

Biologic therapy of liver tumors

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In most cases with multinodular hepatocellular carcinoma (HCC) and liver metastasis of digestive tumors, surgical or ablative interventions are not applicable and the prognosis is dismal, due to the poor response of these tumors to chemotherapy [1,2]. Until very recently, fluorouracil-based regimens were the standard therapy for unresectable metastatic colorectal cancer, but a number of new agents have shown promising activity, including irinotecan, oxaliplatin, raltitrexed, and capecitabine. In fact, adding either irinotecan [3] or oxaliplatin [4] to biomodulated fluorouracil prolongs median survival significant though modestly (3 months and 2 months, respectively). Other strategies, such as chronomodulated schedules based on oxaliplatin-5-fluorouracil/folinic acid, increase the survival of patients that were rescued for surgery after the administration of these chemotherapeutic agents [5].

The great advances in the understanding of tumor biology, combined with progress in molecular and cell biology, have opened novel avenues to treat cancer. In addition to surgery, percutaneous ablation, chemotherapy and radiotherapy, and other procedures, which can be collectively named biological therapies, have been considered to control hepatic and digestive tumors. These therapies are directed to activate antitumoral immune responses, or to interfere with the basic biological requirements for the tumor to grow.

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Biological therapies for liver cancer

Hormonal intervention

Estrogen receptors are frequently present in the nuclei of HCC cells. Tamoxifen, an antiestrogen compound, was claimed to prolong survival in early clinical trials conducted in the 1980s; however, placebo-controlled trials showed that administration of tamoxifen to patients with advanced HCC does not affect their outcome. Androgen receptors are even more frequently detected in malignant hepatocytes, and correlate with the aggressiveness of the illness. Yet neither flutamide (a pure antiandrogen) nor triptorelin (a luteinizing hormone-releasing hormone analog) or the combination of both are effective in prolonging survival of patients with HCC [6]. Megestrol acetate, a semisynthetic progestational steroid, has recently been claimed to improve quality of life in a significant proportion of patients with HCC included in a Phase II, noncontrolled trial [7]. Moreover, megestrol prolonged survival of a selected group of patients with HCC and variant estrogen receptors in a randomized, placebo-controlled study [8].

The fact that HCC cells frequently express somatostatin receptors has prompted a randomized clinical trial exploring the effect of the somatostatin analog octreotide among patients with advanced HCC [9]. When compared with patients receiving placebo, those treated with octreotide had a statistically and clinically relevant increase in survival, irrespective of the receptor status. These results await confirmation by currently ongoing, large clinical trials.

Inhibiting growth signals

The epidermal growth factor receptor (EGFR) is a plasma membrane glycoprotein that possesses an intracellular tyrosine kinase domain. After ligand binding, receptor dimerization leads to tyrosine kinase activation and the recruitment and phosphorylation of intracellular substrates, leading to cell proliferation, motility, adhesion, invasion, survival, and angiogenesis [10]. The EGFR pathway is essential for the regulation of normal cell growth and differentiation, but also promotes proliferation of malignant cells. Approximately 65% to 70% of human colon carcinomas express the EGFR, and its expression carries a poorer prognosis. Procedures to block EGFR function include anti-EGFR monoclonal antibodies, immunotoxin conjugates, and EGFR tyrosine kinase inhibitors [11].

ZD1839 (Iressa, AstraZeneca) is a selective EGFR tyrosine kinase inhibitor that exerts substantial antitumor activity, either as a single agent or in combination with conventional chemotherapy and radiation. In Phase I clinical trials, ZD1839 showed a favorable toxic profile and resulted in clinically meaningful disease stabilization in patients with a variety of tumor types, including colorectal cancer (CRC) [12].

On the other hand, retinoids are vitamin A derivatives essential for epithelial differentiation that have recently gained interest as antineoplastic agents. In preclinical studies, tretinoin, isotretinoin, and the aromatic retinoids etretinate and acitretin have shown preventive and therapeutic effects on carcinogen-induced premalignant and malignant lesions. This therapeutic effect is mediated by retinoids binding to two different nuclear retinoid acid receptors (RAR and RXR) that results in inhibition of cell proliferation and induction of differentiation [13]. With respect to the clinical efficacy of retinoids, some positive effects have been observed in the treatment and secondary prophylaxis of oropharyngeal cancer, and particularly in the treatment of acute promyelocytic leukemia [14].

Polyprenoic acid, an acyclic retinoid, has been shown to inhibit chemically induced hepatocarcinogenesis in rats and suppress cell growth in human hepatoma-derived cell lines. In a controlled trial involving patients with HCC (mostly carrying hepatitis C virus infection) in complete response after surgery or alcohol ablation, polyprenoic acid significantly reduced the incidence of relapses and improved survival [15]. In a noncontrolled trial, however, oral therapy with beta-tretinoin was unable to induce objective tumor remissions and induced significant toxicity to patients with advanced HCC [16].

Finally, colorectal and pancreatic tumor cell lines and human cancers may express a number of isoforms of the gastrin/CCKB receptors and produce their own gastrin. Because tumor cells can respond to gastrin, the therapeutic potential of antigastrin hormone therapy has been explored. Gastrimmune (Aphtron Corp., Miami, Florida) is an immunoconjugate consisting of the nine NH₂-terminal amino acids of human gastrin 17 linked to diphtheria toxoid. The latter acts as the immunogenic carrier, and the gastrin 17 sequence acts as a B-cell epitope. Induction by Gastrimmune of neutralizing anti-G17 antibodies production has been shown to be effective in the treatment of colorectal carcinoma in preclinical models. Results from early clinical trials showed that Gastrimmune lacked substantial activity against human colorectal cancer [17], but induced stabilization of the disease in patients with pancreatic cancer that developed an antibody response [18].

Antibody therapy

Cancer cells sometimes need the constant signaling from mutated or nonmutated surface receptors for survival or progression in the cell cycle. There is an extensive field of investigation around the use of antibodies targeting specific antigens overexpressed in tumor cells. Monoclonal antibodies can be used for therapeutic purpose, either directly due to their ability to mediate tumor cell lysis, or indirectly if they are conjugated with radioisotopes, cytotoxic agents, or toxins.

Unlabeled monoclonal antibodies

EGFR (epidermal growth factor receptor) is expressed in approximately 65% of human colon carcinomas, and its presence is associated with more

aggressive disease. An anti-EGFR antibody (IMC-C225) has shown efficacy in head and neck cancer and is being tested in clinical trials for colorectal carcinomas in combination with conventional chemotherapy [11]. A similar type of mechanism of action is postulated for the murine 17-1A mAb anti-human tumor-associated antigen Ep-CAM (epithelial adhesion molecule), edrecolomab, that binds to CD17-1A, a surface glycoprotein expressed in adenocarcinomas. Although preliminary results of a Phase I/II clinical trial were encouraging, the addition of this antibody to standard adjuvant therapy for patients with Stage III CRC did not modify survival or time to progression [19].

Radiolabeled monoclonal antibodies

Anti-ferritin antibodies labeled with ^{131}I were shown to induce responses in unresectable HCC [20] and ^{131}I -antibodies against tumor-specific antigens such as carcinoembryonic (CEA) [21] and by tumor-associated antigen 72 (TAG-72) [22] induced tumor remission when given to patients with cholangiocarcinoma and liver metastases, respectively. Immunoradiotherapy has not achieved clinical success, however, due to its inconsistency and inconvenience.

Antiangiogenesis

To ensure constant oxygen and nutrient supplies, a tumor must become vascularized. Angiogenesis is a multistep process mainly consisting of endothelial cell proliferation, migration, basement membrane dissolution, and new lumen formation. This process is tightly regulated by both positive and negative signals [23,24]. The continuous expansion of the tumor is accompanied by overproduction of angiogenic stimulators and down-regulation of endogenous angiogenesis inhibitors [24,25]. Among positive angiogenic stimuli, vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs) are the most relevant [26]. Inhibiting angiogenesis has an attractive feature in that, whereas tumoral cells exhibit genetic instability and may develop resistance to chemotherapy, endothelial cells are genetically stable and therefore more predictable in response to therapeutic agents.

Various antiangiogenic compounds have been developed to treat cancer. These products can be classified as direct or indirect inhibitors of angiogenesis, depending on their mechanisms of action. Direct inhibitors prevent the response of vascular endothelial cells to angiogenic factors such as VEGF and FGF, and include angiostatin, endostatin, integrin $\alpha\text{v}\beta_3$, thrombospondin, and antibodies against VEGF. Endostatin (an endogenous cleavage fragment of collagen XIII) was identified in 1997 and has shown strong antitumoral activity in a number of experimental tumors, via inhibition of tumor angiogenesis [27]. In the clinical setting, the recombinant protein administered as a 20-minute daily intravenous (IV) injection at doses up to 240 mg/m² showed no significant toxicities [28]. Among 15 treated

patients, 1 patient with a pancreatic neuroendocrine tumor had a minor response and 2 patients showed stable disease [28]. The humanized anti-VEGF antibody, bevacizumab, has shown antitumor activity against a number of human tumor xenografts in mice. A randomized, double-blind, placebo-controlled clinical trial with bevacizumab in patients with renal cell cancer showed prolonged disease-free survival with minimal toxicity [29]. Experience in cancer patients with HuMV833, an antibody against VEGF, showed that this antibody was generally well tolerated, although the trial could not identify the optimum biologically active dose. Antibody distribution and clearance showed an important heterogeneity between patients and tumor types [30].

Vitaxin (MedImmune, Gaithersburg, Maryland) is a humanized anti- $\alpha_v\beta_3$ antibody that interferes with blood vessel formation by inducing apoptosis in newly generated endothelial cells. Among 17 patients enrolled in a Phase I clinical trial, 14 were finally evaluable for response. In general, treatment was well tolerated, with little or no toxicity. One partial response and 7 patients with stable disease were observed. At the doses and schedule studied, Vitaxin appears safe and potentially active, suggesting that vascular integrin $\alpha_v\beta_3$ represents a clinically relevant antiangiogenic target for cancer therapy [31].

Interferons alpha and beta (which display antiangiogenic activity by reducing the expression of b-FGF [32]) and the inhibitor of EGFR tyrosine kinase ZD1893 (which blocks angiogenesis by acting directly on the EGFR of microvascular endothelial cells and by decreasing production of angiogenic factor by tumor cells [33]) are among the indirect angiogenesis inhibitors. The role of the latter in the therapy of liver tumors awaits results from clinical studies.

Activation of antitumoral immunity

To date there is very limited clinical experience with respect to the use of immunotherapy strategies against gastrointestinal tumors as compared with other malignancies such as melanoma, lymphoma, or renal cell carcinoma. In the last 2 decades, a number of procedures aiming at stimulating immunity against liver tumors have been developed. They include non-specific stimulation by cytokines (including IL-2 and IFN- α), generic immune stimulants (Bacille Calmette-Guerin (BCG), thymosin, OK-432, levamisole), and lymphokine-activated killer cells or tumor-infiltrating lymphocytes. The immunotherapy strategies that are being developed for cancer can be divided into the categories below (Fig. 1).

Systemic administration of cytokines

Cytokines are soluble protein molecules that mediate autocrine, paracrine, and endocrine functions during immune and inflammatory responses. Some of them have effects that interfere with the progression of tumors by

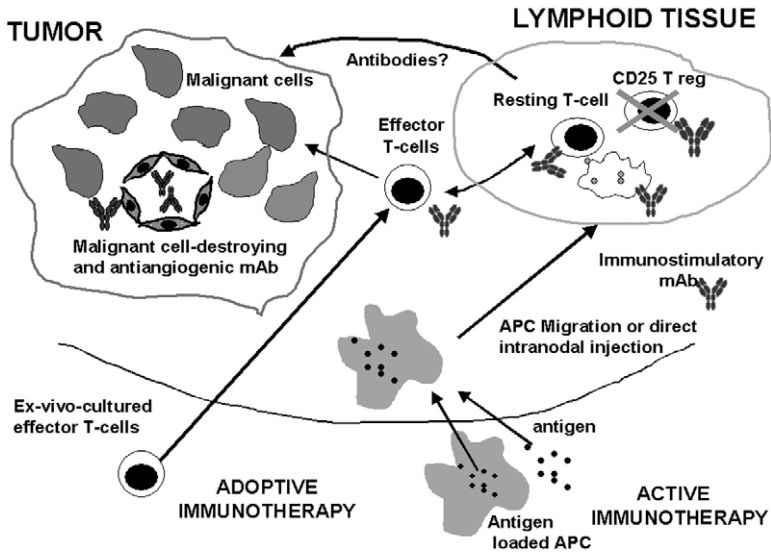


Fig. 1. Concepts in immunotherapy of liver tumors. APC, antigen presenting cells; CD25 T-cell, regulatory cells; mAb, monoclonal antibodies.

augmenting antitumor immune responses, inducing apoptosis in malignant cells, by interfering with the uncontrolled proliferation of cancer cells, or by impairing neovascularization.

The cytokine that has been studied more intensively is Interferon- α . This family of cytokines is primarily involved in fighting viral infections, but its biological properties also interfere with tumor growth by activating immune and nonimmune mechanisms. Interferon-beta [34] and interferon-gamma [35] have shown some activity against advanced HCC in noncontrolled trials, but the two controlled studies comparing interferon-alpha with symptomatic treatment among HCC patients have produced conflicting results. On one hand, high doses (50 MU/m², tiw) produced a 36% response rate and prolonged survival of Japanese patients [36]. On the other, low doses (3 MU tiw) produced a 7% response rate and did not prolong survival of Spanish patients [37]. This difference could be due to dose level as well as to patient population, but in the end, side effects of high doses are simply unaffordable, particularly for cirrhotics.

Also, there is published evidence that IFN α [38] or IFN β [39] regimes after surgical resection or tumor ablation by other techniques prevent or at least delay tumor relapses [40]. Interferon-alpha has also been used extensively in combination with cytotoxic agents, most commonly fluorouracil, for the treatment of HCC [41,42] and liver metastases from colorectal cancer [43]; however, randomized trials have consistently failed to prove a superiority of the combination compared with chemotherapy alone [44,45]. The antitumor mechanism of action of IFN α is probably manifold

and encompasses direct effects on tumor cells and augmentation of lymphocyte and macrophage cytotoxic activities against malignant cells. As we mentioned before, antiangiogenic effect of this molecule might also be involved.

Other cytokines with potential as anticancer agents, such as interleukin-12, TNF α , or TRAIL (tumor necrosis factor [TNF]-related apoptosis inducing ligand), have not been tested yet for primary or metastatic liver cancer in humans, although systemic treatment with IL-12 and TNF α may cause serious toxicity problems. TRAIL, as an agonist of death receptors that is quite selective of tumor cells, is making progress toward being tested clinically for HCC [46]. With respect to interleukin 2, despite the fact that this cytokine has been used against liver tumors in several combinations, the noncontrolled nature of most studies precludes any definitive conclusion. Systemic interleukin 2 was able to produce objective responses against HCC when given alone [47] or in combination with melatonin [48] or lymphokine-activated killer (LAK) cells [49]. On the other hand, hepatic artery infusion of interleukin 2 with or without chemotherapy-induced objective remissions in 5% to 15% of liver metastases from colorectal cancer [50–52]. Clinical development of interleukin 2 has proved unsuitable, however, because efficacy parallels severe toxicity, including systemic vascular leak syndrome.

Immunostimulating monoclonal antibodies

These monoclonal antibodies (mabs) work against murine tumors by amplifying a weak but ongoing immune response. These agents bind molecules on the surface of the cells of the immune system instead of interacting with tumor tissue antigens, providing activatory signals to lymphocytes or antigen presenting cells. Alternatively, they can block the action of surface receptors that normally downregulate immune responses. In combined regimes of immunotherapy, these antibodies are expected to potentiate therapeutic immunizations against tumors, as has been observed in preclinical models. Anti-CTLA-4 mabs that block the inhibitory function of CTLA-4 on T-cells have already started clinical trials against prostate cancer [53]. Other antibodies of this kind that are in the process of clinical development act by providing activating or apoptosis-preventing signals to lymphocytes or dendritic cells. This is the case of antibodies specific for CD40 [54], CD137 (4-1BB) [55], and anti-CD102 (ICAM-2) [56]. Antibodies that deplete suppressor CD25 + T-cells have also demonstrated preclinical efficacy.

Cancer vaccines

Tumor antigens are in general very poor immunogens and need the help of very potent adjuvants if a therapeutic immune response is to be reached [57]. Tumor antigens can be formulated from identified sequences, but are most often obtained from the tumor as complex mixtures of biomolecules containing the antigenic substances. Many such attempts have been tried in

pilot clinical experiments with marginal efficacy at best. Recently these protocols against cancer were boosted by the identification of dendritic cells (DCs) as the protagonist cells in antigen presentation. DCs can present antigen to induce tolerance or immunity, depending on whether they have undergone activation by inflammatory stimuli or T-helper cells. Several trials using DCs to stimulate antitumoral immunity in solid tumors are being conducted without significant toxicity and with a report showing the augmentation of the cellular immune response toward tumor extracts [58]. Similar strategies are being tested for advanced colon cancer [59]. As an alternative, other active clinical protocols rely on the intratumor injection of dendritic cells to exploit their natural capacities to take up tumor antigens and migrate to regional lymph nodes in order to mount a specific antitumoral response. In general, these strategies are safe and well tolerated, but larger patients series and the optimization of laboratory techniques are necessary.

Adoptive T-cell therapy

The idea of this approach is to raise and infuse in vitro-cultured T cells that mediate specific destruction of tumor cells. The main hurdle in its application is the difficulty of obtaining such T-cell cultures against epithelial tumors. The use of dendritic cells to prime T-cell cultures in vitro can be very helpful. In a randomized clinical trial, Takayama et al showed that adoptive immunotherapy consisting of autologous lymphocytes, activated in vitro and administered to patients who had received curative resection for HCC, is a safe therapeutic approach that reduces postsurgical recurrence at 3 (48% versus 33%) and 5 years (38% versus 22%) [60].

Recent evidence shows remarkable partial efficacy of adoptive T-cell therapy in a number of melanoma cases; this can be incremented if the patients are pretreated with drugs inducing transient lymphopenia, if the cultures also contain tumor specific T-helper cells, and if the postadoptive transfer regime contains IL-2 [61]. It is of note that adoptive transfer of activated T cells, cultured without selection for tumor specificity, to patients undergoing surgery for gastric cancer has been found to decrease the number of relapses, and result in a clear improvement of survival [62]. New techniques of selection and culture of tumor-specific cells, as well as combination therapy with tumor vaccination protocols, are expected to improve the outcome.

New tools and strategies: gene therapy

Gene therapy represents a new and promising therapeutic modality based on the introduction of genetic material into cells to generate a curative biological effect [63,64]. Gene therapy is not limited to hereditary diseases and can be used to treat a broad variety of different acquired diseases, including cancer (Fig. 2).

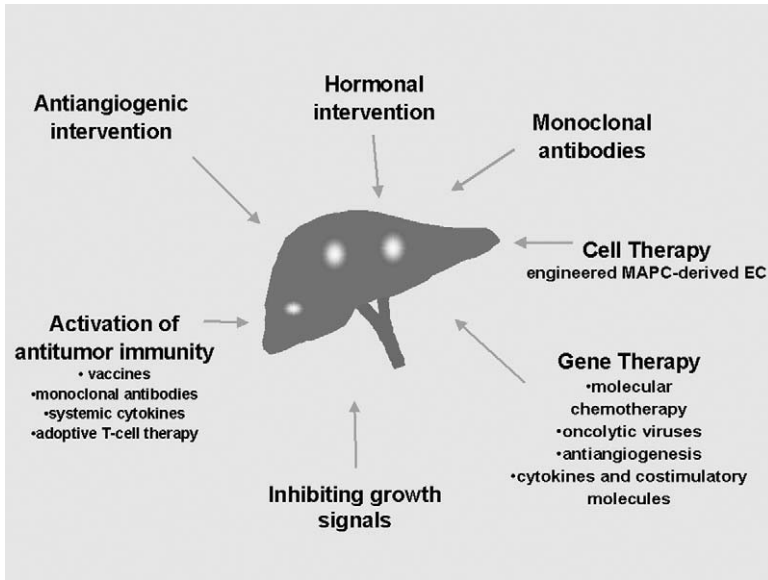


Fig. 2. Biologic therapy for liver tumors. EC, endothelial cells; MAPC, multipotent adult progenitor cell.

The transferred genetic material is most commonly a natural gene, but it can also be a chimeric gene or subgenomic molecule. When the foreign therapeutic gene has penetrated the cell and is functional in its interior, the cells are said to be transduced. To facilitate cell transduction, the genetic material is packaged into molecular constructs named vectors, which can be of viral [65] or nonviral nature [66]. Viral vectors are frequently preferred because of their higher transduction efficiency. These genetic vehicles can be classified as long- and short-term expression vectors. Vectors based on retroviruses, adeno-associated viruses, and gutless adenoviruses belong to the first category, and first generation adenoviruses to the second.

For a gene to be expressed inside a cell, its coding DNA sequence should be linked to appropriate promoters. These regulatory DNA sequences can be categorized as universal promoters, which are those allowing transgene expression in every transduced cell, or tissue-specific promoters, which drive gene transcription only in selected cell types. Tumor-specific promoters contain binding sites for transcription factors that are active in transformed but not in normal cells. Either universal or tissue-specific promoters may induce continuous and fixed expression of the transgene or, alternatively, their activity may depend on the administration of certain drugs that, when given to the patient, can modulate promoter function, allowing regulatable expression of the transgene. The most challenging issues for a successful application of gene therapy concern the choice of efficient therapeutic genes and appropriate promoters, and the selection of vectors allowing an

adequate level and duration of transgene expression. Gene therapy is a matter of genes, vectors, promoters, and regulatory elements. All these materials should be selected to treat efficiently a particular disease without undue risks or side effects for the patient.

Gene therapy of liver tumors

Different gene therapy-based approaches have been tested to treat cancer, including replacement of functional tumor suppressor genes, inhibition of oncogenes, transfer to tumoral cells of genes conferring sensitization to a specific prodrug (“suicide genes”), stimulation of antitumoral immunity, and inhibition of the formation of tumoral neovessels [67].

Antiangiogenic gene therapy

Gene transfer of antiangiogenic factors to solid tumors has aimed to achieve inhibition of new tumor-induced blood vessel formation and represents a promising therapeutic strategy.

One of the most potent angiogenic factors secreted by tumors is vascular endothelial growth factor (VEGF). It has been reported that administration of antisense oligonucleotides against VEGF mRNA was able to reduce tumor growth rate in a rat model of glioblastoma [68]. Another promising approach is to target the two VEGF high-affinity receptors expressed on endothelial cells, Flt-1 and KDR/Flk-1. Genetically modified tumor cells producing native soluble Flt-1 can inhibit VEGF by sequestering this substance, and also by forming inactive heterodimers with membrane-spanning VEGF receptors. It has been shown that survival is prolonged in those tumor-bearing mice that received these genetically modified tumor cells [69].

Recently Lin et al [70] used recombinant adenovirus to deliver a soluble Tie2 receptor that blocked activation of the Tie2 receptor on endothelial cells by angiopoietin. Treatment of tumor-bearing animals with this vector not only significantly inhibited primary tumor growth, but also almost completely inhibited neovascularization and growth of metastasis [70].

Other strategies explore natural antagonists of angiogenesis such as angiostatin and endostatin [27,71]. Gene transfer of angiostatin and endostatin by retroviral and adenoviral vectors has been used for treatment of different tumors in animal models [72,73].

In an important comparative work recently published, Kuo and coworkers demonstrated that a single intravenous injection of an adenovirus-encoding soluble form of FLK-1 or Flt-1 resulted in nearly 80% of inhibition of different cell lines, including human pancreatic carcinoma. Similar adenoviruses encoding for angiostatin, endostatin, or neuropilin were less potent at inducing antitumor effects, however [74]. The studies mentioned here have shown the potential application of antiangiogenic gene transfer in the treatment of liver tumors.

Transfer of suicide genes and tumor suppressor genes. Oncolytic viruses

The Herpes simplex virus thymidine kinase (HSV-tk) is the best-characterized suicide gene [75]. Expression of functional nontoxic enzyme HSV-tk in transduced cells induces the conversion of the nontoxic prodrug ganciclovir (GCV) into a toxic phosphorylated compound that inhibits both nuclear and mitochondrial DNA synthesis [75]. A feature of the suicide genes is the so-called “bystander effect,” which is caused by the diffusion of the toxic drug metabolite around the transduced cells, thus increasing the killing of neighboring tumor [76,77]. The bystander effect also stems from the necrosis of tumoral tissue that induces local inflammation, attraction of dendritic cells, and stimulation of antitumoral immunity. It is not surprising that a synergistic antitumoral effect was observed when combining suicide genes and gene transfer of immunostimulatory molecules [78].

Several studies have demonstrated the efficacy of the HSV-tk/GCV system for the treatment of HCC [79–81] and metastatic colorectal cancer [82] in animal models. One of the main obstacles limiting the clinical application of this therapy is related to toxic side effects affecting nontumoral tissue [83]. These side effects can be reduced by injecting the vector locally inside the tumor, or by employing tumor-specific promoters (such as alpha-fetoprotein [84] and CEA [85] promoters for HCC and CRC, respectively) to restrict HSV-tk expression to tumor tissue.

Gene transfer of *Escherichia coli* cytosine deaminase (CD) into cancer cells transforms the nontoxic prodrug 5-fluorocytosine into the toxic compound 5-fluorouracil [86]. As a result, transduced tumor cells are killed. The effects of cytosine-deaminase/5-fluorocytosine (CD/5-FC) system were tested in an s.c. model using human HT29 cells in nude mice by Hirschowitz et al [87].

Virotherapy with oncolytic viruses is based on the use of tumor-selective replicating virus. In this approach, transduced cells are killed as a consequence of virus infection and replication, and they become cell factories for producing new infectious virus particles that will infect surrounding tumor cells and spread throughout the tumor. The first such vector that has been developed, named mutant *d11520* or ONYX-015, was based on deletion of the E1B 55K gene of adenovirus [88]. E1B 55K binds p53 tumor suppressor gene and allows adenovirus replication in the infected cells. Because ONYX-015 lacks E1B 55K, it cannot replicate in cells with normal function of the p53 pathway, but it can do so in tumor cells without p53 activity.

Gene transfer of immunostimulatory molecules and genetic vaccination

Gene transfer of immunostimulatory cytokines can overcome the immune tolerance against tumoral antigens and facilitate tumor rejection. Different cytokines (IL-2, IL-4, IL-6, IL-7; IL-12, IL-15, IL-18, INF- γ , TNF- α , GM-CSF) have been used to activate antitumoral reactivity by either ex vivo or in vivo gene transfer [89,90].

Interleukin 12 (IL-12) is among the cytokines with most potent antitumoral activity. It acts by activating NK cells and cytotoxic T

lymphocytes and inducing a TH1 type of response [91]. It also inhibits tumoral neoangiogenesis and enhances the expression of adhesion molecules on endothelial cells, thus facilitating the traffic of lymphocytes to the tumor [92]. This cytokine, however, is toxic when administered systemically as a recombinant protein (Phase II clinical trial) [93]. IL-12-based gene therapy allows local production of the cytokine at the tumor site, resulting in high intratumoral or peritumoral levels with low serum concentration. 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 (Ad.IL-12) to rats with orthotopic HCC caused complete tumor eradication in most of the animals and increased long-term survival [94,95]. Ad.IL-12 was also shown to be efficient in the treatment of a very aggressive model of multifocal hepatocellular carcinoma in rats (induced by diethylnitrosamine (DENa)) [94], as well as in mice with colorectal cancer metastatic to the liver [92].

Gene therapy with IL-12 has the risk of toxicity, due to the ability of this cytokine to induce IFN- γ production and a hypercytokinemic syndrome [96]. Intratumoral administration of AdIL-12, together with an adenovirus encoding the chemokine IP-10 (AdIP-10), caused potent antitumoral responses using doses of AdIL-12 that were not effective when given alone [97]. The rationale for combining AdIL-12 and AdIP-10 is based on the activation of lymphocytes by IL-12 and their attraction by IP-10. This strategy allowed reduction of the dose of AdIL-12 without losing antitumor efficacy, but with less risk of toxicity [97]. The combination of IL-12 and MIP3 α has similar synergistic activity [98].

Dendritic cells are powerful inducers of immune responses, and the activation of these cells is essential to establish an antitumoral immune reaction [99]. A possible way to take advantage of the therapeutic effect of IL-12 is to infect dendritic cells with AdIL-12 *ex vivo* and to inject the engineered cells into the tumor. In animal models of colon cancer, this strategy has proved to be extremely potent at eliminating neoplastic lesions and at eliciting antitumoral immunity [99]. Stimulation of dendritic cells is widely dependent on activation by costimulatory molecules such as B7 and CD40 ligand. Gene transfer of CD40 ligand by intratumoral injection of first-generation adenoviral vector was able to eradicate orthotopic HCC in rats and to induce protective antitumor immunity without apparent toxicity [94].

Genetic vaccination has been shown to inhibit tumor growth in animals. In a murine model of subcutaneous HCC, DNA vaccination with a cDNA-encoding murine AFP induced tumor regression and prolonged animal survival. The antitumoral effect was mediated by cytotoxic T cells, and protective antitumoral immunity was shown to depend on both CD-4 + and CD-8 + T cells [100]. Recently, improved immunization protocols alternating DNA priming and recombinant vaccinia Ankara booster generates a multiplied higher immune response than any of these strategies

alone [101]. These concepts have not yet been applied in the clinical setting for the induction of anti-HCC immunity, however.

Gene therapy: data from clinical trials

Gene therapy is still an experimental procedure reserved for clinical trials of serious, frequently deadly, human diseases lacking effective treatment. A substantial proportion of gene therapy clinical trials being conducted have been designed to treat advanced cancer. These pilot studies are Phase I/II studies using escalating doses of the vector to determine toxicity primarily and efficacy secondarily.

Rubin et al showed that direct intratumoral gene transfer of HLA-B7 and β 2-microglobulin into the liver of patients with metastatic CRC is a feasible and safe procedure. They used a single plasmid construction that encodes for both genes in a lipid formulation (Allovectin-7). Genes transfected into tumors were detected by PCR in 14 out of 15 patients, but efficacy was not reported [102].

With respect to primary and metastatic liver cancer, various studies based on p53 gene transfer have been published. In a preliminary report, Habib et al showed that 3 out of 5 patients with HCC treated by intratumoral injection of plasmidic DNA carrying the p53 gene experienced an objective response, with reduction of the tumor volume and a decrease of AFP levels [103]. In another study conducted by Horowitz et al, the authors used replication-defective adenoviruses encoding p53 to treat 30 patients with HCC by intrahepatic artery infusion of doses up to 7.5×10^{13} viral particles (v.p.) [104]. They found a maximum tolerated dose of 2.5×10^{13} v.p. Doses of 7.5×10^{13} v.p. caused severe hemodynamic disturbances. Repeated intra-arterial doses of up to 2.5×10^{12} v. p. were well tolerated. Expression of the transgene in the tumor by reverse-transcription polymerase chain reaction (RT-PCR) could be detected at the 2.5×10^{11} v.p. dose level. Clinical outcome was not commented in this study.

Sung et al [105] have treated hepatic metastases from colorectal cancer by intratumoral administration of adenovirus carrying the suicide gene HSV-tk in doses of up to 1.0×10^{13} v.p. Hepatic toxicity was mild, with transient grade 1 elevations in serum aminotransferase levels in 3 out of 16 patients. Other toxicities, including fever, thrombocytopenia, and leucopenia, were also transient and occurred in few patients. No complete or partial responses were seen. As in other clinical studies with suicide gene therapy, this procedure appears to be safe, but not active enough. Intratumor injection of an adenovirus encoding CD gene was tested in patients with metastatic CRC but results have not been published yet.

An oncolytic adenovirus, *d11520* or ONYX-015, which possesses the ability to replicate selectively in p53-deficient malignant cells, has been employed to treat different types of tumors, including head and neck cancer, pancreatic cancer, liver metastases of colorectal carcinoma, and hepatocellular carcinoma. Habib et al [106] treated 9 patients with HCC or liver

metastases of colorectal carcinoma by intratumoral, intra-arterial, and intravenous injection of *d/1520* in doses up to 6×10^{12} v.p. *d/1520* was well tolerated in the three different routes of administration up to a dose of 3×10^{11} plaque forming units (pfu), but antitumoral activity could not be observed in this trial. In a subsequent clinical study [107], 5 patients with HCC were treated by *d/1520* at day 1 with 6×10^{12} v.p. by intravenous injection in the arm vein at day 1 and by intratumoral injection under ultrasound guidance at same dose on days 2, 15, 16, 29 and 30. No significant toxicity was observed, but only 1 patient experienced a partial response, and disease progression was observed in the remaining 4. In a further study performed by Reid et al [108] with intra-arterial administration of *d/1520* for treatment of liver metastases of colorectal carcinoma, 11 patients were treated with escalating doses up to 2×10^{12} v.p. given twice on days 1 and 8. Subsequent cycles of ONYX-015 were administered in combination with intravenous 5-fluorouracil (5-FU) and leucovorin (LV). Mild to moderate fever and rigors were the most common adverse events, and these effects were transient and not dose-limiting. This study has demonstrated that the hepatic arterial infusion of *d/1520* was well tolerated at doses up to 2×10^{12} v.p. (10^{11} pfu), both as a single agent and in combination with chemotherapy. Viral replication occurred in patients treated with the higher doses ($\geq 2 \times 10^{11}$ v.p.); however, antitumoral activity was mild, with one partial response and two cases with stable disease after treatment with a combination of high doses of ONYX-015 and 5-FU/LV.

Two clinical trials have been designed to enhance antitumoral immunity using intratumoral injection of adenoviruses encoding interleukin-12 (Ad.IL-12). Although the results from these studies are not yet available, in a trial from our group employing Ad.IL12 given intratumorally to treat multinodular HCC, metastatic colon cancer, and advanced pancreatic cancer, the therapy was well tolerated up to 3×10^{12} v.p., but partial regression occurred in only one HCC patient. Another clinical study from our group with intratumoral injection of DC engineered with Ad.IL12 is now in progress.

Morse et al reported the results of a Phase I clinical trial consisting of the administration of autologous dendritic cells loaded with carcinoembryonic antigen RNA (peptide CAP-1) into 21 patients resected of liver metastases of CRC. The procedure was well tolerated, and 1 patient had a minor response, and 1 had stable disease [109]. Fong et al used the hematopoietic growth factor Flt3 ligand to expand dendritic cells in vivo, and subsequent injection of CEA-derived, peptide-loaded DCs into 12 patients with colon or CEA + non small-cell lung cancer enrolled in a Phase I study. Two objective responses, one mixed response, and two stable diseases were observed [110].

Lessons from clinical trials and future avenues in gene therapy of liver cancer

As shown above, the high efficacy of gene therapy at eliminating solid tumors in animal models is in contrast with the poor results observed in the

patients with the presently available vectors. It is clear therefore that progress in cancer gene therapy depends basically on data obtained in humans. Contribution of clinical trials to improvements in the design of vectors and strategies will be especially important if study protocols include *in vivo* molecular imaging techniques, such as positron emission tomography (PET), to allow noninvasive visualization of transgene expression of vectors containing HSV1-tk in human subjects [111]. This procedure will also be important to establish comparisons of different vectors and routes of vector administration with respect to their ability to transduce a given human neoplasm. At the birth of cancer gene therapy, this information is essential for the rational progress of the field.

The reasons for the mentioned discrepancies between clinical and preclinical data are multiple, but poor infectivity of adenoviruses for malignant cells and short duration of transgene expression appear to be important factors. Thus, efforts should be directed to enhance transduction and to prolong expression.

The aim of cancer gene therapy is to achieve an intratumoral or peritumoral environment with high concentration of the therapeutic gene product. When treating a tumoral liver, the vector can be given by intra-arterial or intratumoral injection. The first route is appropriate to transduce the normal liver parenchyma surrounding tumor nodules, but the penetration of the vector into the tumor mass by this route is very poor, at least in animal models, due to the presence of an efficient blood-tumor barrier [112]. When nonreplicating adenoviruses are administered by intratumoral injection, tumor transduction is poor and takes place mainly along the injection tract. Hopes for improvement of tumor transduction may come from the use of replicating vectors, such as Semliki Forest Virus (SFV) or tumor-specific replicating adenoviruses. Examples of the latter are adenoviruses in which a tumor-specific promoter such as the telomerase promoter controls the E1 gene. These adenoviruses could also be constructed to allocate a therapeutic gene in the E3 region to combine replication and transgene expression, thus enhancing the synthesis of the therapeutic product inside the tumor mass.

Long-term expression vectors are promising tools to treat liver cancer, because they allow sustained transgene expression in liver cells surrounding tumor nodules for long periods of time. Two main types of such vectors have been used in preclinical studies: (1) vectors that become integrated into the cell genome (eg, retroviruses or adeno-associated virus (AAV)), and (2) vectors that remain episomal. The latter variety is of particular interest, being mainly represented by the so-called “high-capacity” or gutless adenoviruses. High-capacity adenoviruses lack all viral genes and fail to elicit antivector immunity persisting in the liver for more than 1 year [113]. When using high-capacity adenoviral vectors, the synthesis of the therapeutic product should be controlled with regulatable promoters responding to specific inducers, such as mifepristone or doxycycline. The activity of these promoters can be adjusted at the desired level by modifying the dose of the

inducer substance, or can be silenced by withholding the inducer. Preliminary data from our group indicate that the sustained and controlled expression of antitumoral cytokines such as IL-12 around tumor nodules completely blocks the expansion of experimental liver tumors by displaying antiangiogenic activity and potentiation of antitumor immunity.

New tools and strategies: cell therapy

Progress in the field of stem cell biology has opened promising new avenues to treat nonresectable, chemotherapy-resistant, solid tumors. Stem cells have been identified in many tissues, including bone marrow, nervous system, gastrointestinal tract, epidermis, and the liver [114]. Different stem cell compartments have been described in the bone marrow including hematopoietic stem cells (HSC), and mesenchymal stem cells (MSC) [115]. Whereas HSC give rise to hematopoietic cells, MSC has the potential of generating bone, cartilage, adipocytes, and skeletal myocytes. In addition, the bone marrow harbors a reservoir of angioblasts or endothelial progenitor cells (EPC). These cells are found not only in the bone marrow, but can circulate in blood, playing a fundamental role in the repair of damaged endothelium and in angiogenesis. In the embryo, a common precursor of HSC and angioblasts, the so-called hemangioblast, has been recognized, although its existence in the mature individual has not been proved.

Recently, Reyes and coworkers have identified a very primitive stem cell in the bone marrow, termed multipotent adult progenitor cell (MAPC) that may precede the hemangioblast in ontogeny and that can differentiate to other mesodermal cell types (including cardiac, skeletal, and smooth muscle) as well as neuroectodermal cells (including neurons, astrocytes, and oligodendrocytes) and to endodermal lineage (including hepatocytes and GI epithelium) [116,117]. Multipotent adult progenitor cells (MAP) copurify with MSC and were selected by depleting adult bone marrow of CD45 and glycophorin-positive cells, followed by long-term culture under serum-free conditions. MAPCs express AC133 and low levels of VEGFR2/Flk1 and VEGF1/Flit1, but are HLA Class I and II negative and lack such stem cell markers as CD34 and *c-kit*. MAPCs display telomerase activity and can be expanded in vitro without obvious senescence for more than 80 population doublings. Because large amounts of MAPCs can be obtained from donors over a wide age range, these cells may represent a useful tool for diverse therapeutic purposes.

It has been shown that MAPCs can be easily differentiated in vitro to CD34⁺, VE-cadherine⁺, AC133⁻, and Flk1⁺ “angioblasts,” and subsequently to more mature endothelial cells (EC) that are fully functional both in vitro and in vivo. Reyes et al have demonstrated that injection of 0.25×10^6 of in vitro-generated, MAPC-derived EC of human origin to non-obese diabetic/severe combined immune deficiency (NOD-SCID) mice with

growing murine Lewis lung carcinoma leads to their incorporation into the tumor vessels in such a way that about 35% of the tumoral vascular cells were derived from injected endothelial cells [115]. The fact that MAPC-derived EC respond to angiogenic stimuli by migrating to tumor sites and contributing to tumor vascularization [117] opens the possibility of using these cells as a Trojan horse to treat solid neoplasms. A possible strategy would be to administer to patients with HCC or other tumors repeated transfusions of genetically engineered MAPC-derived EC, in order to populate with cells the tumoral neovessels of all actively growing neoplastic lesions. The genetic equipment of MAPC-derived EC should include both a reporter gene (such as HSV-tk, allowing visualization of the fate of the EC by PET) and a therapeutic gene under the control of an endothelium-specific regulatable promoter. Once the cells have homed at the tumoral nodules (which can be ascertained using PET imaging) the expression of the therapeutic gene (eg, IL-12) can be activated by administration of the inducer of the regulatable promoter. Because this therapy would rely on the use of autologous cells that can be generated from the bone marrow in virtually unlimited amounts, the procedure can be repeated as many times as necessary to achieve tumor control.

Summary

Nonresectable primary and metastatic liver tumors are common malignancies that lack therapies allowing substantial prolongation of survival. Recent progress in molecular and cell biology has opened the way to novel therapies based on biological modifiers, gene transfer, and autologous stem cells. It is now possible to transfer therapeutic genes to the tumor or pericancerous tissue, and to control their expression for long periods of time. It is also feasible to generate autologous endothelial progenitor cells that can be recruited by tumoral vessels acting as vehicles to convey therapeutic genes to the interior of the tumor mass. Combination of biological modifiers, gene therapy, and cell therapy will hopefully provide efficient means to combat inoperable neoplasms in a not-very-distant future.

References

- [1] Bruix J, Sherman M, Llovet JM, Beaugrand M, Lencioni R, Burroughs AK, et al. Clinical management of hepatocellular carcinoma. Conclusions of the Barcelona-2000 EASL conference. European Association for the Study of the Liver. *J Hepatol* 2001;35:421–30.
- [2] Lorenz M, Staib-Sebler E, Hochmuth K, Heinrich S, Gog C, Vetter G, et al. Surgical resection of liver metastases of colorectal carcinoma: short and long-term results. *Semin Oncol* 2000;27:112–9.
- [3] Saltz LB, Douillard JY, Pirodda N, Alakl M, Gruia G, Awad L, et al. Irinotecan plus fluorouracil/leucovorin for metastatic colorectal cancer: a new survival standard. *Oncologist* 2001;6:81–91.

- [4] de Gramont A, Figer A, Seymour M, Homerin M, Hmissi A, Cassidy J, et al. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. *J Clin Oncol* 2000;18:2938–47.
- [5] Bismuth H, Adam R. Reduction of nonresectable liver metastasis from colorectal cancer after oxaliplatin chemotherapy. *Semin Oncol* 1998;25:40–6.
- [6] Manesis EK, Giannoulis G, Zouboulis P, Vafiadou I, Hadziyannis SJ. Treatment of hepatocellular carcinoma with combined suppression and inhibition of sex hormones: a randomized, controlled trial. *Hepatology* 1995;21:1535–42.
- [7] Chao Y, Chan WK, Wang SS, Lai KH, Chi CW, Lin CY, et al. Phase II study of megestrol acetate in the treatment of hepatocellular carcinoma. *J Gastroenterol Hepatol* 1997;12:277–81.
- [8] Villa E, Ferretti I, Grottola A, Buttafoco P, Buono MG, Giannini F, et al. Hormonal therapy with megestrol in inoperable hepatocellular carcinoma characterized by variant oestrogen receptors. *Br J Cancer* 2001;84:881–5.
- [9] Reubi JC, Zimmermann A, Jonas S, Waser B, Neuhaus P, Laderach U, et al. Regulatory peptide receptors in human hepatocellular carcinomas. *Gut* 1999;45:766–74.
- [10] Woodburn JR. The epidermal growth factor receptor and its inhibition in cancer therapy. *Pharmacol Ther* 1999;82:241–50.
- [11] O'Dwyer PJ, Benson AB 3rd. Epidermal growth factor receptor-targeted therapy in colorectal cancer. *Semin Oncol* 2002;29:10–7.
- [12] Baselga J, Rischin D, Ranson M, Calvert H, Raymond E, Kieback DG, et al. Phase I safety, pharmacokinetic, and pharmacodynamic trial of ZD1839, a selective oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with five selected solid tumor types. *J Clin Oncol* 2002;20:4292–302.
- [13] Marill J, Idres N, Capron CC, Nguyen E, Chabot GG. Retinoic acid metabolism and mechanism of action: a review. *Curr Drug Metab* 2003;4:1–10.
- [14] Bollag W, Holdener EE. Retinoids in cancer prevention and therapy. *Ann Oncol* 1992;3: 513–26.
- [15] Muto Y, Moriwaki H, Ninomiya M, Adachi S, Saito A, Takasaki KT, et al. Prevention of second primary tumors by an acyclic retinoid, polyprenoic acid, in patients with hepatocellular carcinoma. Hepatoma Prevention Study Group. *N Engl J Med* 1996;334:1561–7.
- [16] Meyskens FL Jr, Jacobson J, Nguyen B, Weiss GR, Gandara DR, MacDonald JS. Phase II trial of oral beta-all trans-retinoic acid in hepatocellular carcinoma (SWOG 9157). *Invest New Drugs* 1998;16:171–3.
- [17] Smith AM, Justin T, Michaeli D, Watson SA. Phase I/II study of G17-DT, an anti-gastrin immunogen, in advanced colorectal cancer. *Clin Cancer Res* 2000;6:4719–24.
- [18] Brett BT, Smith SC, Bouvier CV, Michaeli D, Hochhauser D, Davidson BR, et al. Phase II study of anti-gastrin-17 antibodies, raised to G17DT, in advanced pancreatic cancer. *J Clin Oncol* 2002;20:4225–31.
- [19] Punt CJ, Nagy A, Douillard JY, Figer A, Skovsgaard T, Monson J, et al. Edercolomab alone or in combination with fluorouracil and folinic acid in the adjuvant treatment of stage III colon cancer: a randomized study. *Lancet* 2002;360:671–7.
- [20] Order SE, Klein JL, Leichner PK. Hepatoma: model for radiolabeled antibody in cancer treatment, NCI Monogr 7–4., 1987.
- [21] Stillwagon GB, Order SE, Klein JL, Leichner PK, Leibel SA, Siegelman SS, et al. Multimodality treatment of primary nonresectable intrahepatic cholangiocarcinoma with 131I anti-CEA—a Radiation Therapy Oncology Group Study. *Int J Radiat Oncol Biol Phys* 1987;13:687–95.
- [22] Divgi CR, Scott AM, Gulec S, Broussard EK, Levy N, Young C, et al. Pilot radioimmunotherapy trial with 131I-labeled murine monoclonal antibody CC49 and deoxyspergualin in metastatic colon carcinoma. *Clin Cancer Res* 1995;1:1503–10.
- [23] Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* 2002;2:727–39.

- [24] Folkman J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med* 1995;1:27–31.
- [25] Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996;86:353–64.
- [26] Kandel J, Bossy-Wetzel E, Radvanyi F, Klagsbrun M, Folkman J, Hanahan D. Neovascularization is associated with a switch to the export of bFGF in the multistep development of fibrosarcoma. *Cell* 1991;66:1095–104.
- [27] O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997;88:277–85.
- [28] Eder JP Jr, Supko JG, Clark JW, Puchalski TA, Garcia-Carbonero R, Ryan DP, et al. Phase I clinical trial of recombinant human endostatin administered as a short intravenous infusion repeated daily. *J Clin Oncol* 2002;20:3772–84.
- [29] Yang JC, Haworth L, Steinberg SM, Rosenberg SA, Novotny W. A randomized trial of bevacizumab, an anti-vascular endothelial growth factor antibody, for metastatic renal cancer. *N Engl J Med* 2003;349:427–34.
- [30] Jayson GC, Zweit J, Jackson A, Mulatero C, Julyan P, Ranson M, et al. Molecular imaging and biological evaluation of HuMV833 anti-VEGF antibody: implications for trial design of antiangiogenic antibodies. *J Natl Cancer Inst* 2002;94:1484–93.
- [31] Gutheil JC, Campbell TN, Pierce PR, Watkins JD, Huse WD, Bodkin DJ, et al. Targeted antiangiogenic therapy for cancer using Vitaxin: a humanized monoclonal antibody to the integrin $\alpha v\beta 3$. *Clin Cancer Res* 2000;6:3056–61.
- [32] Singh RK, Gutman M, Bucana CD, Sanchez R, Llansa N, Fidler IJ. Interferons alpha and beta down-regulate the expression of basic fibroblast growth factor in human carcinomas. *Proc Natl Acad Sci U S A* 1995;92:4562–6.
- [33] Hirata A, Ogawa S, Kometani T, Kuwano T, Naito S, Kuwano M, et al. ZD1839 (Iressa) induces antiangiogenic effects through inhibition of epidermal growth factor receptor tyrosine kinase. *Cancer Res* 2002;62:2554–60.
- [34] Colleoni M, Buzzoni R, Bajetta E, Bochicchio AM, Bartoli C, Audisio R, et al. A phase II study of mitoxantrone combined with beta-interferon in unresectable hepatocellular carcinoma. *Cancer* 1993;72:3196–201.
- [35] Forbes A, Johnson PJ, Williams R. Recombinant human gamma-interferon in primary hepatocellular carcinoma. *J R Soc Med* 1985;78:826–9.
- [36] Lai CL, Lau JY, Wu PC, Ngan H, Chung HT, Mitchell SJ, et al. Recombinant interferon-alpha in inoperable hepatocellular carcinoma: a randomized controlled trial. *Hepatology* 1993;17:389–94.
- [37] Llovet JM, Sala M, Castells L, Suarez Y, Vilana R, Bianchi L, et al. Randomized controlled trial of interferon treatment for advanced hepatocellular carcinoma. *Hepatology* 2000;31:54–8.
- [38] Kubo S, Nishiguchi S, Hirohashi K, Tanaka H, Shuto T, Yamazaki O, et al. Effects of long-term postoperative interferon-alpha therapy on intrahepatic recurrence after resection of hepatitis C virus-related hepatocellular carcinoma. A randomized, controlled trial. *Ann Intern Med* 2001;134:963–7.
- [39] Ikeda K, Arase Y, Saitoh S, Kobayashi M, Suzuki Y, Suzuki F, et al. Interferon beta prevents recurrence of hepatocellular carcinoma after complete resection or ablation of the primary tumor—A prospective randomized study of hepatitis C virus-related liver cancer. *Hepatology* 2000;32:228–32.
- [40] Shiratori Y, Shiina S, Teratani T, Imamura M, Obi S, Sato S, et al. Interferon therapy after tumor ablation improves prognosis in patients with hepatocellular carcinoma associated with hepatitis C virus. *Ann Intern Med* 2003;138:299–306.
- [41] Patt YZ, Hassan MM, Lozano RD, Brown TD, Vauthey JN, Curley SA, et al. Phase II trial of systemic continuous fluorouracil and subcutaneous recombinant interferon Alfa-2b for treatment of hepatocellular carcinoma. *J Clin Oncol* 2003;21:421–7.

- [42] Sakon M, Nagano H, Dono K, Nakamori S, Umeshita K, Yamada A, et al. Combined intraarterial 5-fluorouracil and subcutaneous interferon-alpha therapy for advanced hepatocellular carcinoma with tumor thrombi in the major portal branches. *Cancer* 2002; 94:435–42.
- [43] Grem JL, Jordan E, Robson ME, Binder RA, Hamilton JM, Steinberg SM, et al. Phase II study of fluorouracil, leucovorin, and interferon alfa-2a in metastatic colorectal carcinoma. *J Clin Oncol* 1993;11:1737–45.
- [44] Hausmaninger H, Moser R, Samonigg H, Mlineritsch B, Schmidt H, Pecherstorfer M, et al. Biochemical modulation of 5-fluorouracil by leucovorin with or without interferon-alpha-2c in patients with advanced colorectal cancer: final results of a randomised phase III study. *Eur J Cancer* 1999;35:380–5.
- [45] Fountzilas G, Zisiadis A, Dafni U, Konstantaras C, Hatzitheoharis G, Papavramidis S, et al. Fluorouracil and leucovorin with or without interferon alfa-2a as adjuvant treatment, in patients with high-risk colon cancer: a randomized phase III study conducted by the Hellenic Cooperative Oncology Group. *Oncology* 2000;58:227–36.
- [46] Jo M, Kim TH, Seol DW, Esplen JE, Dorko K, Billiar TR, et al. Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. *Nat Med* 2000;6:564–7.
- [47] Palmieri G, Montella L, Milo M, Fiore R, Biondi E, Bianco AR, et al. Ultra-low-dose interleukin-2 in unresectable hepatocellular carcinoma. *Am J Clin Oncol* 2002;25: 224–6.
- [48] Aldeghi R, Lissoni P, Barni S, Ardizzoia A, Tancini G, Piperno A, et al. Low-dose interleukin-2 subcutaneous immunotherapy in association with the pineal hormone melatonin as a first-line therapy in locally advanced or metastatic hepatocellular carcinoma. *Eur J Cancer* 1994;30A:167–70.
- [49] Ishikawa T, Imawari M, Moriyama T, Ohnishi S, Matsushashi N, Suzuki G, et al. Immunotherapy of hepatocellular carcinoma with autologous lymphokine-activated killer cells and/or recombinant interleukin-2. *J Cancer Res Clin Oncol* 1988;114:283–90.
- [50] Patt YZ, Mavligit GM. Arterial chemotherapy in the management of colorectal cancer: an overview. *Semin Oncol* 1991;18:478–90.
- [51] Okuno K, Hirohata T, Nakamura K, Jinnai H, Shigeoka H, Koh K, et al. Hepatic arterial infusions of interleukin-2-based immunochemotherapy in the treatment of unresectable liver metastases from colorectal cancer. *Clin Ther* 1993;15:672–83.
- [52] Lygidakis NJ, Savanis G, Pothoulakis J, Kapetanakis A. Transarterial locoregional immunostimulation and chemotherapy in patients with unresectable secondary liver tumours. *Anticancer Res* 1994;14:643–6.
- [53] Kuhns MS, Epshteyn V, Sobel RA, Allison JP. Cytotoxic T lymphocyte antigen-4 (CTLA-4) regulates the size, reactivity, and function of a primed pool of CD4 + T cells. *Proc Natl Acad Sci U S A* 2000;97:12711–6.
- [54] Todryk SM, Tutt AL, Green MH, Smallwood JA, Halanek N, Dalgleish AG, et al. CD40 ligation for immunotherapy of solid tumours. *J Immunol Methods* 2001;248:139–47.
- [55] Melero I, Shuford WW, Newby SA, Aruffo A, Ledbetter JA, Hellstrom KE, et al. Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. *Nat Med* 1997;3:682–5.
- [56] Melero I, Gabari I, Corbi AL, Relloso M, Mazzolini G, Schmitz V, et al. An anti-ICAM-2 (CD102) monoclonal antibody induces immune-mediated regressions of transplanted ICAM-2-negative colon carcinomas. *Cancer Res* 2002;62:3167–74.
- [57] Pardoll DM. Therapeutic vaccination for cancer. *Clin Immunol* 2000;95:S44–62.
- [58] Stift A, Friedl J, Dubsy P, Bachleitner-Hofmann T, Schueller G, Zontsich T, et al. Dendritic cell-based vaccination in solid cancer. *J Clin Oncol* 2003;21:135–42.
- [59] Nair SK, Morse M, Boczkowski D, Cumming RI, Vasovic L, Gilboa E, et al. Induction of tumor-specific cytotoxic T lymphocytes in cancer patients by autologous tumor RNA-transfected dendritic cells. *Ann Surg* 2002;235:540–9.

- [60] Takayama T, Sekine T, Makuuchi M, Yamasaki S, Kosuge T, Yamamoto J, et al. Adoptive immunotherapy to lower postsurgical recurrence rates of hepatocellular carcinoma: a randomised trial. *Lancet* 2000;356:802–7.
- [61] Dudley ME, Wunderlich JR, Robbins PF, Yang JC, Hwu P, Schwartzentruber DJ, et al. Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002;298:850–4.
- [62] Kono K, Takahashi A, Ichihara F, Amemiya H, Iizuka H, Fujii H, et al. Prognostic significance of adoptive immunotherapy with tumor-associated lymphocytes in patients with advanced gastric cancer: a randomized trial. *Clin Cancer Res* 2002;8:1767–71.
- [63] Mulligan RC. The basic science of gene therapy. *Science* 1993;260:926–32.
- [64] Miller AD. Human gene therapy comes of age. *Nature* 1992;357:455–60.
- [65] Lotze MT, Kost TA. Viruses as gene delivery vectors: application to gene function, target validation, and assay development. *Cancer Gene Ther* 2002;9:692–9.
- [66] Davis ME. Non-viral gene delivery systems. *Curr Opin Biotechnol* 2002;13:128–31.
- [67] Ruiz J, Mazzolini G, Sangro B, Qian C, Prieto J. Gene therapy of hepatocellular carcinoma. *Dig Dis* 2001;19:324–32.
- [68] Saleh M, Stacker SA, Wilks AF. Inhibition of growth of C6 glioma cells in vivo by expression of antisense vascular endothelial growth factor sequence. *Cancer Res* 1996;56:393–401.
- [69] Goldman CK, Kendall RL, Cabrera G, Soroceanu L, Heike Y, Gillespie GY, et al. Paracrine expression of a native soluble vascular endothelial growth factor receptor inhibits tumor growth, metastasis, and mortality rate. *Proc Natl Acad Sci U S A* 1998;95:8795–800.
- [70] Lin P, Buxton JA, Acheson A, Radziejewski C, Maisonpierre PC, Yancopoulos GD, et al. Antiangiogenic gene therapy targeting the endothelium-specific receptor tyrosine kinase Tie2. *Proc Natl Acad Sci U S A* 1998;95:8829–34.
- [71] O'Reilly MS, Holmgren L, Chen C, Folkman J. Angiostatin induces and sustains dormancy of human primary tumors in mice. *Nat Med* 1996;2:689–92.
- [72] Griscelli F, Li H, Bennaceur-Griscelli A, Soria J, Opolon P, Soria C, et al. Angiostatin gene transfer: inhibition of tumor growth in vivo by blockage of endothelial cell proliferation associated with a mitosis arrest. *Proc Natl Acad Sci U S A* 1998;95:6367–72.
- [73] Schmitz V, Wang L, Barajas M, Peng D, Prieto J, Qian C. A novel strategy for the generation of angiostatic kringle regions from a precursor derived from plasminogen. *Gene Ther* 2002;9:1600–6.
- [74] Kuo CJ, Farnebo F, Yu EY, Christofferson R, Swearingen RA, Carter R, et al. Comparative evaluation of the antitumor activity of antiangiogenic proteins delivered by gene transfer. *Proc Natl Acad Sci U S A* 2001;98:4605–10.
- [75] Fillat C, Carrio M, Cascante A, Sangro B. Suicide gene therapy mediated by the Herpes simplex virus thymidine kinase gene/Ganciclovir system: fifteen years of application. *Curr Gene Ther* 2003;3:13–26.
- [76] Mesnil M, Yamasaki H. Bystander effect in Herpes simplex virus-thymidine kinase/ganciclovir cancer gene therapy: role of gap-junctional intercellular communication. *Cancer Res* 2000;60:3989–99.
- [77] Kianmanesh AR, Perrin H, Panis Y, Fabre M, Nagy HJ, Houssin D, et al. A “distant” bystander effect of suicide gene therapy: regression of nontransduced tumors together with a distant transduced tumor. *Hum Gene Ther* 1997;8:1807–14.
- [78] Drozdziak M, Qian C, Xie X, Peng D, Bilbao R, Mazzolini G, et al. Combined gene therapy with suicide gene and interleukin-12 is more efficient than therapy with one gene alone in a murine model of hepatocellular carcinoma. *J Hepatol* 2000;32:279–86.
- [79] Bilbao R, Gerolami R, Bralet MP, Qian C, Tran PL, Tennant B, et al. Transduction efficacy, antitumoral effect, and toxicity of adenovirus-mediated herpes simplex virus thymidine kinase/ ganciclovir therapy of hepatocellular carcinoma: the woodchuck animal model. *Cancer Gene Ther* 2000;7:657–62.

- [80] Qian C, Idoate M, Bilbao R, Sangro B, Bruna O, Vazquez J, et al. Gene transfer and therapy with adenoviral vector in rats with diethylnitrosamine-induced hepatocellular carcinoma. *Hum Gene Ther* 1997;8:349–58.
- [81] Qian C, Bilbao R, Bruna 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;22:118–23.
- [82] Wildner O, Blaese RM, Candotti F. Enzyme prodrug gene therapy: synergistic use of the herpes simplex virus-cellular thymidine kinase/ganciclovir system and thymidylate synthase inhibitors for the treatment of colon cancer. *Cancer Res* 1999;59:5233–8.
- [83] van der Eb MM, Cramer SJ, Vergouwe Y, Schagen FH, van Krieken JH, van der Eb AJ, et al. Severe hepatic dysfunction after adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene and ganciclovir administration. *Gene Ther* 1998;5:451–8.
- [84] Kaneko S, Hallenbeck P, Kotani T, Nakabayashi H, McGarrity G, Tamaoki T, et al. Adenovirus-mediated gene therapy of hepatocellular carcinoma using cancer-specific gene expression. *Cancer Res* 1995;55:5283–7.
- [85] Qiao J, Doubrovin M, Sauter BV, Huang Y, Guo ZS, Balatoni J, et al. Tumor-specific transcriptional targeting of suicide gene therapy. *Gene Ther* 2002;9:168–75.
- [86] Ohwada A, Hirschowitz EA, Crystal RG. Regional delivery of an adenovirus vector containing the *Escherichia coli* cytosine deaminase gene to provide local activation of 5-fluorocytosine to suppress the growth of colon carcinoma metastatic to liver. *Hum Gene Ther* 1996;7:1567–76.
- [87] Hirschowitz EA, Ohwada A, Pascal WR, Russi TJ, Crystal RG. In vivo adenovirus-mediated gene transfer of the *Escherichia coli* cytosine deaminase gene to human colon carcinoma-derived tumors induces chemosensitivity to 5-fluorocytosine. *Hum Gene Ther* 1995;6:1055–63.
- [88] Heise C, Sampson-Johannes A, Williams A, McCormick F, Von Hoff DD, Kirn DH. ONYX-015, an E1B gene-attenuated adenovirus, causes tumor-specific cytolysis and antitumoral efficacy that can be augmented by standard chemotherapeutic agents. *Nat Med* 1997;3:639–45.
- [89] Reilly RT, Machiels JP, Emens LA, Jaffee EM. Cytokine gene-modified cell-based cancer vaccines. *Methods Mol Med* 2002;69:233–57.
- [90] Cao L, Kulmburg P, Veelken H, Mackensen A, Mezes B, Lindemann A, et al. Cytokine gene transfer in cancer therapy. *Stem Cells* 1998;16(Suppl 1):251–60.
- [91] Trinchieri G. Interleukin-12: a cytokine at the interface of inflammation and immunity. *Adv Immunol* 1998;70:83–243.
- [92] Mazzolini G, Qian C, Narvaiza I, Barajas M, Borrás-Cuesta F, Xie X, 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;11:113–25.
- [93] Leonard JP, Sherman ML, Fisher GL, Buchanan LJ, Larsen G, Atkins MB, et al. Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production. *Blood* 1997;90:2541–8.
- [94] Barajas M, Mazzolini G, Genove G, Bilbao R, Narvaiza I, Schmitz V, et al. Gene therapy of orthotopic hepatocellular carcinoma in rats using adenovirus coding for interleukin 12. *Hepatology* 2001;33:52–61.
- [95] Mazzolini G, Narvaiza I, Bustos M, Duarte M, Tirapu I, Bilbao R, 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;3:665–72.
- [96] Mazzolini G, Narvaiza I, Perez-Diez A, Rodriguez-Calvillo M, Qian C, Sangro B, et al. Genetic heterogeneity in the toxicity to systemic adenoviral gene transfer of interleukin-12. *Gene Ther* 2001;8:259–67.

- [97] Narvaiza I, Mazzolini G, Barajas M, Duarte M, Zaratiegui M, Qian C, et al. Intratumoral coinjection of two adenoviruses, one encoding the chemokine IFN-gamma-inducible protein-10 and another encoding IL-12, results in marked antitumoral synergy. *J Immunol* 2000;164:3112–22.
- [98] Mazzolini G, Narvaiza I, Martinez-Cruz A, Arina A, Barajas M, Galofre JC, et al. Pancreatic cancer escape variants that evade immunogene therapy through loss of sensitivity to IFN γ -induced apoptosis. *Gene Ther* 2003;164:1067–78.
- [99] Melero I, Duarte M, Ruiz J, Sangro B, Galofre J, Mazzolini G, 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;6:1779–84.
- [100] Grimm CF, Ortmann D, Mohr L, Michalak S, Krohne TU, Meckel S, et al. Mouse alpha-fetoprotein-specific DNA-based immunotherapy of hepatocellular carcinoma leads to tumor regression in mice. *Gastroenterology* 2000;119:1104–12.
- [101] Woodberry T, Gardner J, Elliott SL, Leyrer S, Purdie DM, Chaplin P, et al. Prime boost vaccination strategies: CD8 T cell numbers, protection, and Th1 bias. *J Immunol* 2003;170:2599–604.
- [102] Rubin J, Galanis E, Pitot HC, Richardson RL, Burch PA, Charboneau JW, et al. Phase I study of immunotherapy of hepatic metastases of colorectal carcinoma by direct gene transfer of an allogeneic histocompatibility antigen, HLA-B7. *Gene Ther* 1997;4:419–25.
- [103] Habib NA, Ding SF, el-Masry R, Mitry RR, Honda K, Michail NE, et al. Preliminary report: the short-term effects of direct p53 DNA injection in primary hepatocellular carcinomas. *Cancer Detect Prev* 1996;20:103–7.
- [104] Horowitz JA, Maneval DC, Ryback ME, Johnson D, Harris MP, Demers WG, et al. Intra-arterial p53 gene therapy of liver malignancies: preclinical studies and initial clinical observations. *Cancer Gene Ther* 1997;4:S12.
- [105] Sung MW, Yeh HC, Thung SN, Schwartz ME, Mandeli JP, Chen SH, et al. Intratumoral adenovirus-mediated suicide gene transfer for hepatic metastases from colorectal adenocarcinoma: results of a phase I clinical trial. *Mol Ther* 2001;4:182–91.
- [106] Habib NA, Sarraf CE, Mitry RR, Havlik R, Nicholls J, Kelly M, et al. E1B-deleted adenovirus (dl1520) gene therapy for patients with primary and secondary liver tumors. *Hum Gene Ther* 2001;12:219–26.
- [107] Habib N, Salama H, Abd El Latif Abu Median A, Isac Anis I, Abd Al Aziz RA, Sarraf C, et al. Clinical trial of E1B-deleted adenovirus (dl1520) gene therapy for hepatocellular carcinoma. *Cancer Gene Ther* 2002;9:254–9.
- [108] Reid T, Galanis E, Abbruzzese J, Sze D, Wein LM, Andrews J, et al. Hepatic arterial infusion of a replication-selective oncolytic adenovirus (dl1520): phase II viral, immunologic, and clinical endpoints. *Cancer Res* 2002;62:6070–9.
- [109] Morse MA, Deng Y, Coleman D, Hull S, Kitrell-Fisher E, Nair S, et al. A Phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. *Clin Cancer Res* 1999;5:1331–8.
- [110] Fong L, Hou Y, Rivas A, Benike C, Yuen A, Fisher GA, et al. Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. *Proc Natl Acad Sci U S A* 2001;98:8809–14.
- [111] Sangro B, Qian C, Ruiz J, Prieto J. Tracing transgene expression in cancer gene therapy: a requirement for rational progress in the field. *Mol Imaging Biol* 2002;4:27–33.
- [112] Bilbao R, Bustos M, Alzuguren P, Pajares MJ, Drozdik M, Qian C, et al. A blood-tumor barrier limits gene transfer to experimental liver cancer: the effect of vasoactive compounds. *Gene Ther* 2000;7:1824–32.
- [113] Kondo M, Wagers AJ, Manz MG, Prohaska SS, Scherer DC, Beilhack GF, et al. Biology of hematopoietic stem cells and progenitors: implications for clinical application. *Annu Rev Immunol* 2003;21:759–806.

- [114] Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol* 2000;28:875–84.
- [115] Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM. Origin of endothelial progenitors in human postnatal bone marrow. *J Clin Invest* 2002;109:337–46.
- [116] Reyes M, Lund T, Lenvik T, Aguiar D, Koodie L, Verfaillie CM. Purification and e vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood* 2001;98:2615–25.
- [117] Moore MA. Putting the neo into neoangiogenesis. *J Clin Invest* 2002;109:313–5.