Review

Gene Therapy of Cancer: Induction of Anti-Tumor Immunity

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Many malignancies lack satisfactory treatment and new therapeutic options are urgently needed. Gene therapy is a new modality to treat both inherited and acquired diseases based on the transfer of genetic material to the tissues. Different gene therapy strategies against cancers have been developed. A considerable number of preclinical studies indicate that a great variety of cancers are amenable to gene therapy. Among these strategies, induction of anti-tumor immunity is the most promising approach. Gene therapy with cytokines has reached unprecedented success in preclinical models of cancer. Synergistic rather than additive effects have been demonstrated by combination of gene transfer of cytokines/chemokines, costimulatory molecules or adoptive cell therapy. Recent progress in vector technology and in imaging techniques allowing *in vivo* assessment of gene expression will facilitate the development of clinical applications of gene therapy, a procedure which may have a notorious impact in the management of cancers lacking effective treatment. *Cellular & Molecular Immunology*. 2004;1(2):105-111.

Key Words: gene therapy, anti-tumor immunity, cytokines, costimulatory molecules, dendritic cell

Introduction

Despite the impressive progress in biomedical sciences during the last decades, the therapy of many malignancies remains unsatisfactory (1, 2). There is evidently an urgent need for efficient alternative therapeutic approaches. In recent years gene therapy has emerged as a new and promising method to treat cancers (3-5). The underlying principle is based on the introduction of genetic material into cells in order to generate a curative biological effect (6). Gene therapy is not limited to hereditary diseases but can be used for a broad variety of different acquired diseases like infections, degenerative disorders and cancer (3-6). The most challenging issues for a successful application of gene therapy to human diseases concern 1) the choice of the relevant therapeutic gene, 2) the choice of promoter and regulatory sequences driving the expression of the transgene and 3) the vector used for the delivery of the transgene into cells. Promoter, regulatory elements and vector characteristics determine the transduction efficacy (that is the number of target cells expressing the transgene and the intensity of gene expression per cell), the specificity of the transduction, the time of transgene expression, the host's immune response against the vector and eventually the undesired side effects. At present important efforts are focused on the search for vectors with less toxicity and prolonged and controlled transgene expression thereby widening the potential application of gene therapy to a high spectrum of medical fields.

This article deals with the main features of gene therapy and focuses on recent gene therapeutic strategies for the treatment of cancer based on induction of anti-tumor immunity.

Gene delivery systems

High efficient gene transfer is essential for gene therapy. A number of gene transfer methods have been developed. They are divided into two catalogues: viral and non-viral vectors.

Viral vectors

The viral vectors are based on the genetic modification of viruses. So far viral vectors are the most efficient means for the transfer of foreign genes into target cells (7). Murine retroviruses are single-stranded RNA viruses that consist of two long-terminal repeat sequences and structure genes (8, 9). Foreign genes can be inserted into retroviral vectors that have been deleted of structural genes. Retroviral vectors transducer dividing cells with high efficiency *in vitro* cell culture and stably integrate into the host genome (8, 9). This property allows retroviruses to be used for *in vitro* transduction of tumor cells, which can afterwards be injected into the host. This *ex-vivo* gene transfer method has been used to transduce tumor cells

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Abbreviations: HBV, Hepatitis B virus; AFP, alpha-fetoprotein; AAV, adeno-associated virus; TNF, tumor necrosis factor; PET, Positron Emission Tomography.

very efficiently with different foreign genes (8). One important disadvantage of retroviruses is the low titer of the vector that can be obtained with current methodologies, a fact which limits the *in vivo* gene transfer using retroviruses.

The adenoviruses are double-stranded DNA viruses with a natural tropism for the liver when administrated systemically (10, 11). The first generation adenoviral vectors are constructed with deletion of the E1 gene, a manoeuvre that renders the virus replication-deficient and eliminates the possibility of transforming host-cells. Adenoviral vectors are stable and can be easily purified at high titer. They transduce dividing and non-dividing cells in a variety of tissues with high efficiency (10, 11). These vectors have been used to transduce foreign genes into different types of tumor cells (12-14). In contrast to retroviral vectors, adenoviral vectors do not integrate their genome into host chromosomes being transient the expression of the transgene. The disadvantage is that they induce immune responses against adenoviral antigens expressed on transduced cells, a fact which contributes to limit the duration of transgene expression (15). However, the short-term expression of the therapeutic gene may be an advantage for treatment of cancer, since the expression of the transgene may no longer be needed after killing tumoral cells with suicide genes or after stimulation of antitumoral responses with cytokines. Second and third generation adenoviruses include changes in the E2-region or deletion of E4, respectively (11). Recently, the so-called gutless adenovirus has been developed. These vectors lack all the viral sequences except the inverted terminal repeats (ITR) and packaging signal (16, 17). As a result they have low toxicity, very high capacity and do not elicit immunological responses against the vector thus allowing prolonged transgene expression (16, 17). Other research avenues aim to construct tumor specific oncolytic adenoviruses by modifying the E1A and/or E1B regions of adenoviruses (18). In this way, conditional replicating adenoviruses can be constructed by placing the E1 gene under the control of a tumor specific promoter such as alpha-fetoprotein (AFP) or TERT promoter. Since AFP expression can be reactivated in hepatocellular carcinoma cells or TERT is activated in the most of tumor cells, this would restrict adenovirus replication and cytopathic effect only to these tumoral cells (19, 20).

Adeno-associated virus (AAV) is a safe vector, because the virus is naturally non-pathogenic and replication deficient (21). Previous work has shown that rAAV vector can transduce a variety of cells. However, rAAV transduces some kinds of cells with low efficiency in the absence of helper virus (22). It has been reported that treatment of cells with DNA damaging agents such as etoposide and γ-irradiation, caused a marked increase *in vitro* cell transduction by rAAV (22). This enhancement was related to conversion of single-stranded AAV DNA into transcriptional active double-stranded forms of the AAV vector (22). Our studies have shown that rAAV can infect human, rat and mouse HCC cells and that adenovirus or DNA damaging agents (γ-irradiation and etoposide) are very efficient in enhancing transgene expression *in vitro* and *in vivo* (23).

Lentiviral vectors have emerged as a promising new tool in gene therapy. Lentiviruses belong to the retrovirus family, but they are able to infect both dividing and non-dividing cells. After injection into rodent muscle, brain, liver or pancreatic islet cells the lentiviral vector permits a sustained expression of the transgene for over six months (24). Furthermore, it seems that there is no potent humoral response directed against this vector. So, at present, lentiviral vectors seem to offer an excellent opportunity for *in vivo* gene delivery with sustained expression (25).

Hepatitis B virus (HBV) has been investigated as a carrier for transfer of therapeutic genes and transgene could be expressed in HCC cells based on this vector (26). Baculovirus has been used as a vector for efficient delivery of genes into cultured cells with a strong preference for hepatocytes of different origin with high level of expression (27). In addition to these viruses, the vectors based on herpes simplex virus, SV40, and alphavirus have been explored in various studies.

Although viral vectors hold promise for cancer gene therapy, attempts at re-treating an immunocompetent host with same viral vector may result in diminished transgene expression. This is because of neutralizing antibodies against viral antigens, which are produced after the first injection of the vector. These antibodies can eliminate the viral particles after a second dose of the vector (3). However, this might not be always the case. A recent study showed that neutralizing antibodies to adenovirus did not reduce transgene expression in the tumor cells when the adenoviral vector was repeatedly injected locally into the tumor nodules, while markedly reduced the access of the virus to non-tumoral liver tissue and the expression of the transgene in normal hepatocytes (3).

Non-viral vectors

Apart from vectors based on recombinant viruses transgenes can be introduced into plasmids and these can be administered as naked DNA or complexed to different compounds such as cationic liposomes or poly-lysine. Naked DNA plasmids can be taken up efficiently by muscle or liver cells (28, 29). For the transduction of other cells plasmid DNA should be complexed to facilitate incorporation and expression into the cells (30). One strategy to provide non-viral vectors with affinity for certain tissues or organs is the construction of complexes of DNA with ligands for specific receptors such as the asialoglycoprotein receptor, whose expression is limited to hepatocytes and HCC cells (31). Other ligands that have been used in similar conjugates include insulin, lectin and transferrin. Similarly, antibodies could be incorporated to liposomes to endow these vectors with the tropism conferred by the antibodies. The recently developed "gene gun" device uses DNA-coated gold particles that are accelerated by pressurized helium gas to supersonic velocity for DNA transfer into living cells (3).

Non-viral vectors offer some advantages as compared to viral vectors. They can transfer larger pieces of DNA of interest, they are weakly immunogenic and easier to use and to produce than viral vectors. However, a disadvantage is that gene transfer efficiency is low and the expression of the transgene is of short duration (3).

The choice of the appropriate vector depends on characteristics of target cells or tissues and specific gene therapy strategies that will be adopted.

Gene transfer of cytokines and chemokines for cancer therapy

Malignant cells in tumors harbor mutated or overexpressed protein sequences that can be recognized by cell-destroying mechanisms of the immune system (32). Unfortunately, the basal immune response against tumor antigens is absent or very weak in most cases. Tumors can evade antitumoral immunity due to poor antigenicity of tumoral antigens or to the lack of expression of MHC molecules by the transformed cells and/or to the secretion of immunosuppressive factors (such as TGF-β or VEGF) by the tumor (32, 33). Gene transfer of immunostimulatory cytokines or chemokines can overcome the immune tolerance against tumoral antigens and facilitate tumor rejection (34, 35). Different cytokines or chemokines (IL-2, IL-4, IL-6, IL-7; IL-12, INF-γ, TNF-α, GM-CSF) have been used to activate antitumoral activity. Basically, the following approaches have been applied: the *in vivo* injection of vector expressing cytokines or chemokines into tumor lesions and the *ex vivo* transduction of DCs or tumor cells with vector expressing cytokines or chemokines (35, 36). The overexpression of cytokines or chemokines causes tumor infiltration by host leukocytes, and divergent anti-tumor activity can be induced depending on the cytokines or chemokines and tumor model used. Here we focus on some of potential cytokines that have been reported to exert strong anti-tumor activity.

Interluekin-12 (IL-12)

IL-12 is among the cytokines with most potent antitumoral activity. IL-12 acts by inducing a Th1 type of response, activating NK cells and cytotoxic T lymphocytes (37). It also inhibits tumoral neoangiogenesis and enhances the expression of adhesion molecules on endothelial cells thus facilitating the traffic of lymphocytes to the tumor (37, 38). This cytokine, however, is toxic when administered systemically as a recombinant protein (39). The rational for IL-12 gene therapy is to allow local production of the cytokine at the tumor site thus achieving high intratumour or peritumoral levels with low serum concentration (34, 35). This procedure would therefore maximize the antitumoral effect of the cytokine while minimizing its systemic toxicity.

It has been shown that intratumoral administration of a recombinant adenovirus encoding IL-12 (AdIL-12) to animals with different types of carcinoma caused complete tumor eradication in most of animals and increased long-term survival (40-42). Moreover, in rats with two tumor nodules the injection of AdIL-12 in only one tumor nodule caused regression of the distant tumor (42). This effect has been attributed to the fact that a proportion of the adenovirus injected intratumorally escapes to the general circulation and, due to the intense hepatotropism of adenoviruses, the circulating virions infect the hepatic tissue surrounding the tumor nodules (43). The IL-12 produced by the tumor and by hepatocytes adjacent the neoplastic nodules strongly activates NK cells, induces specific anti-tumor immunity, stimulates expression of adhesion molecules in the tumor vessels and displays a powerful anti-angiogenic effect with resulting tumor regression (42, 43). AdIL-12 given by intra-hepatic arterial route was also shown to be efficient in the treatment of a very aggressive model of multifocal hepatocellular carcinoma in rats (induced by carcinogen) causing a significant reduction of tumor burden and prolongation of survival (42).

Costimulatory molecules (B7 and CD40 ligand)

Lymphocyte activation requires recognition of an MHC/antigen complex and costimulatory signals. The encounter of T cells with the antigen in the absence of these costimulatory signals leads to T cell unresponsiveness or apoptotic cell death (31). Many tumours express class I antigens but lack the B7 costimulatory molecules that are expressed on antigen presenting cells (APCs) and thus they may fail to stimulate an anti-tumor response. The importance of B7 in antitumoral immunity is illustrated by reports showing that immunization of mice with B7-expressing tumor led to the regression of established B7-negative tumours (44). Results from Tatsumi et al. (45) demonstrated that transfection of human HCC cell line Hep3B with a plasmid containing human B7-1 gene substantially augmented primary cytolytic activity against parental Hep3B cells. We also found that mouse HCC cells partially lost their tumorigenicity after transduction with retroviral vector encoding B7-1 (46). These findings suggest that tumoral immunogenicity can be enhanced by transfer of B7-1 gene into HCC cells.

CD40L is a member of the tumor necrosis factor (TNF) family, which is expressed on activated T cells and binds to CD40 present on the membrane of antigen-presenting cells (APCs) (47). CD40-CD40L interaction plays a crucial role in the activation of APC and in the initiation of both humoral and cellular immune responses. The rationale for transducing tumor cells with CD40L is to convert these cells into stimulators of APCs, an effect leading to enhanced presentation of tumor antigens to T cells and activation of anti-tumor immune responses. In fact, CD40-CD40L interaction has been demonstrated to overcome tumor specific $CD4^+$ and $CD8^+$ tolerance and induce anti-tumor immunity. Treatment of lymphoma with CD40 antibody induces a rapid cytotoxic T cell response independent of T helper cells, leading to eradication of the lymphoma and protection against tumor cell rechallenge. *Ex vivo* transduction of tumor cells with CD40L gene was able to induce anti-tumor immunity against different tumor cell lines in subcutaneous mouse models (48). *In vivo* transfer of CD40L gene mediated by adenoviral vector led to complete tumor eradication and long-term survival in different types of tumors (49-51). We have found that gene therapy with adenovirus expressing CD40L induced strong lymphocytic infiltration of the tumoral tissue and increased apoptosis of malignant cells. The observed antitumoral effect was mediated by $CD8^+$ T cells and was associated with increased IL-12 serum levels and enhanced NK cells activity. Animals that eliminated the tumor after *in vivo* gene therapy developed protective anti-tumor immunity being resistant to rechallenge with neoplastic cells. Toxicity of the therapy with AdCMVmCD40L was slight with only a transient increase in the level of serum transaminases and minor lymphocyte infiltration of normal liver tissue (51).

Genetic engineering dendritic cells (DCs) for cancer therapy

The antigen presenting cells play an important role in the generation and regulation of the immune response against tumors. Dendritic cells are professional and very effective antigen presenting cells that can be manipulated *in vitro* to present tumor antigens by incubation with antigen-derived peptides or tumor lysates or by transfer of genes coding for relevant antigens such as alpha-fetoprotein or for cytokines involved in self-activation and proliferation such as IL-12. Subcutaneous or intra-lymph node administration of these manipulated dendritic cells can be used to enhance anti-tumoral activity (36).

Two groups realized that IL-12 transfected dendritic cells, either with adenovirus or retrovirus, when injected inside malignant tissue induced potent anti-tumor immune responses that resulted in complete eradication in many models (52, 53). In this case, tumor antigens were acquired by DCs in the tumor environment and transported avidly to lymph nodes. IL-12 carries out its function by promoting the effect of several types of lymphocytes among which the most prominent role is played by $CD8^+$ T-cells. In addition, IL-12 has autocrine effects on DCs (54) and locally activates NK cells (55). Migration of DCs to lymphoid organs is a phenomenon induced by direct contact of DCs with tumor cells that cause the expression of CCR7 on the membrane of DCs as shown in cocultures (56). Effects of intratumor injection of IL-12-producing DCs have been confirmed in mice by at least two other groups (57, 58).

After intratumor injection, antigen uptake can be mediated by phagocytosis of malignant cell debris released from those cells dying by necrotic or apoptotic processes (59, 60). Mechanical damage by the injection seems to be a potential inducer of cell death. Besides, tumor antigen release can be caused by dendritic cells that have been shown to be mediators of direct cellular cytotoxicity (61, 62). In addition, they could also enhance, when secreting IL-12, NK-mediated cytolysis (63). Studies analyzing requirements for CTL *in vitro* priming have shown a clear role for NK cells in favouring the release of processable tumor antigens to be presented by dendritic cells (64). In addition to IL-12, other cytokines work in this setting of intratumor injection of dendritic cells transfected to express their genes. This is the case of IL-7 (65, 66), IL-2 to some extent (58) and SLC (67). Expression of CD40L by adenoviral transfection into DCs is also effective when those DCs are injected intratumorally (68). In this case efficacy depends on dendritic cell activation by direct membrane contact among dendritic cells (some of them artificially expressing CD40L). It is still unclear whether a combination of some of these factors transfected together would display better immune-promoting effects.

Synergistic effect of combined therapy

Preclinical studies have indicated that viral vectors expressing cytokines induced strong anti-tumor immunity. However, anti-tumor activity was quite limited when these vector were used in patients with different types of cancers (69-71). Thus, new approaches that combine cytokines or chemokines with different properties on activation of immune system are being investigated. The group of Graham pioneered the combination of two adenoviruses one encoding IL-2 and the other IL-12, which were coinjected into tumor nodules. As a result of synergistic effects, they observed over 60% complete regressions of established mammary carcinomas and induction of anti-tumor CTL activity (72). There are recent data with the close related cytokine IL-15, that also synergizes with IL-12 as seen with stable transfectants co-expressing both cytokines in human lung cancer cells xenografted in nude mice. The anti-tumor mechanisms studied in this artificial system are independent of the immune system including NK cells but unravel the intriguing involvement of neutrophils in the rejections (73).

Interleukin-18 was also identified as a potent inducer of IFN. Importantly, IL-18 upregulates the expression of IL-12 receptors. In a poorly immunogenic tumor (MC205), it was observed that there was a clear synergy in the anti-tumor effects mainly mediated by NK cells in this case (74). However the administration of recombinant proteins has also a threatening synergistic effect at inducing lethal levels of IFN-γ.

Genes encoding lymphocyte attracting chemokines can be also successfully combined with IL-12. The approach has shown remarkable efficacy with adenoviruses encoding for the chemokines lymphotactin (75), IP-10 (76) and MIP-3α (77). Lymphotactin by itself induced lymphoid infiltration but that was clinically meaningless since transplanted mammary carcinomas progressed. Similarly IP-10 transduced by adenovirus into experimental colon carcinomas had very little anti-tumor effect but caused hemorragic necrosis consistent with its antiangiogenic properties. Combined therapy with Lymphotactin, IP-10 or MIP-3α and IL-12 showed great potency in a scenario where $CD4^+$ T-cells, $CD8^+$ T-cells and NK cells were showing prominent roles. One order of magnitude reduction in the dose of the IL-12 adenovirus eradicated the tumors when combined with IP-10, thus reducing potential IL-12 toxicity (76). Interestingly, colocalization of both adenoviruses in the same tumor nodule was required for the systemic anti-tumor effect that was not seen if given separately into two distantly implanted malignant nodules (76).

Apart from soluble mediators certain membrane attached proteins are important to ignite and sustain the immune response. Such molecules are typically restricted to the surface of professional antigen presenting cells and are transfected to tumors in an attempt to make them more immunogenic.

B7 molecules are an expanding family of proteins interacting with counterreceptors on T-cells, which provide signals that modulate the immune response. Transfection of B7-1 (CD80) and B7-2 (CD86) makes tumor cells more immunogenic and combination with IL-12 has been proved beneficial in certain models (78), but not in others (46).

4-1BB-Ligand is a TNF family member expressed on the membrane of mature dendritic cells and other cell types that upon transfection enhances the immunogenecity of tumors. This molecule interacts with 4-1BB which is a surface differentiatiation antigen restricted to activated T-cells and NK cells. Intratumor injections with adenovirus encoding 4-1BB-Ligand and IL-12 mutually potentiated their effect against large and well established liver

metastasis of experimental colon cancer (79). In addition tumor immunity can be triggered by agonistic monoclonal antibodies acting on 4-1BB. Regimes of intratumor gene transfer of IL-12 and anti-4-1BB mAbs also show synergizing properties (80), which are more potent than those observed with the natural ligand. This is explained because antibodies distribute widely reaching and stimulating every available 4-1BB$^+$ lymphocyte, in contrast to the membrane bound 4-1BB natural Ligand.

IL-12 induces CTL expansion that facilitates the culture of effector cells for adoptive cell therapy regimes. Surprisingly IL-12 synergy with adoptive T-cell therapy is not restricted to simplify the obtention of CTLs, but also operates at the effector level. These phenomena were unveiled with experiments observing the outcome of tumor-bearing mice in which some malignant nodules were adenovirally transduced with IL-12 and subsequently given anti-tumor CTLs intravenously (38). Later experimentation has shown a key role for inflammatory adhesion molecules for CTL that are induced on the endothelium of peritumoral capilaries by virtue of IL-12 gene transfer (43).

Outlook

Gene therapy has emerged as a powerful and very plastic tool to regulate biological functions in diseased tissues with application in practically all medical fields. An increasing number of experimental and clinical studies clearly underline the important role of genes to serve as a curative drug in future. However, intense research is needed to evaluate the potential of gene therapy, to improve the efficacy and minimize the toxicity of the procedure. Future efforts should be directed to: 1) the development of vectors with high transduction efficiency, high transgene capacity, and acceptable toxicity profile; 2) development of systems allowing desired duration and regulation of the gene expression; 3) identification of the ideal therapeutic gene or gene combinations for each therapeutic indication; 4) testing of different routes for vector administration and 5) development of innovative methods for large-scale industrial production to allow accessible prices for wide access to these new drugs. Intensive testing in animal models to ensure drug safety and functionality is needed before contemplating clinical applications. On the other hand, carefully designed clinical trials under strict regulatory conditions, can provide very useful information on the potential of gene therapy in humans, specially in devastating diseases lacking effective therapy, as in cases of non-resectable chemo-resistant tumors. New imaging techniques such as Positron Emission Tomography (PET) have the potential of detecting and quantitating the function of the transferred gene to the tissues *in vivo*, thus providing a powerful tool to guide the progress of clinical gene therapy. In summary, genes can be transferred to tissues and the transferred gene is functional *in vivo*; this opens a new field of medical applications, which is just at its beginning.

References

1. Okuda K. Hepatocellular carcinoma. J Hepatol. 2000;32:225-237.
2. Llovet JM, Bruix J. Systematic review of randomized trials for unresectable hepatocellular carcinoma: Chemoembolization improves survival. Hepatology. 2003;37:429-442.
3. Qian C, Drozdzik M, Caselmann WH, Prieto J. The potential of gene therapy in the treatment of hepatocellular carcinoma. J Hepatol. 2000;32:344-351.
4. Mohr L, Geissler M, Blum HE. Gene therapy for malignant liver disease. Expert Opin Biol Ther. 2002;2:163-175.
5. Sangro B, Qian C, Schmitz V, Prieto J. Gene therapy of hepatocellular carcinoma and gastrointerstinal tumors. Ann N Y Acad Sci. 2002;963:6-12.
6. Mulligan RC. The basic science of gene therapy. Science. 1993;260:926-932.
7. Kay MA, Glorioso JC, Naldini L. Viral vectors for gene therapy: the art of turning infectious agents into vehicles of therapeutics. Nat Med. 2001;7:33-40.
8. Gordon EM, Anderson WF. Gene therapy using retroviral vectors. Curr Opin Biotechnol. 1995;6:611-616.
9. Grossman M, Rader DJ, Muller DW, et al. A pilot study of ex vivo gene therapy for homozygous familiar hypercholesterolaemia. Nat Genet. 1994;6:335-341.
10. Graham FL. Adenovirus vectors for high-efficiency gene transfer into mammalian cells. Immunol Today. 2000;21:426-428.
11. Benihoud K, Yeh P, Perricaudet M. Adenovirus vectors for gene delivery. Curr Opin Biotechnol. 1999;10:440-447.
12. Zhang WW. Development and application of adenoviral vectors for gene therapy of cancer. Cancer Gene Ther. 1999;6:113-118.
13. Qian C, Bilbao R, Bruña O, Prieto J. Induction of sensitivity to ganciclovir in human hepatocellular carcinoma cells by adenovirus-mediated gene transfer of herpes simplex virus thymidine kinase. Hepatology. 1995;22:118-123.
14. Qian C, Iodate M, Bilbao R, et al. Gene transfer and therapy with adenoviral vector in rats with diethylnitrosamine-induced hepatocellular carcinoma. Hum Gene Ther 1997;8:349-358.
15. Yang Y, Jooss KU, Su Q, Ertl HC, Wilson JM. Immune responses to viral antigens versus transgene product in the elimination of recombinant adenovirus-infected hepatocytes *in vivo*. Gene Ther. 1996;3:137-144.
16. Kochanek S. High-capacity adenoviral vectors for gene transfer and somatic gene therapy. Hum Gene Ther. 1999;10:2451-2459.
17. Kim IH, Jozkowicz A, Piedra PA, Oka K, Chan L. Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. Proc Natl Acad Sci U S A. 2001;98:13282-13287.
18. Alemany R, Lai S, Lou YC, Jan HY, Fang X, Zhang WW. Complementary adenoviral vectors for oncolysis. Cancer Gene Ther. 1999;6:21-25.
19. Li Y, Yu DC, Chen Y, et al. A hepatocellular carcinoma-specific adenovirus variant, CV890, eliminates distant human liver tumors in combination with doxorubicin. Cancer Res. 2001;61:6428-6436.
20. Takakura M, Kyo S, Kanaya T, et al. Cloning of human telomerase catalytic subunit (hTERT) gene promoter and identification of proximal core promoter sequences essential for transcriptional activation in immortalized and cancer cells. Cancer Res. 1999;59:551-557.
21. Patijn GA, Kay MA. Hepatic gene therapy using adeno-associated virus vectors. Semin Liver Dis. 1999;19:61-69.
22. Alexander IE, Russell DW, Spence AM, Miller AD. Effects of gamma irradiation on the transduction of dividing and nondividing cells in brain and muscle of rats by adeno-associated virus vectors. Hum Gene Ther. 1996;7:841-850.
23. Peng D, Qian C, Sun Y, Barajas MA, Prieto J. Transduction of hepatocellular carcinoma (HCC) using recombinant adeno-

associated virus (rAAV): *in vitro* and *in vivo* effects of genotoxic agents. J Hepatol. 2000;32:975-985.
24. Kafri T, Blomer U, Peterson DA, Gage FH, Verma IM. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat Genet. 1997;17:314-317.
25. Galimi F, Verma IM. Opportunities for the use of lentiviral vectors in human gene therapy. Curr Top Microbiol Immunol. 2002;261:245-254.
26. Chaisomchit S, Tyrrell DL, Chang LJ. Development of replicative and nonreplicative hepatitis B virus vectors. Gene Ther. 1997;4:1330-1340.
27. Sandig V, Hofman C, Steinert S, Jennings G, Schlag P, Strauss M. Gene transfer into hepatocytes and human liver tissue by baculovirus vectors. Hum Gene Ther. 1996; 7:1937-1945.
28. Wolff JA, Malone RW, Williams P, et al. Direct gene transfer into mouse muscle *in vivo*. Science. 1990;247:1465-1468.
29. Hickman MA, Malone RW, Lehmann-Bruinsma K, et al. Gene expression following direct injection of DNA into liver. Human Gene Ther. 1994;4:1477-1483.
30. Crespo J, Blaya C, Crespo A, Aliño SF. Long-term expression of the α1-antitrypsin gene in mice employing anionic and cationic liposome vectors. Biochem Pharmacol. 1996;51: 1309-1314.
31. Wu GY, Wu CH. Receptor mediated gene delivery and expression *in vivo*. J Biol Chem. 1988;263:14621-14624.
32. Roth C, Rochlitz C, Kourilsky P. Immune response against tumors. Adv Immunol. 1994;57:281-351.
33. Gabrilovich DI, Chen HL, Girgis KR, et al. Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. Nat Med. 1996;2: 1096-1103.
34. Musiani P, Modesti A, Giovarella M, et al. Cytokine, tumor-cell death and immunogenicity: a question of choice. Immunol Today. 1997;18:32-36.
35. Melero I, Mazzolini G, Narvaiza I, Qian C, Chen L, Prieto J. IL-12 gene therapy for cancer: in synergy with other immunotherapies. Trends Immunol. 2001;22:113-115.
36. Tirapu I, Rodriguez-Calvillo M, Qian C, et al. Cytokine gene transfer into dendritic cells for cancer treatment. Curr Gene Ther. 2002;2:79-89.
37. Trinchieri G. Interleukin-12: a cytokine at the interface of inflammation and immunity. Adv Immunol. 1998;70:83-243.
38. Mazzolini G, Qian C, Narvaiza I, et al. Adenoviral gene transfer of interleukin 12 into tumors synergizes with adoptive T cell therapy both at the induction and effector level. Hum Gene Ther. 2000;11:113-125.
39. Leonard JP, Sherman ML, Fisher GL, et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-γ production. Blood. 1997;90:2541-2548.
40. Caruso M, Pham-Nguyen K, Kwong YL, et al. Adenovirus-mediated interleukin-12 gene therapy for metastatic colon carcinoma. Proc Natl Acad Sci U S A. 1996;93:11302-11306.
41. Mazzolini G, Qian C, Xie X, Sun Y, Lasarte J, Drozdzik M Prieto J. Tumor regression and long-term protection for colon cancer after intratumoral injection of adenovirus expressing IL-12. Cancer Gene Ther. 1999;5:514-522.
42. Barajas M, Mazzolini G, Genové G, et al. Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus coding for interleukin-12 (IL-12). Hepatology. 2001;33:52-61.
43. Mazzolini G, Narvaiza I, Bustos M, et al. Alpha(v)beta(3) integrin-mediated adenoviral transfer of interleukin-12 at the periphery of hepatic colon cancer metastases induces VCAM-1 expression and T-cell recruitment. Mol Ther. 2001;3: 665-672.
44. Chen L, Ashe S, Brady WA, et al. Costimulation of anti-tumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD8 and CTLA-4. Cell. 1992;71:1093-1102.
45. Tatsumi T, Takehara T, Katayama K, Mochizuki K, Yamamoto M, Kanto T. Expression of costimulatory molecules B7-1 (CD80) and B7-2 (CD86) on human hepatocellular carcinoma. Hepatology. 1997; 25:1108-1114.
46. Sun Y, Qian C, Peng D, Prieto J. Gene transfer to liver cancer cells of B7-1 plus interleukin 12 changes immunoeffector mechanisms and suppresses helper T cell type 1 cytokine production induced by interleukin 12 alone. Hum Gene Ther. 2000;11:127-138.
47. Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. Annu Rev Immunol. 1998;16:111-135.
48. Grossmann ME, Brown MP and Bernner MK. Anti-tumor responses induced by transgenic expression of CD40 ligand. Hum Gene Ther. 1997;8:1935-1943.
49. Kikuchi T, Crystal RG. Anti-tumor immunity induced by in vivo adenovirus vector-mediated expression of CD40 ligand in tumor cells. Hum Gene Ther. 1999;10:1375-1387.
50. Sun Y, Peng D, Lecanda J, et al. *In vivo* gene transfer of CD40 ligand into colon cancer cells induces local production of cytokines and chemokines, tumor eradication and protective anti-tumor immunity. Gene Ther. 2000;7:1467-1476.
51. Schmitz V, Barajas M, Wang L, et al. Adenovirus mediated CD40 ligand gene therapy in a rat model of orthotopic hepatocellular carcinoma. Hepatology. 2001;34:72-81.
52. Melero I, Duarte M, Ruiz J, et al. Intratumoral injection of bone-marrow derived dendritic cells engineered to produce interleukin-12 induces complete regression of established murine transplantable colon adenocarcinomas. Gene Ther. 1999;6:1779-1784.
53. Nishioka Y, Hirao M, Robbins PD, Lotze MT, Tahara H. Induction of systemic and therapeutic anti-tumor immunity using intratumoral injection of dendritic cells genetically modified to express interleukin 12. Cancer Res. 1999;59: 4035-4041.
54. Grohmann U, Belladonna ML, Bianchi R, et al. IL-12 acts directly on DC to promote nuclear localization of NF-κB and primes DC for IL-12 production. Immunity. 1998;9:315-323.
55. Rodriguez-Calvillo M, Duarte M, Tirapu I, et al. Upregulation of natural killer cells functions underlies the efficacy of intratumorally injected dendritic cells engineered to produce interleukin-12. Exp Hematol. 2002;30:195-204.
56. Hirao M, Onai N, Hiroishi K, et al. CC chemokine receptor-7 on dendritic cells is induced after interaction with apoptotic tumor cells: critical role in migration from the tumor site to draining lymph nodes. Cancer Res. 2000;60:2209-2217.
57. Furumoto K, Arii S, Yamasaki S, et al. Spleen-derived dendritic cells engineered to enhance interleukin-12 production elicit therapeutic anti-tumor immune responses. Int J Cancer 2000;87:665-672.
58. Akiyama Y, Watanabe M, Maruyama K, Ruscetti FW, Wiltrout RH, Yamaguchi K. Enhancement of anti-tumor immunity against B16 melanoma tumor using genetically modified dendritic cells to produce cytokines. Gene Ther. 2000;7:2113-2121.
59. Melero I, Vile RG, Colombo MP. Feeding dendritic cells with tumor antigens: self-service buffet or a la carte? Gene Ther. 2000;7:1167-1170.
60. Larsson M, Fonteneau JF, Bhardwaj N. Dendritic cells resurrect antigens from dead cells. Trends Immunol. 2001;22: 141-148.
61. Chapoval AI, Tamada K, Chen L. *In vitro* growth inhibition of a broad spectrum of tumor cell lines by activated human dendritic cells. Blood. 2000;95:2346-2351.
62. Fanger NA, Maliszewski CR, Schooley K, Griffith TS. Human dendritic cells mediate cellular apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). J Exp Med. 1999;190:1155-1164.
63. Fernandez NC, Lozier A, Flament C, et al. Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses *in vivo*. Nat Med. 1999;5:405-

411.
64. Tanaka F, Hashimoto W, Okamura H, Robbins PD, Lotze MT, Tahara, H. Rapid generation of potent and tumor-specific cytotoxic T lymphocytes by interleukin 18 using dendritic cells and natural killer cells. Cancer Res. 2000;60:4838-4844.
65. Westermann J, Aicher A, Qin Z, et al. Retroviral interleukin-7 gene transfer into human dendritic cells enhances T cell activation. Gene Ther. 1988;5:264-271.
66. Miller PW, Sharma S, Stolina M, et al. Intratumoral administration of adenoviral interleukin 7 gene-modified dendritic cells augments specific anti-tumor immunity and achieves tumor eradication. Hum Gene Ther. 2000;11:53-65.
67. Kirk CJ, Hartigan-O'Connor D, Nickoloff BJ, et al. T cell-dependent anti-tumor immunity mediated by secondary lymphoid tissue chemokine: augmentation of dendritic cell-based immunotherapy. Cancer Res. 2001;61:2062-2070.
68. Kikuchi T, Moore MA, Crystal RG. Dendritic cells modified to express CD40 ligand elicit therapeutic immunity against preexisting murine tumors. Blood. 2000;96:91-99.
69. Stewart AK, Lassam NJ, Quirt IC, et al. Adenovector-mediated gene delivery of interleukin-2 in metastatic breast cancer and melanoma: results of a phase 1 clinical trial. Gene Ther. 1999;6:350-363.
70. Khorana AA, Rosenblatt JD, Sahasrabudhe DM, et al. A phase I trial of immunotherapy with intratumoral adenovirus-interferon-γ (TG1041) in patients with malignant melanoma. Cancer Gene Ther. 2003;10:251-259.
71. Bruno Sangro, Mazzolini G, Ruiz J, et al. A phase I clinical trial of intratumoral injection of an adenovirus encoding interleukin 12 for advanced digestive tumors. J Clin Oncol. In press.
72. Addison CL, Bramson JL, Hitt MM, Muller WJ, Gauldie J, Graham FL. Intratumoral coinjection of adenoviral vectors expressing IL-2 and IL-12 results in enhanced frequency of regression of injected and untreated distal tumors. Gene Ther. 1998;5:1400-1409.
73. Di Carlo E, Comes A, Basso S, et al. The combined action of IL-15 and IL-12 gene transfer can induce tumor cell rejection without T and NK cell involvement. J Immunol. 2000;165: 3111-3118.
74. Osaki T, Hashimoto W, Gambotto A, et al. Potent anti-tumor effects mediated by local expression of the mature form of the interferon-γ inducing factor, interleukin-18 (IL-18). Gene Ther. 1999;6:808-815.
75. Emtage PC, Wan Y, Hitt M, et al. Adenoviral vectors expressing lymphotactin and interleukin 2 or lymphotactin and interleukin 12 synergize to facilitate tumor regression in murine breast cancer models. Hum Gene Ther. 1999;10:697-709.
76. Narvaiza I, Mazzolini G, Barajas M, et al. Intratumoral coinjection of two adenoviruses, one encoding the chemokine IFN-γ-inducible protein-10 and another encoding IL-12, results in marked antitumoral synergy. J Immunol. 2000;164: 3112-3122.
77. Mazzolini G, Narvaiza I, Martinez-Cruz LA, et al. Pancreatic cancer escape variants that evade immunogene therapy through loss of sensitivity to IFNγ-induced apoptosis. Gene Ther. 2003;10:1067-1078.
78. Zitvogel L, Robbins PD, Storkus WJ, et al. Interleukin-12 and B7.1 co-stimulation cooperate in the induction of effective anti-tumor immunity and therapy of established tumors. Eur J Immunol. 1996;26:1335-1341.
79. Martinet O, Ermekova V, Qiao JQ, et al. Immunomodulatory gene therapy with interleukin 12 and 4-1BB ligand: long- term remission of liver metastases in a mouse model. J Natl Cancer Inst. 2000;92:931-936.
80. Martinet O, Divino CM, Zang Y, et al. T cell activation with systemic agonistic antibody versus local 4-1BB ligand gene delivery combined with interleukin-12 eradicate liver metastases of breast cancer. Gene Ther. 2002;9:786-792.