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Cell-specific delivery of biologicals: problems, pitfalls and possibilities of antifibrotic compounds in the liver

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Liver fibrosis is a complex disease affecting millions of people world-wide. It involves the activation of several cell types whose activities are tightly controlled by endogenous mediators. No pharmacotherapy is available for this disease, despite the fact that many experimental drugs are very effective *in vitro* and the liver is easily accessible for most drugs. Our review provides arguments showing that cell-selectivity is essential for most antifibrotics. Several cell-specific drug carriers targeting the key pathogenic liver cells are discussed with special focus on hepatic stellate cells and fibroblast-like cells. Since endogenous mediators represent a powerful set of tools to modify the pathogenic process, this review focuses on these mediators as therapeutics and the problems and pitfalls associated with the use of such biologicals.

The aiming points

We have entered the era of biologicals [1]. Although new chemical entities are still produced and successfully reach the market, many new biological products like antibodies and their derivatives, siRNA, cytokines, enzymes and other therapeutic peptides are now being developed. Already a third of all new therapeutic products in 2011 were biologicals rather than chemical derivatives [1].

These biologicals provide many new exciting opportunities but also new challenges. New opportunities include manipulation of complex biological processes using endogenous substances with potent pleiotropic activities. Cytokines for instance provide powerful tools that are effective in the picomolar range within the key effector cells of diseases [2–4]. Within the liver field Interferon α2a and α2b (Pegasys respectively PegIntron) have revolutionized the treatment of Hepatitis B and C, providing an unmet medical need at the time of their introduction [5,6].

However, native endogenous substances are subjected to endogenous clearance mechanisms that affect their pharmacokinetic profile considerably. Most cytokines and lipid mediators are locally acting mediators due to rapid inactivation and degradation in plasma, renal clearance or uptake by different cell types. Their limited action radius is important because receptors for these mediators are often expressed throughout the body. Systemic administration either leads to lack of efficacy due to the aforementioned clearance mechanisms or, after increased dosing compensating for these mechanisms, cause multiple adverse effects. Pegylation of cytokines [7], leading to increased plasma stability and reduced renal clearance, is an efficient way to overrule these clearance mechanisms, but although the efficacy of pegylated or other long-circulating biologicals is in many cases significantly enhanced, adverse effects may also be enhanced.

In addition, it is becoming increasingly clear that the pleiotropic effects of cytokines often lead to a combination of local effects depending on the cell types present. It may be difficult, if not impossible, to modulate this overall effect simply by increasing the concentration of a cytokine in the diseased tissue. For instance, Il-10 has powerful anti-inflammatory effects in dendritic cells, B-cells, CD4+ and Cd8+ cells and M1 macrophages [8], thus preventing fibrogenesis but it also exerts pro-fibrotic effects in M2c macrophages [9,10] thus stimulating fibrogenesis. In a mixed cell population the outcome of treatment will depend on the dominant cell type that is present in the tissue. An exciting new line of compounds in the field of biologicals is siRNA. SiRNA has been very effective in blocking pathological responses in effector cells *in vitro* but its intracellular delivery and delivery to the target cells *in vivo* are key problems that significantly hamper their clinical development [11–13].

In this review we aim to summarize the possibilities to deliver anti-fibrotic agents to the fibrotic liver. We specifically focus on the use of biological products because these represent an exciting

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new group of compounds whose therapeutic application becomes within reach after modification of their *in vivo* distribution profile.

Options for improvement

To prevent the rapid clearance of biologicals, long-circulating compounds have been developed. In particular pegylation of cytokines has proven to be a valuable strategy. Interferonα has been pegylated (Pegasus and PegIntron) leading to a prolonged circulation time; the circulation time of Interferon α, which is only a few hours [14], was increased to several days after Pegylation [15] allowing dosing schedules of once or twice a week [14,15]. This was associated with an increased therapeutic efficacy compared to native Interferon α. The long circulation time led to an increased uptake into hepatocytes, the target cells for this disease. These compounds provided therefore a substantial improvement over standard therapies with chemical drugs, although adverse effects of these long circulating compounds were still evident [14]. Recently, new direct-antiviral drugs with high efficacy against Hepatitis B and C and less adverse effects have been developed [16,17], demonstrating that the chemical approach is not made redundant. Nevertheless, pegylation of cytokines is nowadays a widely pursued approach. Another approach is the coupling of compounds to albumin or incorporation in liposomes which prolongs the plasma half-life and thereby the efficacy of drugs and biologicals [18]. Doxil (Caelyx) is based on doxorubicin incorporated in pegylated liposomes and this compound has less adverse effects than the free drug in cancer patients [19]. The use of pegylated liposomes also has been explored for antifibrotic therapies and successful delivery of Hepatocyte Growth Factor to the fibrotic liver has been achieved in rats [20], but this approach has not led to any follow up yet.

This strategy of prolonging plasma half-life is particularly successful in diseases characterized by an increased local vascular permeability. Angiogenesis in tumors, associated with poorly developed microvessels, or acute and chronic inflammatory processes, associated with local release of many vasoactive compounds, are characterized by local extravasation of compounds [21,22]. The low local extravascular pressure will subsequently lead to a relative retention of blood-derived compounds, in particular of high-molecular weight compounds like immunoglobulins, albumin-bound substances and exogenous products like liposomes, polymers, or pegylated compounds. Due to this enhanced permeability and retention (EPR) effect, a prolonged plasma half-life of such compounds leads to higher drug concentrations in diseased areas and therefore to higher efficacy of drugs.

However, this approach is not always a proper solution. A prolonged circulation-time also leads to a prolonged exposure of non-target cells to the drug, and may thus also lead to enhanced adverse effects. In addition, a prolonged circulation time by increasing size generally means reduced renal clearance. This will result in higher non-specific uptake by other cells endowed with multiple receptor-mediated uptake mechanisms, such as hepatocytes or antigen presenting cells, including macrophages. *Via* this mechanism, immune responses were elicited against PEG in long-circulating Pegylated liposomes [23–25].

As stated above, many cytokines have pleiotropic activities with effects in one cell type being counterbalanced by effects in a neighboring cell type. These balanced effects cannot be modulated by longer circulation times. The differential effects of Il-10 have been mentioned already but many other therapeutic cytokines may encounter the same pitfall. Interferon-γ (INF-γ) for instance inhibits fibroblast-like cells [26], it stimulates polarization of macrophages into an antifibrotic phenotype [9,27], and it enhances NK-mediated apoptosis of hepatic stellate cells [28], all leading to reduced fibrogenic activity. However, it also stimulates production of the macrophage chemoattractant MCP-1 [29], thereby stimulating inflammation. In addition, INFγ-stimulated NK-cells in non-alcoholic steatohepatitis (NASH) seem to drive the progression of hepatitis towards fibrosis [30]. So, *via* effects on different cell types a mixed result on fibrogenesis is achieved upon systemic administration of Interferon-γ. Similarly, TGFβ is one of the most potent stimulators of fibrosis in fibroblasts [31], yet it also has anti-inflammatory effects on macrophages [8,9]. In these cells TGFβ induces an enhanced IL-10 and PGE2 production, both of which can induce subsequently pro- and antifibrotic effects [32,33], thus balancing TGFβ-mediated effects. Thus, for all these cytokines and mediators the local effects seem to depend on the composition of local cell types that respond to the mediator. Therefore even enhanced local delivery of cytokines and other mediators to diseased tissues, that is, by prolongation of their circulation time, may yield mixed responses and hence low efficacy, despite their potency *in vitro*.

In addition, some diseases are not characterized by an enhanced vascular permeability. A hallmark of fibrotic and sclerotic diseases is an increased deposition of extracellular matrix constituents. And although enhanced angiogenesis is found during for instance liver fibrosis [34], this disease is associated with a reduced size of endothelial fenestrae and capillarisation of hepatic sinusoids caused by the collagen deposition in the space of Disse and thus to a reduced vascular permeability in the diseased areas [34]. Similarly, some solid tumors have increased pressure inside the mass due to rapid cell proliferation, thus limiting the advantage of long circulating compounds.

Liver fibrosis

In order to overcome these problems, cell-specificity may be essential for therapeutic success. For anti-tumor therapies, cell-specific approaches are widely pursued but also for liver fibrosis, such an approach may be quite relevant. It is a complex disease, induced by the concerted action of many cell types. It can be induced by viruses (Hepatitis B and C), genetic disorders (*e.g.* Wilson disease), autoimmune-mediated disorders (Primary Biliary Cirrhosis, Primary Sclerosing Cholangitis), toxins, alcohol and obesity (NASH) and it affects millions of people world-wide. To date, there is no pharmacotherapy available to treat liver fibrosis or its end-stage cirrhosis [35]. The only options are liver transplantation or removal of the inciting stimulus.

As outlined above, much progress has been achieved with potent new antiviral drugs thus preventing the cause of this disease, yet liver fibrosis as such cannot be treated yet [36,37]. This is also true for other fibrotic and sclerotic diseases like Idiopathic pulmonary fibrosis and renal fibrosis. The reversal of fibrosis seen in some rodent models and in patients with sustained viral reduction [38], demonstrates that scar tissue formation is not an irreversible process, which provides opportunities for therapeutics.

The key cells in the pathogenesis of liver fibrosis are hepatic stellate cells and portal fibroblasts [39]. These cells proliferate upon

activation after tissue damage and transform into (myo)fibroblasts that are highly proliferative and contractile cells and these cells produce extracellular matrix constituents, mostly collagens type I and III. This process is mostly initiated by mediators produced by damaged hepatocytes, activated Kupffer cells and infiltrating macrophages.

Antifibrotic drugs

Many antifibrotic drugs have been tested, however, none of these has reached the clinic [35]. Various reasons have been listed for this lack of success: the chronic nature of liver fibrosis that may comprise decades; the long lag-time between the inciting stimulus and significant disease activity with only subclinical symptoms until the end-stage; and the lack of clear disease parameters. All this imposes severe demands on drugs and clinical trials. In addition, the disease is complex, involving many cell types. Fibrosis basically is a dysregulated tissue remodeling and wound healing process that is part of normal tissue turnover and repair. These processes are tightly regulated and pharmacological interventions that were often very successful *in vitro* or in specific animal models yielded negative results in patients.

There are many examples of antifibrotic drugs that have beneficial effects in one cell type, yet display completely opposite effects in other cell types. The differential effects of cytokines (INFγ, TGFβ, Il-10) have been outlined above but this is also true for many drugs. For instance, cyclooxygenase inhibitors exert anti-inflammatory effects in Kupffer cells, thereby inhibiting fibrogenesis associated with chronic inflammation [40]. However, these very same products potentiate the response of hepatic stellate cells on profibrogenic mediators thereby stimulating the fibrogenic process [41]. The net inhibition of liver fibrosis is at best modest or highly variable, depending on the local composition of inflammatory cells at the time of treatment. In addition, drugs that prevent apoptosis may be beneficial in liver diseases associated with hepatocyte death [42], but detrimental to liver fibrosis where regression is induced by apoptosis of the collagen-producing cells [43,44]. Similarly, inhibition of TGFβ-mediated effects by the use of kinase inhibitors may yield antifibrotic effects [45], but these inhibitors may induce transformation of hepatocytes into hepatocellular carcinoma cells [8,46], which implies a high risk in cirrhotic patients who already have an increased incidence of liver cancer.

These examples illustrate the need for cell-specific delivery of antifibrotic compounds. The key cells for such compounds are hepatic stellate cells, portal fibroblasts or myo-fibroblasts that all produce extracellular matrix constituents. Liver uptake of drugs mostly represents uptake by hepatocytes and Kupffer cells which are endowed with multiple receptor-mediated endocytotic mechanisms. In Kupffer cells such compounds are mostly degraded intracellularly, providing a problem for biological compounds. In addition, Kupffer cells are not the designated target cells to attenuate collagen production, although recent studies do show a significant regulatory role for macrophages during fibrogenesis and regression of fibrosis. New antifibrotic drugs affecting specific macrophage activities can therefore be envisioned in the future, but inhibition of ECM production can only be achieved by modulation of fibroblast activities. Cell-specific approaches are needed to achieve that goal.

Magic bullets

Monoclonal antibodies are highly cell-specific magic bullets. They can have very high affinity for a particular receptor or protein and they can be long to very-long circulating compounds, with the benefits related to this (see above). In the past decade, these cell-specific compounds have provided major breakthroughs in many areas, in particular in the cancer field [47]. Antibodies against growth factor receptors (Herceptin, Avastin, *etc.*) are representatives of successful biologicals that have reached the market in recent years. Hundreds of monoclonal antibodies are now in clinical trials and many new antibodies will reach the market the coming years.

However, the use of antibodies as drug carriers is characterized by intrinsic problems. Their long half-life may be a disadvantage when their final uptake by antigen-presenting cells leads to immunogenicity. Many antibodies bind to extracellular targets that are not linked to endocytotic mechanisms, which seriously limits their use as transporters for drugs that act intracellularly or that need intracellular proteolytic enzymes for their release from the carrier. Also the payload of drugs, that is, the total amount that can be delivered into the target cell, may be limited because the structure of immunoglobulins does not allow attachment of high amounts of drugs. Only very potent drugs are suitable for this approach. The development of derivatives of immunoglobulins such as Fab- and single chain-fragments does not solve most of these problems. In addition, the costly development of monoclonal antibodies and the complex synthesis of drug-immunoglobulin constructs comprising a drug, a linker and the protein molecule are serious limitations for any product to reach the market. The cost-of-goods, intellectual property rights on different constituents and the combination of a chemical drug and a protein creates serious hurdles for GMP production and market introduction of such constructs. In the cancer field the first drug-monoclonal antibody constructs, after decades of research, now have reached the market [1,48]. In the liver field, as in many other clinical relevant areas, such constructs are still non-existing.

Towards liver cell-specific antifibrotic drugs

In the past years, a few groups have developed several cell-specific constructs that accumulate in activated hepatic stellate cells [49,50]. Coupling of mannose-6-phosphate (M6P) to albumin creating a molecule that binds to M6P/insulin-like growth factor II receptor [51] and a peptide that binds to the Platelet Derived Growth Factor receptor [52,53] have provided the first opportunities to reach this target cell. Activated hepatic stellate cells are characterized by high expression of both of these receptors. Meanwhile, many different drugs have been coupled to these carriers [49,50]. Both carriers are rapidly and massively taken up by the liver (>50–70% of the injected dose in some cases [51,52] and double stainings have demonstrated that the majority of this liver uptake reflects uptake in activated hepatic stellate cells and fibroblasts. Targeted drugs were in most cases significantly more effective in models of liver fibrosis in rats and mice than untargeted drugs [53–55]. These M6P-R and PDGF-R directed proteins have also been successfully used to deliver an adenoviral construct [56] and liposomes [57] to the hepatic stellate cells. Mahato *et al.* applied these M6P-modified albumin molecules to deliver triplex-forming oligonucleotides to the hepatic stellate cells [58].

These triplex-forming oligonucleotides are able to bind to their target DNA, thus effectively blocking transcription of relevant genes. Attachment of these molecules to M6P-Albumin led to effective delivery into hepatic stellate cells, but this was associated with immunogenicity against the construct. Replacement of albumin with HPMA polymers with lower immunogenicity yielded effective attenuation of the fibrotic process in rats. The group of Wright *et al*. developed a single chain antibody that binds to the synaptophysin receptor on hepatic stellate cells (C1–3) and this also has shown to represent an effective approach in delivering apoptotic drugs to this cell type [59]. In addition, vitamin A-coupled liposomes have been applied to deliver siRNA against the rat homolog of human Heat Shock Protein 47 into hepatic stellate cells [60]. These liposomes successfully delivered siRNA into the designated target cells in rat models of fibrosis.

These studies show that much progress has been obtained in delivering drugs and biologicals to hepatic stellate cells. Which hepatic stellate cell-directed drug carrier is the most effective remains to be established. All these carriers achieve effective targeting of the key target cells during liver fibrosis. They differ with respect to uptake by non-target cells, the rate of endocytosis (high for M6P-based carriers, lower for others) and chemical composition. M6P-derivatized-proteins represent neoglycoproteins, with their own benefits and disadvantages with respect to synthesis and pharmacokinetic profile compared to peptide-modified proteins and antibodies. The chemical synthesis of neoglycoproteins involves a complex, multi-step process [51] and they may be immunogenic [58], which is an important consideration for long term treatments. Peptide-modified proteins are easier to produce and less immunogenic, in particular when an endogenous sequence is applied as homing device (such as PPB [52]), but their stability in plasma may be potential hurdles. Antibodies are more stable, yet the above-mentioned problems with monoclonal antibodies in general may hamper further development of such constructs. Also the route administration of such constructs is an important factor for success. Most biological constructs require systemic administration, which is a disadvantage for chronic diseases like liver fibrosis. Treatment may comprise months or even years, making the use of intravenous therapeutics unrealistic.

Some years ago we produced a small peptide binding to PDGF-β receptors on activated hepatic stellate cells [52]. We coupled this peptide to the signaling moiety of Interferon γ [61], thereby inducing interferon-γ effects in PDGF-positive cells and thus avoiding adverse effects in other cells that express INFγ receptors. In this way pro-inflammatory effects on immune competent cells are separated from antifibrotic effects on fibrogenic cells. Significant antifibrotic and anti-angiogenic effects were obtained in mouse models of fibrosis and in tumor models, without the adverse effects that characterize INFγ-based therapies [53,61]. This minimalized construct, containing a small peptide-based homing device and a peptide-based therapeutic entity can easily be produced by recombinant techniques and chronically administered, amongst others subcutaneously. Therefore it lacks the disadvantages of most drug-carrier constructs. Its size, structure and short plasma half-life also confer a low risk for immunogenicity although this remains to be examined. This approach may open the door to the clinical use of cytokines or derivatives thereof that are left unexploited due to adverse effects despite their potency and key regulatory activities during disease progression.

Other important cells in the pathogenesis of liver fibrosis include cholangiocytes and macrophages. Cholangiocytes that reside in bile ducts are an important source of fibroblast-activating mediators upon damage to bile ducts [62,63]. These cells contribute importantly to tissue remodeling in primary biliary cirrhosis, primary sclerosing cholangitis and other bile duct related diseases. Recent work has shown that the integrin αvβ3 receptor is highly and selectively expressed on activated cholangiocytes and a peptide has been developed that selectively binds to this receptor [64]. This represents the first effective drug carrier to this important cell type.

There is also growing evidence for an important regulatory role for macrophages in the development of liver fibrosis [65,66]. Several subtypes of macrophages have been identified in recent years, with different, even opposing activities during fibrogenesis. On the one hand macrophages can adopt a pro-inflammatory phenotype that regulates host defense mechanisms, but they can also adopt anti-inflammatory and profibrogenic activities that regulate tissue repair and remodeling processes [65,66]. Macrophages can be reached by a variety of mechanisms; through delivery of constructs to the mannose receptor [67], the scavenger receptor [68], the folate receptor [69] or by administration of high molecular weight compounds such as liposomes, polymers (*e.g.* dendrimers) or micelles [49,50,70,71]. These constructs have been available for quite a while but the specificity of all these constructs needs to be re-evaluated with respect to the different macrophage phenotypes that have been identified now. The preference of a macrophage-specific carrier for a particular phenotype is quite relevant in view of the different key activities that macrophages can perform during fibrogenesis [67]. Indeed, we have previously shown the importance of choosing the right drug for the right phenotype when we found aggravated fibrosis after delivery of the anti-inflammatory drug dexamethasone to the mannose-receptor [9]. This drug was found to induce a pro-fibrogenic phenotype in this type of macrophages in rats. This highlights the need for macrophage phenotype-specific delivery of compounds when trying to modulate macrophage behavior and this represents an important area of research the coming years.

Finally, the use of adenoviruses that bind to the coxsackie and adenovirus receptor (CAR), or galactose-containing proteins, polymers (dendrimers) and liposomes that bind to the Asialoglycoprotein receptor (ASGPR), or Apolipoprotein A-1 containing carriers that bind to the scavenger receptor B1 (SR-B1) [72] complete the set of carriers available for cell-specific liver treatments [73,74]. CAR, ASGPR and SR-B1 receptors are abundantly expressed on hepatocytes and carriers that bind to these receptors can therefore be used to deliver drugs to this cell type. SiRNA for instance has successfully been delivered to hepatocytes using dendrimers binding to the ASGP-R [75] and liposomes binding to the SRB1 receptor [72]. Antiviral compounds or cytoprotective drugs can thus be selectively delivered to the hepatocyte. Liver fibrosis starts with damage to these cells and therefore delivery of therapeutic entities to these cells may be relevant as an antifibrotic therapy [38] although such an approach does not target the fibrogenic process itself. Many different drugs have been delivered to hepatocytes using one of these approaches but none has reached the market yet.

Future perspectives

Liver diseases represent a major cause of morbidity and mortality world-wide and despite the high uptake of drugs in this organ that is equipped with multiple uptake mechanism for exogenous compounds, many liver diseases are untreatable with the currently available drugs. In fact, liver diseases are characterized by a growing incidence in morbidity and mortality world-wide, mainly due to hepatitis C infections and the metabolic syndrome. The recent development of new antiviral drugs may halt this ever-increasing incidence, but the lack of effective therapies is intriguing. It cannot be explained by difficulties in reaching the liver or lack of effective compounds. Many of the experimental drugs were very effective *in vitro* and the liver is easily accessible for most compounds. As argued in this review, maybe cell-selectivity is the key factor here. This is particularly true for biologicals like cytokines and siRNA, that on the one hand represent a powerful set of tools, but on the other hand require cell-specific delivery. The development of new homing devices to each and every hepatic cell type in recent years has provided an important step towards a cell-specific treatment. Now, different carriers need to be compared in animal models of disease. Most carriers are biologicals which are characterized by a specific set of problems that are quite different from small chemical entities. Fortunately, experience in this area is rapidly growing. However, in the coming years maybe not the successes and benefits of the different carriers need to be heralded, but the disadvantages and major hurdles for clinical applications of each type of construct need to be listed. These hurdles need to be faced and addressed step by step in order to obtain a clinical successful product. This review aimed to present the first steps in this process.

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