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TARGETING HEPATIC STELLATE CELLS FOR CELL-SPECIFIC TREATMENT OF LIVER FIBROSIS

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1. ABSTRACT

Since hepatic stellate cells (HSC) play a crucial role in the development of liver fibrosis, this cell is the major target for anti-fibrotic drugs. Most of the experimental drugs that influenced the HSC activity showed however low efficacy in vivo. Either a low uptake of the compounds in the cells that cause disease might account for this lack of effect, or side-effects in other cells may limit the dosage of the drugs. These side-effects may even counteract the beneficial effects. Therefore a selective delivery of drugs to the HSC may comprise a promising new way to improve liver fibrosis. The targeting to HSC has become a feasible option, because albumin-based carriers have been developed that preferentially distribute to HSC in fibrotic rat livers. In addition to the targeting of drugs, also the selective delivery of genes to HSC in fibrotic livers is of interest for therapeutic purposes and a start is made in this respect. The present review discusses the drugs to be targeted to HSC and summarizes some of the problems encountered during this novel strategy in the treatment of liver fibrosis.

2. INTRODUCTION

In the last decade, significant advances have been made in the development of new anti-fibrotic agents, mostly because of a better understanding of the cellular and molecular mechanisms that cause liver cirrhosis. The most promising classes of anti-fibrotic drugs have recently been summarized (1,2). Yet, only a few agents have reached the clinical testing stage, and successful pharmacological treatment of liver cirrhosis in patients is not yet feasible. In fact, at present only a liver transplantation can lead to satisfactory results. Probably, the best pharmacological treatment for cirrhosis nowadays is the removal of the injurious event, for example through a reduction of the viral load in patients with viral hepatitis. However, a complete cure remains an exception. By the time cirrhosis is diagnosed, the fibrotic process has usually progressed beyond 'the-point-of-no-return', and removal of the injurious event will only have limited effects. Agents acting at both the inflammatory and fibrogenic level are therefore needed.

The problems with drugs that influence the fibrogenic or inflammatory activity are, among others, a lack of cell specificity *in vivo* and consequently the occurrence of side effects. Due to these adverse reactions, dose limitations prevent effective treatment. For example, corticosteroids can be very effective in the management of several types of autoimmune and alcoholic hepatitis (3,4). However, they can only be applied for a short period of time in order to minimize the inherent side effects. For example, in the treatment of primary biliary cirrhosis, corticosteriods turned out to be rather toxic and not really effective (5). Therefore, a selective delivery of drugs to the diseased tissue and cell types therein may provide a solution for minimizing the adverse reactions and at the same time optimize the efficacy. The targeting of the corticosteroid dexamethasone to endothelial and Kupffer cells in fibrotic livers was studied by Melgert *et al* (6,7). However, although the delivery of this potent drug to these cells was successful in itself, the supposed anti-fibrotic effects that were expected on the basis of its anti-

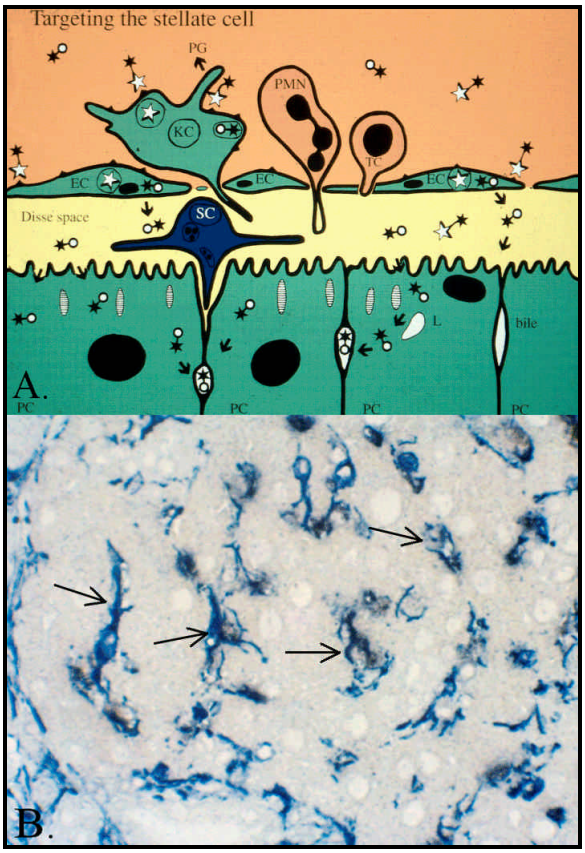

Figure 1. Targeting of drugs to different cell types in the liver. *A.* Drugs can be specifically delivered to various hepatic cell types, including the hepatic stellate cell. After modification of albumin with the sugar lactose a selective binding is obtained with the asialoglycoprotein receptor on hepatocytes (PC). Mannosylated albumin is used for targeting to Kupffer cells (KC), whereas negatively charged albumins are predominantly taken up by scavenger receptors (type A) present on the cell membrane of hepatic endothelial cells (EC). Targeting to hepatic stellate cells (HSC) is obtained with mannose-6-phosphate (M6P) modified albumin or albumin modified with cyclic peptides. *B.* Immunohistochemical demonstration of the co-localization of the carrier M6P-HSA, identified with anti-HSA IgG (red), and HSC, identified with anti-desmin and GFAP IgG (blue), in fibrotic rat livers (BDL3). Double positive cells are indicated with arrows (original magnification 400x).

inflammatory properties were disappointing. This was probably due to the fact that also the release of mediators with a negative feedback on the fibrotic process was blocked. In liver fibrosis, in particular targeting of drugs to the HSC (figure 1) could be more promising for the pharmacological treatment of this chronic liver disease, as will be outlined in this review.

3. WHY ARE HSC TARGET CELLS?

The liver consists of various cell types, i.e. parenchymal cells (also called hepatocytes), endothelial cells (sinusoidal as well as vascular type), Kupffer cells (macrophages), bile duct epithelial cells, pit cells, and hepatic stellate cells, that are all to some extent involved in the processes of initiation and perpetuation of liver fibrosis. However, the HSC are considered crucial, because of their prominent role in extracellular matrix production, regulation of vascular tone, and production of inflammatory mediators such as transforming growth factor-β (TGF-β) and platelet derived growth factor (PDGF). During fibrosis in particular these three processes are de-arranged. At a certain point in the whole process, the HSC perpetuate the fibrogenesis by creating several autocrine loops, thus maintaining the process even without contribution of the other cell types (8-13).

4. DRUG TARGETING

4.1. Drug targeting methodology

Drug targeting is an approach in which the body distribution of a drug is manipulated in order to increase the efficacy and reduce the toxicity of the drug. In this way, it is not the drug itself that determines the pharmacokinetics and cellular distribution, but the carrier molecule to which the drug is coupled. Some call this a magic bullet, but the magic is not so much in the carrier but rather in the target cell as will be pointed out in the next sections. The concept of drug targeting and the recent developments up to clinical perspectives are extensively described in the book 'Drug Targeting - Organ-Specific Strategies' (14).

The carrier modality chosen is often a macromolecular device. In our lab, we have frequently used the plasma protein human serum albumin (HSA), as well as sugar, charge and peptide modified forms (15-18). Albumin is abundantly present in blood (±4 g per 100 ml). The protein is readily water soluble, biodegradable, and biocompatible in contrast to various polymeric or lipidic carriers. Other macromolecular carriers used are liposomes or certain particles (nanospheres, microspheres, and viruses). As intrinsically active carriers certain antibodies and enzymes can be used (16,19). Two types of drug targeting strategies can be distinguished: passive and active targeting. During passive targeting, the strategy is to avoid side effects by preventing major distribution to a particular organ or cell type. For example, the cardiotoxicity of doxorubicin can be decreased by including it in a liposomal formulation (20,21). Normal (conventional) liposomes can release drugs slowly into the general circulation preventing toxic peak concentrations, but at the same time accumulate in macrophages or other cells of the reticuloendothelial system, due to the phagocytic activity of these cells. This may induce another type of toxicity (22). The size of a carrier protein is a factor that influences the body distribution, i.e. compounds with a relatively low molecular weight (<30-50 kD) will be filtrated in the kidneys, whereas proteins with a higher molecular weight generally are not removed by kidneys and are finally taken up by the liver (16,23). More specific targeting of carriers can be achieved by incorporation of site-directed ligands (homing devices) to the macromolecular backbone, thereby redirecting the construct to specific binding sites on cell membranes. This active targeting strategy can lead to higher therapeutic concentrations at the desired site of

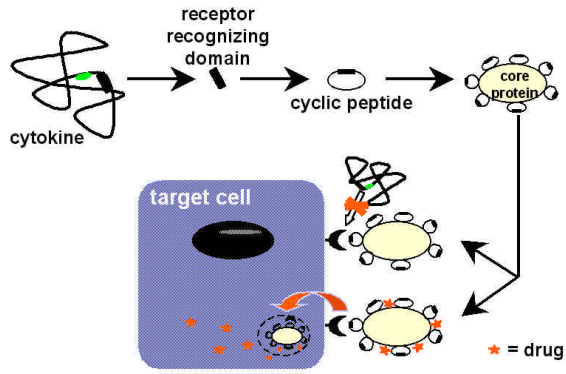

Figure 2. Schematic representation of the concept of drug targeting using receptor recognizing peptides. These cyclic peptides consist of the stretch of amino acids within a cytokine molecule that is specific for receptor binding, and serve as homing ligands for a macromolecular protein by covalent attachment to the protein backbone. The resulting carrier can subsequently be conjugated with drug molecules. Besides delivering the drug at or into the target cells, carrier or conjugate binding to the cytokine receptor may be able to inhibit activation of signal transduction pathways. The target cell in case of liver fibrosis is the hepatic stellate cell.

action. The homing devices with specificity for a certain membrane receptor can be carbohydrate ligands, functional groups bearing multiple negative or positive charges, antibodies, or peptide ligands. Since drug targeting preparations are applied in the diseased state, the cell-selectivity of the carriers should preferably be assessed under diseased conditions because in pathological situations often changes in receptor expression occur (24-26).

Proteins contain many different functional groups that can be used for conjugation of the chosen homing device or drug. For instance, the primary amino group of lysine and the thiol group of cysteins have been frequently used (27). Many conjugation procedures are based on nucleophilic substitution reactions in which an activated electrophilic group of the drug reacts with a nucleophilic group of the protein. In addition to the chemical modification of carriers, recombinant DNA approaches can be exploited for the preparation of suitable carrier molecules (27). Apart from conventional drugs, also anti-sense oligonucleotides, and genes are attractive candidates for cell specific delivery. At present a spectrum of gene modulating substances that interfere with multiple regulatory sites within the cell are under development, but often lack cell-specificity. In addition, radioactive ligands can be coupled to the HSC selective-carriers for diagnostic purposes of fibrosis, similar to the developed hepatocyte-directed ligands to detect fibrosis (28).

The majority of the drug targeting strategies employ receptor-specific ligands attached to the carrier-drug complex to deliver the drug to the target cell of choice. Depending on the subsequent routing of the receptor complex after internalization, the drug will end up in a specific compartment in the cell. Because most of the compounds are taken up via receptor mediated endocytosis, the degradation of the complex occurs within the endosomal/lysosomal compartments of the cell. The release of the active drug from the carrier and the subsequent transfer to the cytoplasm and/or other organelles is crucial to obtain a final therapeutic effect. If the rate of release of the drug from the carrier is not adequate, this can be improved by using a biodegradable spacer between the carrier and the drug. Various peptide-like and acid-sensitive spacers are described in literature, that can be split via enzymatic reactions or in a relatively acidic environment. The intracellular release profile of the targeted drug can in this manner be tailored to the therapeutic need (27,29,30).

4.2. HSC-selective drug carriers

Until a few years ago the targeting to HSC was not studied, and HSC-specific carriers were not described. At that time, we developed several macromolecules that can be recognized by HSC, and in particular by the HSC in fibrotic livers. Realizing that a number of receptors become overexpressed on the cell membrane of HSC after activation or proliferation, we designed three modified proteins with increased affinity for the HSC. The first target receptor chosen was the mannose 6-phosphate/ insulin-like growth factor II (M6P/IGFII) receptor, because it was reported to be highly upregulated on the cell membranes of activated HSC (31,32). HSA was modified with the sugar mannose 6-phosphate and *in vivo* experiments showed that this modified albumin accumulated rapidly in livers of rats with liver fibrosis (about 60% of the dose already at 10 min after iv injection). More importantly, the major part of the hepatic content was found in the HSC (33). This cell-specific distribution in fibrotic livers is both due to marked proliferation of HSC as well as the much higher expression of the particular receptor on this cell type. The specificity of binding to HSC was confirmed in *in vitro* studies. M6P-HSA bound in particular to the activated HSC and a rapid internalization of the protein occurred via a receptor-mediated endocytotic route (34).

In addition, two other HSC-selective carriers were obtained (18,35). Instead of the derivatisation of albumin with specific sugars, albumin was now modified with cyclic peptide moieties (minimized proteins) that represented the binding domains of cytokines/growth factors responsible for binding to the activated HSC (figure 2). In order to mimic the binding but not induce the subsequent signaling/effects, the binding sequence was separated from the domain that triggers the cellular effects. We prepared two different types of peptide-modified albumin, each of them bearing cyclic peptide groups that contained the receptor recognizing peptide sequence of either collagen type VI or PDGF-BB. In case of the binding to collagen type VI receptors, we modified the lysine groups of HSA with the cyclic RGD containing peptide C*GRGDSPC*, designated as pCVI-HSA (18). This albumin bound avidly to cultured rat HSC, in particular to the activated cell type, and also internalization was found in vitro, although not to the same extent as the M6P-HSA (34). *In vivo* a preferential distribution of pCVI-HSA to HSC in rats with liver fibrosis (3 weeks after bile duct

ligation) was noted. Ten min after intravenous injection 62±6% of the administered dose accumulated in the fibrotic rat liver, mostly in the HSC (18). Studies with PDGF receptor recognizing peptides, aiming for an even more selective interaction with the HSC and myofibroblasts of the fibrotic liver are in progress. Of note, both receptors play an important role in cell attachment and proliferation implying that the peptide modified albumin not only can specifically bind to the stellate cells but also may act as peptidic antagonists that can block cellular activity.

4.3. Drugs to be targeted

With the recently developed HSC-specific carriers, targeting of anti-fibrotic and anti-inflammatory drugs has become a feasible option. To couple a drug to a carrier, the drug does need some special features. First of all, a chemical group must be available in the drug that allows the coupling to the protein backbone, thereby causing no change in the specificity of the carrier. Moreover, the drug linkage should allow the release of the drug in a therapeutic active form after internalization in the target cell. Therapeutic agents that are brought to lysosomes by their carriers should be resistant to proteolytic enzymes and acidic conditions present in the endosomal and lysosomal cell compartment. Peptides and oligonucleotides generally do not meet this latter requirement.

Recently, comprehensive reviews on drugs with a potential beneficial effect in HSC have appeared (1,2). Some of these promising drugs that may benefit from targeting will be outlined below. Many drugs display potent anti-fibrotic effects *in vitro*, but are rather ineffective in the *vivo* situation, even at relatively high doses. One explanation for this is that the pharmacokinetic profile of these drugs, including cellular distribution *in vivo*, is not favorable. The challenge therefore is to manipulate the whole body distribution such that a selective delivery of the particular drug to the required cell type is attained. Drug delivery to a certain organ may not be sufficient. For instance, some drugs exert therapeutic effects in one hepatic cell type and can induce adverse effects in a neighboring one. A cell-specific targeting obviously can enhance the net effect of such drugs and reduce their side effects.

Pentoxifylline is a good example of a promising drug that may benefit from drug targeting strategies. It is seen as a powerful agent to inhibit liver fibrosis, since it reduces tumor necrosis factor-α production, prevents PDGF-related signaling in HSC, decreases collagen deposition and HSC proliferation, and positively influences the micro-circulation in livers (36-40). *In vivo*, the effects of pentoxifylline, however, vary from no effect at all to a minor inhibition of the development of liver fibrosis (in various rat models) (41-44). It has been postulated that the disappointing results of pentoxifylline *in vivo* were caused by an upregulation of the production of tissue inhibitor of metalloproteinase (TIMP) in Kupffer cells (45). This profibrotic effect may counteract the anti-fibrotic effects induced in HSC. Studies with targeted pentoxifylline in rats with liver fibrosis may prove this point.

Another candidate for cell specific delivery is gliotoxin. Driving activated HSC into apoptosis may be seen as a way to resolve fibrosis (46-48). In a study of Wright *et al* (49), fibrotic rats were treated with gliotoxin to induce apoptosis in HSC and this resulted in a reduced number of activated HSC and a decreased deposition of extracellular matrix. Although gliotoxin is a rather unspecific fungal toxin, no adverse reactions of the treatment were measured in hepatocytes (49). Potential effects on Kupffer and endothelial cells, however, were not reported. Other studies showed major effects of gliotoxin in several cells of the immune system. The drug has been shown to induce apoptosis in macrophages, thymocytes, and T lymphocytes (50). Since no cell-specific uptake mechanism in cells is reported for gliotoxin, also apoptotic effects in liver macrophages (e.g. Kupffer cells) may be expected *in vivo*, in addition to those seen in HSC. Therefore, it may be necessary to target gliotoxin to the HSC, at least to circumvent uptake of this compound by cells of the immune system.

Cytokines are pleiotropic molecules that have many activities in many cell types. A cytokine may have beneficial effects in one cell type, but may be harmful in another cell. Compounds that also may benefit from drug targeting are cytokine antagonists. For example, TGF-β is a key mediator in the induction of liver fibrosis and the inhibition of TGF-β thus appears to be an attractive intervention (51). TGF-β is involved in matrix deposition via stimulation of collagen type I and TIMP production and by inhibition of metalloproteinase production. TGF-β activates HSC in combination with PDGF, has chemo-attractive properties, and it induces apoptosis. Hepatocytes are reported to be more sensitive to its apoptosis inducing effect than non-parenchymal cells such as HSC. Furthermore, TGF-β modulates numerous genes relevant to tumor initiation and progression. A number of reports have proposed to inhibit TGF-β production to treat fibrosis (52-56). However, concerns may be raised on the safety of a prolonged inhibition of TGF-β activity. This because of general growth dysregulation, increased risk of neoplasia, and the ability to trigger autoimmune diseases (51,57). Since its receptors are expressed on many cell types, only a targeting approach seems feasible for TGF-β antagonists.

Other agents that are currently of interest are peroxisome proliferator-activated receptor (PPAR)-γ agonists (58,59). Although PPAR-γ is predominantly expressed in adipose tissue, where this nuclear factor regulates lipid metabolism and adipocyte differentiation, the liver also contains PPAR-γ. Within the liver, PPAR-γ is both expressed in Kupffer cells and HSC. Its gene expression in HSC is reduced during cellular activation as observed in fibrosis. Beneficial effects of PPAR-γ agonists in stellate cells are now reported *in vitro* (60,61). However, care is needed for the application *in vivo*, because peroxisome proliferators are also associated with carcinogenicity especially during long-term treatment, and the Kupffer cell is related to this toxicity (58). Cell specific targeting may be a solution to confine the actions of PPAR-γ agonists to the HSC.

During fibrosis the balance between the production and the degradation of matrix constituents is affected, resulting in increased deposition of extracellular matrix in fibrotic livers. This process is an important target for those anti-fibrotic agents that either reduce matrix production or enhance matrix degradation. The latter may occur via increased activity of metalloproteinases or inhibition of TIMP. Reducing the expression of TIMP-1 activity appears to be a powerful way to enhance collagenase activity within diseased livers (62,63). A small increase in TIMP-1 expression in fibrotic livers induced by, for instance, dexamethasone was associated with a strong increase in collagen deposition (7,64). Interference with matrix turnover as a way to treat liver fibrosis should obviously be confined to the liver to avoid serious problems in other organs.

Vasoconstriction also contributes to the deterioration of liver function during liver fibrosis (11,65,66). Both endothelial cells and HSC control the vascular tone within the liver, and the most important mediators in this respect appear to be nitric oxide (NO) and endothelin (ET) (67-73). NO is an important vasodilator and its production is reduced during cirrhosis (74). Several pharmacological interventions aim at enhancing intrahepatic NO production as a way to protect liver function and reduce portal hypertension (75-77). However, systemic vasodilating agents can not be applied, since liver fibrosis is characterized by intrahepatic vasoconstriction combined with extrahepatic vasodilatation (65). The use of Pyrr-NO, which is activated within hepatocytes, is a sophisticated approach to solve this (78). However, as indicated above, it is not the hepatocyte but rather endothelial cells and HSC that are important for the vascular tone. A cell-selective approach in the liver is also needed with other compounds that interfere with hemodynamic parameters, because the systemic vascular tone should not be affected. Examples are angiotensin II agonists, inhibitors of angiotensin converting enzyme (ACE), and ET-antagonists, all of which are emerging compounds whose anti-fibrotic effects are explored now (79-83). Opposite pharmacological effects with these types of drugs are needed in the liver and the systemic vasculature, which may be troublesome. At our lab, the ACE inhibitor captopril was selectively targeted to the kidney in normal rats and in rats with proteinuria (84,85), thereby avoiding systemic ACE inhibition. Endothelin induces constriction of HSC, via ET-A receptor activation, and relaxation of EC via triggering of ET-B receptors and subsequent release of NO. Upon activation, HSC express ET-A and ET-B receptors (86), and in particular ET-A plays an important role during liver fibrosis (87). In recent years many attempts have been explored to attenuate hepatic ET production or antagonize its effects (82,88-90). In particular vasoactive substances may benefit from a targeting strategy.

Until now we are not able to report on the effectivity of targeted anti-fibrotic agents mentioned above. The bottleneck of the targeting approach is the preparation of chemical constructs. The chemical bond between the drug and the drug carrier should be stable outside the cell, but on the same time should allow the release of the active drugs within the cell. Furthermore, the substitution of a drug to the carrier system should not corrupt the cell-specific accumulation of the carrier. To date, the chemical conjugation of various anti-fibrotic drugs to the albumin carrier are the object of extensive studies.

4.4. Gene targeting to HSC

Not only drugs but also genes are of interest to be specifically targeted to HSC. Gene therapy is an elegant way to correct genetic deficiencies or to induce the production of essential proteins in a certain cell type. Adenoviral or lipid based non-viral vectors are options to deliver genes and antisense material to cells. Most therapies aiming at the modulation of gene expression in pathogenic cells suffer from deficient uptake of the active agents in the target cell. For reviews on hepatic gene targeting, the reader is referred to references (91-96).

Weiskirchen *et al* evaluated various methods of transfecting HSC (97). For lipid particles the highest transfection efficiency *in vitro* was obtained with the FuGENE^{TM}6 method, that is, 6% of the rat HSC in culture were transfected, whereas none of the rat myofibroblasts were transfected with the chosen model gene. With an adenoviral-based vector (Ad5), a transfection of 100% of HSC and myofibroblasts was obtained. So, the introduction of genes into target cells via adenoviruses is effective. However, it should be taken into account that adenoviruses also introduce genes in other hepatic cells. In particular, hepatocytes are target cells for this virus, because they express coxsackie-adenovirus receptor (CAR) on the cell membrane, which displays high affinity for adenoviruses (98). The internalization of adenoviral particles is also promoted by integrins present on cell membranes (99-101). HSC and myofibroblasts express high levels of various types of integrins (102,103), which is an advantage with regard to the adenoviral infection. On the cell membrane of endothelial cells, however, integrins are also abundantly present (104,105). The approach of Weiskirchen *et al* to improve the specificity of the adenoviral transduction comprises the addition of a promoter to the viral construct that are recognized by genes that are only present in HSC (106). An example of this gene is CRP2 (cysteine- and glycine-rich protein 2) (107). In this approach, a cell-selective expression is achieved by cell-selective transcription rather than cell-selective delivery.

A few reports on gene delivery to the cirrhotic liver or to HSC have appeared in the past few years, mostly using adenoviral mediated transduction methods. A hepatic delivery of neuronal NOS (Nitric Oxide Synthase) by Yu *et al* (77) resulted in a reduction of the intrahepatic resistance and portal pressure in *vivo* models of liver fibrosis. Qi *et al* (53) delivered a dominant-negative type II transforming growth factor-β receptor gene to the liver and found a blocking of transforming growth factor-β, which attenuated the development of liver fibrosis. Other examples of gene delivery in the context of liver fibrosis are the hepatic delivery of telomerase RNA by Rudolph *et al* (108), and of urokinase-type plasminogen activator by Salgado *et al* (109).

An advantage of adenoviral transduction is its relative specificity for the liver, which means that predominantly hepatocytes but also HSC, Kupffer and endothelial cells will be transfected and consequently the systemic toxicity may thus be reduced. A disadvantage is that the gene expression is generally elevated for a relatively short period of time and repeated administration induces an immune response against the virus. This may eliminate the therapeutic effects and cause serious side effects. An adjuvant therapy with immunosuppressive drugs together with the adenoviral vector might reduce the immune response. But another strategy may be to induce a rapid uptake of viral constructs by target cells and thus to avoid uptake by immune-competent cells.

5. PERSPECTIVES

In the next few years, the targeting of drugs and genes to HSC will be further studied and the real value of this targeting approach compared to conventional therapy will become known. As discussed in the present review, drug targeting may create new opportunities for 'old' drugs that are not sufficiently effective or that display serious extra- or intrahepatic side effects. Although the research concerning gene delivery to HSC is just started, it offers good perspectives and we will hear more of it in the next couple of years. The targeting to HSC may not only be of value in liver fibrosis/cirrhosis, but HSC are also involved in other liver diseases, such as hepatitis (110,111) and hepatocellular carcinoma (HCC) (112-114). These chronic diseases are difficult to treat and drug targeting technology may provide opportunities for its improvement.

Drug targeting also offers possibilities in more pathological or fundamental research areas. Targeting of pharmacological active agents to an individual cell type offers the advantage of selective elimination of this cell type or blockade of a single process within this cell type. After specific intervention, the implications of such manipulation for the development of a particular disease can be studied in the *vivo* situation. In this way, drug targeting methods can be used to gain more insight in the molecular basis of diseases *in vivo*. Drug-targeting preparations may therefore not only improve the efficacy and safety of appropriate drugs they are also instrumental in target finding procedures in drug innovation.

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Abbreviations: HSC = hepatic stellate cells, TGF-β = transforming growth factor beta, PDGF = platelet-derived growth factor, HSA = human serum albumin, M6P/IGFII receptor = mannose 6-phosphate/ insulin-like growth factor II receptor, M6P-HSA = HSA modified with mannose 6-phosphate groups, pCVI-HSA = HSA modified with the cyclic RGD peptide C*GRGDSPC*, TIMP = tissue inhibitor of metalloproteinase, PPAR = peroxisome proliferator-activated receptor, NO = nitric oxide, ET = endothelin, ACE = angiotensin converting enzyme

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