free survival in over 80% of the animals [74]. This in turn was associated with resolution of pre-established metastases in the lung (distant site) and was T cell-mediated [72]. A clinical trial using an IL-12 expression vector in patients with metastatic lesions from breast and colon cancer has been authorised by the FDA and is awaiting the GMP product. In animal models, the autoradiographic imaging of I [133]-labelled viral vector showed maximal bio-distribution in the injected tumour site with only low levels of activity in normal liver, possibly related to leakage to bile ducts through the needle puncture site.

IL-2 has a proven record of improving cancer vaccinations by expanding T cells [1]. DNA-lipid complex encoding the interleukin 2 (IL-2) gene (Leuvectin; Vical, San Diego, Calif) administered intraprostatically into the hypo-echogenic tumour lesion showed evidence of clinical efficiency based on an increase in the intensity of T-cell infiltration seen on immunohistochemical analysis of tissue samples from injected tumor sites and on increased proliferation rates of peripheral blood lymphocytes. Furthermore, transient decreases in serum prostate-specific antigen (PSA) were seen in 16 of 24 responding patients [75]. Established RM11-PSA tumors ranging in size from 500 to 1,000 mm^3 were efficiently eliminated if Ad5-PSA (adenovirus-5) priming was followed 7 days later by intratumoral injection of recombinant canarypox viruses (ALVAC) encoding interleukin-12 (IL-12), IL-2, and tumor necrosis factor-alpha. This data demonstrates the utility of an Ad5-PSA vaccine combined with cytokine gene delivery to eliminate large established tumours refractory to other intervention [76]. Intratumoral treatment of nude mice with vaccinia virus (VV) expressing interleukin 2 (IL-2) or IL-12 significantly inhibited tumour growth, however there was significant associated toxicity [77]. After four vaccinations with cytokine-transduced melanoma cells, antibodies (Abs) against vaccinating and autologous melanoma cells were generated in 62% of patients. These findings demonstrate that the identification and titration of alloreactive Ab helps to monitor the extent of immunization against cellular vaccines, while the induction of Ab reactive to antigens shared between vaccinating and autologous melanoma cells may contribute to their therapeutic efficacy [78]. The role of cytokines such as GM-CSF and IL-2 in the generation of antitumour immune responses was further demonstrated by their use in association with poxvirus vaccines. While rV-CEA was effective in priming the immune system, avipox-CEA could be given up to eight times with continued increases in CEA T-cell precursors, however further increases in CEA-specific T-cell precursors were seen when local granulocyte-macrophage colony-stimulating factor (GM-CSF) and low-dose interleukin (IL)-2 were given with subsequent vaccinations [79].

Targeted adenoviral transduction to activate cutaneous dendritic cells, was achieved by complexing virus to a bi-specific antibody, thereby neutralizing the virus receptor binding site as well as *agonistically binding to CD40* [80]. This resulted in a more selective insitu transduction of $CD1a^+$ dermal dendritic cells (DC) in a human skin explant model. DC's were shown to prime specific CTL more efficiently in vitro in an autologous restimulation protocol employing HER-2/neu as the model tumour target. However, with as little as 3–10% of tumour cell supernatant even CD40-targeted CTL had a reduced efficiency in the cancer situation. DC differentiation was hampered and cells retained the $CD14^+$ phenotype, an effect partially reversible by GM-CSF treatment. Similarly, in an orthotopic hepatocellular carcinoma model (HCC) in the rat, tumorigenicity could be abrogated by prior transfection with an adenoviral vector carrying the murine CD40 ligand [81, 82]. Tumour rejection was associated with a peak of IL-12 release on day 5 (> 700 pg/ml) and was $CD8^+$ T cell dependent. Animals developed protective immunity. Toxicity consisted of a mild increase in ALT levels with a minor infiltration of lymphocytes into normal liver.

IP10. Synergy between IL-12 and the interferon gamma inducible protein IP10 in cancer treatment was shown using a CT26 tumour model [83]. A one hundred percent eradication of both injected malignant hepatic nodules and distant tumour nodules could be achieved through co-injection of the adenoviral vectors carrying IL-12 and IP10. Antitumour activity was greatly diminished by simultaneous in vivo depletion of CD4 and $CD8^+$ T-cells. The use of the vector carrying IP10 alone or IP10 together with the IV adoptive transfer of antitumour T lymphocytes only eradicated tumour in 35% of cases.

Blockade of both the CD40-CD40L and CD80/CD86-CD28 costimulatory pathways represents a strategy to inhibit the immune response against Adenovirus vectors [84]. The CD80/CD86-CD28 costimulatory pathway can be effectively inhibited by a (stimulatory) CTLA4 fusion protein [84]. The opposite is desirable in cancer treatment

and the co-stimulatory pathway can be activated through blockade of CTLA4 and/or transfer of CD80/CD86 [85, 86]. In early stage clinical trials, the addition of B7.1 to virus-based vaccines showed some improvement in immunological response and in the number of patients with stable disease following vaccination against tumour-associated antigens [65]. ALVAC-CEA B7.1 alone (n = 30) or with GM-CSF (n = 30) was also administered to patients with advanced CEA-expressing tumors to determine whether the addition of the adjuvant GM-CSF could enhance induction of CEA-specific T cells [87]. All of the patients had evidence of leukocytic infiltration and CEA expression in vaccine biopsy sites. In the patients receiving GM-CSF, infiltration by leukocytes but not lymphocytes was greater. Designs of increasing complexity are being currently explored [88]. A diversified prime and boost strategy using a prime with a recombinant vaccinia vector expressing CEA and the triad of costimulatory molecules (designated rV-CEA/TRICOM) and a boost with rF-CEA/TRICOM was more potent in inducing CEA-specific T-cell responses than the repeated use of rF-CEA/TRICOM alone. The addition of GM-CSF-enhanced CEA-specific T-cell responses. These studies demonstrate that the use of cytokines and diversified prime and boost regimens can be combined with the use of recombinant vectors [89, 90].

Replacing defective genes (p53, BRCA1, RB, p16) [35, 38]

Genes that are mutated or deleted in cancer include the cancer susceptibility genes p53 and BRCA1 [91]. Both p53 and BRCA1 appear to inhibit cancer cells that lack mutations in these genes, suggesting that the so-called gene correction strategies may have broader potential than initially believed [92]. p16, also called *MTS1* (multiple tumor suppressive gene 1) is known to be an important tumour suppressor gene especially in nonsmall cell lung cancer [93]. Extensive effort may have been put prematurely into large scale phase III trials which in essence confirmed the excellent tolerance of these vectors, with little clinical activity as single agents, strongly suggesting a need for review of concept [94]. Over 900 patients have been treated by gene transfer products (nonreplication-selective AdV p53, Aventis Pharma) over a period of 5 years. Three phase II studies in patients with recurrent squamous cell carcinoma of the head & neck testing different schedules and doses of administration resulted in stable disease in 26% of patients (NDDO meeting report, Valencia, Spain). No replication competent adenovirus was detected.

Enzymes and prodrugs (TK) [95]

Genetic prodrug activation therapy depends on the conditional expression of a gene encoding an enzyme capable of converting a nontoxic prodrug into an active cytotoxic agent. An alternative strategy is to exploit the transcriptional regulatory elements of genes that display tumour selective patterns of expression [44, 96]. Examples of tissue specific patterns are those of MUC1, CEA, PSA, thyroglobulin, and calcitonin whereas tumour selective patterns include HER2 FGFR4 and VEGF [97]. In a phase I clinical trial of direct intratumour injection of an HER2-promoter-dependent cytosine deaminase (CD) plasmid in patients with skin nodules of recurrent breast cancer, restriction of cytosine deaminase expression to tumour cells was documented. Combination of the MUC1 enhancer and HER2 promoters in pancreatic cancer that expressed both MUC1 and HER2 enhanced the level of expression as shown by cDNA microarray analysis. An adenoviral vector encoding the enzyme *E.coli* nitro-reductase (NR) which reduces the prodrug CB1954 to a powerful alkylating agent under the control of the CMV promoter in primary and secondary liver cancer had some activity in tumour cells which were resistent to Cisplatin. Synergy was shown with Doxorubicin, Cisplatin and Topotecan [98].

TK gene expression has been placed under the control of the alpha-fetoprotein promoter to enhance specificity for HCC cells and to diminish tk/gancyclovir toxicity to normal cells. While 80% of animals died and 20% were cured with the original vector, this modification dramatically increased survival and reduced treatment-related toxicity.

PITFALLS IN GENE THERAPY / IMMUNOTHERAPY OF CANCER

Difficulties encountered in clinical trial design using biologicals are manifold, including the definition of optimal dose, the absence of a correlation between maximally tolerated dose (MTD) and maximal efficacy, and the sequence and frequency of injections over time among others. In addition, the frequently advanced disease stage of patients under consideration means a vast heterogeneity of tumour cells is to be expected with a highly variable expression of tumour antigens by subclones. Moreover, the heterogeneity of the genetic background in a patient population may affect the outcome and the usefulness of a particular product may be difficult to define in particular since clinical benefit is achieved only in a small fraction of patients. Prospective statistical methodologies based on MTD and clinical response are not optimal for making decisions as to whether to develop or reject the gene therapy product. Combinations with reference treatments appear to give added benefit, but synchronising the timing of injection of live viruses with potentially immune suppressive chemotherapy, as well as uncertainty surrounding how to assess the relative contribution of each product separately renders such combinations problematic. It is also well documented that the immune system in late stage disease is compromized, as evidenced by lymphopenia, low circulating $CD4^+$ T lymphocytes, and a Th2 bias in cytokine secretion, resulting in a less efficacious immune response.

T cell dysfunction, defective dendritic cell maturation, and inflammation in cancer patients

T-cell dysfunction in cancer patients has been classified by 120 experts in the field as the number-1 criteria to be evaluated against clinical response. Hallmarks of T-cell dysfunction are absent IFN-γ production, defective T cell proliferative response, low and nonstimulable TCR z chain expression, decreased signalling in T cells (Lck), and low expression of nuclear transcription factors. Dysfunctional T cells appear to be provoked, at least in part, through inadequate stimulation by immature DC [99], lacking costimulatory molecule and CD40 ligand expression. It has been repeatedly demonstrated that tumour culture supernatants contain elements which can inhibit the functional maturation of DCs [1, 100, 101], and that dendritic cells taken from patients with a variety of solid tumours, including breast cancer, have an impaired ability to stimulate allogeneic T-lymphocytes. A number of cytokines, such as IL-10 [102], IL-6 [103, 104], MCS-F (CSF-1) [105] VEGF [106, 107, 108], and soluble IL-2 receptor [109], have been associated with immunosuppression and/or poor patient survival. Ménétrier-Caux et al [1] in a comparative study demonstrated that CSF-1 (macrophage colony stimulating factor) was the dominant immuno-suppressive cytokine in renal cell cancer cell lines. In particular, CSF-1 produced by renal cell carcinoma cell lines inhibited the differentiation of DCs from $CD34^+$ progenitor cells, resulting instead in monocytic cells with a potent phagocytic activity but lacking antigen presenting function. We were further able to show that the CSF-1 induced reduction in allostimulatory function may be mediated through an effect on class-II traffic [110]. Clearly this has implications for immune based therapies. Given its physiological role, CSF-1 is an obvious candidate in the generation of these effects. CSF-1 expression by tumours is associated with extensive macrophage infiltration both in animal, and human models. In a recent publication, Lin et al [111] reported that CSF-1 is a critical factor in tumour progression and metastasis, an effect mediated through recruitment of inflammatory macrophages to the tumour site. In a clinical gene therapy trial, using VV-MUC1-IL-2 to treat patients with breast cancer, 2 out of 4 patients with low CSF-1 serum levels and high $CD4^+$ numbers at the start of treatment responded to therapy, whereas none of the patients with high CSF-1 titers and low $CD4^+$ responded (submitted).

Anti-inflammatory agents in cancer prevention and treatment

The link between chronic inflammation and the subsequent development of cancer is well established, and there is increasing evidence that these effects are mediated, at least in part, through the production of proinflammatory cytokines and other mediators of inflammation [112]. Tumour cells, tumor associated macrophages, tumour infiltrating lymphocytes, and the tumour stroma itself, secrete factors such as TNF, VEGF, GM-CSF, IL-6, and IL-10 which promote tumour progression. Effects include angiogenesis, DNA damage, induction of T cell anergy, production of proteases, and bypass of the tumour suppressor protein p53 [113]. It is because of these deleterious effects of inflammation on cancer pathogenesis that researchers are increasingly looking for ways to modify inflammation as part of cancer treatment. Breaking this cycle of chronic inflammation and immune suppression could thereby render existing therapies more efficacious.

Mediators of inflammation implicated to date include cyclo-oxygenase-2 (COX-2), which is highly induced in many solid tumours [114, 115, 116, 117, 118, 119, 120]. A role for this enzyme in tumour progression, angiogenesis, and the inhibition of apoptosis has been established in animal models [121, 122]. Moreover, epidemiological studies have established that long-term intake of nonsteroidal anti-inflammatory drugs (NSAIDs), which inhibit the enzymatic activity of COX-2, reduces the relative risk of developing colorectal cancer [123]. As a result their use as adjuvant therapeutic agents in cancer clinical trials is currently under assessment.

NSAIDs also inhibit the expression of the nuclear transcription factor NF-κB, which regulates activation of specific genes encoding for diverse proteins involved in the inflammatory response and the host immune response. These include many different cytokines and chemokines, proteins involved in immune recognition, proteins involved in the control of cellular proliferation and apoptosis (c-IAP1, cIAP-2), and cell adhesion proteins (ICAM-1). Through the regulation of genes encoding for matrix metalloproteinase 9, tissue plasminogen activator, and ICAM-1, NF-κB may also play a role in tumour metastasis. High levels of NF-κB have been demonstrated in both haematological and solid tumours, including breast, ovarian, prostate, and colon cancers [124]. In addition, preliminary results suggest that inhibition of NF-κB in association with chemotherapy may be beneficial [125, 126].

FUTURE STRATEGIES FOR CANCER TREATMENT IN PATIENTS

The need to develop adequate trial designs, to choose precisely defined endpoints, and to use methodological strategies which compare favourably with established reference treatments were recently emphasized by M. Papaluca-Amati from the preauthorization unit at the Agency for the Evaluation of Medicinal products for human use in Europe (EMEA). A major obstacle to the pan European development of clinical gene therapy protocols is the multitude of national regulatory bodies and the frequent requirement for translation into at least one other language. Furthermore, according to Dr Papaluca-Amati, common legislation is sometimes rendered problematic by the clash between Saxon and Latin cultures, exemplified in the contrasting attitudes according to which "what's not forbidden is allowed for one, whilst what is not allowed is forbidden for the other."

Future clinical trial design and evaluation of genetic therapies

Gene therapy is still in its infancy, but significant accomplishments have been achieved. The ability to transfer genes safely and successfully into animals and patients has been established and rapidly expanding preclinical evidence suggests that gene therapy will yet deliver on its promise. So far clinical response to cancer vaccines has been infrequent, but the ability to target tumour cells specifically [127] together with interesting results using a variety of vectors and transgenes in early tumour models are intriguing.

The future of cancer treatment could lie in customized treatment [128], based on the molecular properties of the tumour, utilizing combinations of novel and conventional agents. The revolution in molecular methods has allowed the development of approaches whereby cancer-specific changes can be targeted, including mutation compensation for correction of cancer-associated defects and molecular chemotherapy for delivering toxic substances and specific small molecular inhibitors of abnormally activated pathways.

The choice of vector will depend on the result to be achieved. If the expected result is increased immunogenicity, then poxvirus or adenovirus vectors will be favoured. If durable gene transfer is the goal, lentiviral vectors or liposomal vectors are ideally suited. If selective targeting for molecular chemotherapy or viral lytic agents are to be used, selectively replicating adenoviruses are optimally used. Tissue-specific promoters can be engineered into the vector such that they will be expressed in the target tissue.

The choice of the insert will depend on whether correction of cancer-associated defects is molecular chemotherapy for delivering toxic substances or an enhanced immune response against one or several specific tumour antigens is to be engineered. In the latter case, it would be important to know whether tumour MHC class-I expression is adequate or low (suggesting for instance the need for IFN-γ transfer) and whether inflammatory macrophages predominate over dendritic cells (suggesting strategies to decrease inflammation). Synergy of viral vector-based approaches with standard therapies has been documented by a number of authors and diagnosis and correction of cancer associated molecular defects can enhance the effectiveness of standard treatments. Because p53 status influences the expression of microtubule-associated proteins and hence the sensitivity of a tumour to taxanes, it is likely that p53 gene transfer could be useful in taxane refractory patients [129]. Combinations of standard therapies are extremely interesting in preclinical studies and should find their way into early clinical studies [3, 130]. Ad-p53 transfer and Cisplatin administration to GLC-82 cells exerted substantially greater therapeutic effects than the single agent treatment alone [5]. Data from Nishizaki et al suggests that a combination of gene therapy, chemotherapy, and radiation therapy may be an effective strategy for human cancer treatment [131].

Methodological aspects remain to be addressed; while single agent phase I and phase II designs appear not to be productive, the tolerance and the toxicity profile of combinations still need to be evaluated in the first instance. While the MTD is unlikely to be the most active dose, it seems reasonable to test extremes of potentially effective dosages based on preclinical studies. A flexible design allowing progressive association with standard or third biological agents, based on preclinical results, should allow tolerance assessment and a subsequent increase in the number of patients creating a phase II study if a real advantage is suggested. Multiple point surveys of molecular markers at baseline and following therapeutic interventions should shed light on the dynamic aspects of the tumour-host interactions. Finally, the development of tools to evaluate tumour-induced immune escape or drug resistance should be helpful in curbing more advanced disease. A continuous interaction with basic scientists involved in preclinical studies should permit us to define RNA expression profiles predictive of a clinical response. Statistical innovations for clinical trials include the minimax design [132] which assures the patients safety while allowing flexibility in the study.

Immunological monitoring has recently been reviewed by a group of 120 experts in the field [133]. The frequent discrepancy between clinical and immunological response in past trials was underlined and the advantages and disadvantages of the different methods (ease of assay, precision of the test, reliability of the measure) were commented upon. It is evident that immunological response documentation is most relevant at the tumour site as opposed to in peripheral PBMC and to this end, noninvasive imaging of vectors and immune competent cells might not be as futuristic as it first sounds. In vaccine based therapies, a better definition of the patients' genetic polymorphisms and immunological background should narrow the predictive window for an effective immune response.

CONCLUSIONS

Rapid clinical advances in gene therapy of cancer are to be expected. Progress will be achieved through the selection of the most likely effective therapy combinations based both on the molecular analysis of tumours as well as on preclinical studies aiming to correct given biological defects. There is little doubt that we are at the beginning of a new era in cancer treatment.

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