Review Letter

Targeting of antiviral drugs by coupling with protein carriers

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Side effects of antiviral drugs might be circumvented by their selective delivery into infected cells. This targeting can be obtained by conjugation of the drugs to macromolecules which are taken up specifically by the infected cells. The experiments reviewed, on this approach to antiviral chemotherapy, are mainly directed at improving the chemotherapeutic index of adenine arabinoside (ara-A) in the treatment of chronic hepatitis B by its coupling to galactosyl terminating glycoproteins.

Antiviral drug *Drug targeting* *Adenine arabinoside* *Hepatitis B*
Lactosaminated albumin *Asialoglycoprotein receptor*

1. INTRODUCTION

In the therapy of some diseases (e.g., tumors, infections caused by intracellular microorganisms) drug side effects might be circumvented by selectively delivering the drugs into those cells where their action is required. This can be obtained by conjugating the drug to a macromolecular vector which is specifically taken up by target cells. In these cells conjugates will be pharmacologically effective if the bond linking the drug to the carrier does not suppress the activity of the drug or if the bond is broken down in lysosomes with consequent intracellular release of the drug. According to this rationale several attempts have been made in recent years to selectively deliver drugs or toxins to neoplastic cells by binding them with appropriate carriers mainly with antibodies against cancer-specific antigens (for reviews see [1–4]). Here we briefly report experiments where a similar approach was applied to the antiviral chemotherapy. Inhibitors of DNA synthesis were selectively transported to deoxyribovirus-infected macrophages or hepatocytes by conjugation with albumin or with galactosyl-terminating glycoproteins, respectively. Protein conjugates can be active only in cells in direct contact with plasma (endothelial cells, cells of organs with sinusoid-type capillaries) since the wall of non-sinusoid capillaries is a barrier for proteins and does not allow the conjugates to come into contact with the surface of other cells. For this reason, chronic hepatitis B infection appears to be a good target for this approach to antiviral chemotherapy because in this disease virus growth takes place only in hepatocytes which are in contact with plasma. The experiments were consequently mainly directed at selectively delivering adenine arabinoside, which is an antiviral drug used in chronic hepatitis B, into hepatocytes.

2. ALBUMIN CONJUGATES

The idea to selectively deliver antiviral drugs to infected macrophages by conjugation with albumin arose from the finding that the toxic peptide amanitin [5–7] after conjugation with this protein [8], changed its original target (hepatocytes) and produced the typical nuclear changes [9,10] in cells endowed with high pinocytotic activity such as sinusoidal liver cells (endothelial cells and Kupffer cells) [11], macrophages cultured in vitro [12,13] and proximal convoluted tubule cells of rat kidney [14]. Amanitin–albumin conjugate probably exerts its damaging action by releasing the toxin after penetration into the cells and digestion of the albumin moiety in lysosomes [11,15]. The possibility was therefore considered that other

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drugs, covalently linked to albumin, might be released in an active form after penetration of the conjugates into the cells [13,16]. If the drugs are selective inhibitors of DNA synthesis, the conjugates should hinder the replication of DNA viruses in macrophages without damaging these cells as they do not divide, and should not affect proliferating cells which do not take up albumin [17].

Two inhibitors of DNA synthesis, 5-fluorodeoxyuridine (FUDR) and cytosine arabinoside (ara-C) were conjugated to rabbit serum albumin (RSA) by carbodiimide coupling. In experiments in vitro it was found that FUDR–RSA and ara-C–RSA kill pinocytosing and proliferating cells [16,17]. Thymidine and deoxycytidine, which are known to remove the effects of FUDR and ara-C, respectively [18], counteracted the action of FUDR–RSA and ara-C–RSA, indicating that the activity of the conjugates is due to the FUDR and ara-C moieties. Most probably the drugs are released free into the cells; in fact the bonds linking them to albumin involve their primary hydroxyl groups [19], which must be phosphorylated for the drugs to be able to block DNA synthesis [18]. Mice infected with *Ectromelia* virus, the agent of mousepox, were chosen as an experimental model to study the antiviral activity of these conjugates in vivo [17]. *Ectromelia* virus, when injected intravenously into mice, is ingested mainly by the Kupffer cells where it replicates. After about 12 h it infects neighbouring hepatocytes which in turn infect more hepatic cells after each cycle of growth [20,21]. The effect of FUDR–RSA and ara-C–RSA on virus growth in vivo was evaluated according to two criteria: inhibition of virus production in liver, and survival time and number of survivors. The conjugates, injected into mice at the same time as purified *Ectromelia* virus, reduced the virus yield in liver by 1–2 logs 48 h after infection, whereas free FUDR and ara-C were completely ineffective. Moreover ara-C–RSA significantly increased the mean survival time of infected mice and the number of survivors. The conjugates were inactive or only slightly active if administered 12 or 24 h after infection, when virus replication occurs in hepatocytes. These results indicate that the conjugates interfere mainly with the phase of virus infection which takes place in liver macrophages.

3. ASIALOFETUIN CONJUGATES

Albumin conjugates of inhibitors of DNA synthesis can not be used in viral diseases of man because the growth of deoxyriboviruses in human macrophages (poxviruses) occurs only in a presymptomatic stage, when therapy can not be started. However, the results obtained with albumin conjugates of ara-C and FUDR suggested [17,22] a similar approach for the treatment of human chronic hepatitis B, in which the virus replicates in hepatocytes. Inhibitors of DNA synthesis can be coupled to glycoproteins which, after removal of sialic acid and consequent exposure of galactosyl residues, are selectively taken up by hepatocytes where they are digested in lysosomes [23–29]. Trifluorothymidine (F_3T), an inhibitor of DNA synthesis which is active in mice in very low doses and can be easily conjugated to proteins, was linked [22] to desialylated fetuin, a carrier which has been previously used to deliver proteins [30] and liposomes [31] to parenchymal liver cells. F_3T was first converted to its glutarate which was subsequently coupled via its hydroxysuccinimide ester to ϵ-NH_2 groups of lysine residues of asialofetuin (AF). The molar ratio F_3T:AF in the conjugate was 8. Coupling with F_3T did not change the ability of AF to interact with the specific receptors on the surface of hepatocytes. Indeed, the clearance of ^{14}C-labelled AF from mouse blood was inhibited to the same extent by F_3T–AF or by an equal amount of non-conjugated AF. F_3T and F_3T–AF were injected in mice 44 h after *Ectromelia* virus infection and their effect on deoxy[5-^{3}H]cytidine incorporation into DNA was determined in liver and in bone marrow [22]. Forty-four h after *Ectromelia* virus infection the greater part of deoxycytidine incorporation in liver is probably due to virus DNA synthesis [22,32] most of which occurs in hepatocytes [32]. In liver, F_3T coupled to AF caused inhibitions of deoxycytidine incorporation 3-times higher than those produced by equal doses of the free drug. On the contrary, in bone marrow the free or coupled drug inhibited the incorporation to the same extent. This result indicates that after injection of the conjugate, F_3T is concentrated in a pharmacologically active form in liver. Evidence has been obtained [22] that the inhibition of DNA synthesis in bone marrow of mice, injected with F_3T–AF, was due to

F_3T which after release from the conjugate in hepatocytes escaped from these cells into the blood as a free drug.

F_3T is a very toxic compound and is not used as a systemic drug in man. Therefore in subsequent experiments, adenine-9-β-D-arabinofuranoside (ara-A), which is a less toxic inhibitor of DNA synthesis, was coupled to AF [33]. Ara-A has been used in recent years in the treatment of chronic hepatitis B. It reduces, in a dose-dependent fashion, the serum levels of virus DNA-polymerase [34–38], with a loss of infectivity [39] and an overall improvement of liver disease [40] in some patients. However, ara-A also produces side-effects, mostly gastrointestinal and neurological disturbances [41,42]; at doses higher than 25 $mg.kg^{-1}.day^{-1}$, it also causes severe depression of bone marrow functions [43].

Two different coupling procedures were employed to conjugate ara-A with AF. In the first, ara-A glutarate was linked to the ϵ-NH_2 lysine groups of AF via its hydroxysuccinimide ester. The molar ratio ara-A:AF in this conjugate was 8. In the second procedure ara-A monophosphate (ara-AMP) was coupled to AF using a water soluble carbodiimide. In this coupling ara-A is linked to AF probably by the formation of a phosphoamide bond between the ϵ-NH_2 lysine groups of the protein and the phosphate group of ara-AMP. The molar ratio ara-A:AF was 4. Conjugation with ara-A glutarate or ara-AMP did not change the ability of AF to interact with the specific receptors on the surface of hepatocytes.

Free ara-A, free ara-AMP and ara-A conjugates were injected in mice 44 h after *Ectromelia* virus infection and their effects on thymidine incorporation into DNA in liver and intestine were determined [33]. Since ara-A is more than 100-times less active in rodents than in primates due to its rapid inactivation [44], infected mice were also given 2′-deoxycoformicin, which blocks ara-A deamination [45,46] in order to obtain an inhibition of DNA synthesis by free or conjugated ara-A. The inhibition of DNA synthesis in liver produced by free ara-A or ara-AMP ranged from 42–77% according to the doses administered. Greater inhibitions (60–89%) were found in intestine. On the contrary, AF conjugates of ara-A inhibited DNA synthesis in liver (26–59%) but produced only small inhibitions in the intestine (0–19%).

4. LACTOSAMINATED ALBUMIN CONJUGATES

A drawback for the clinical use of AF conjugates of ara-A is their immunogenicity. These conjugates are strong inducers of antibodies [47] which can both inactivate the conjugates and produce allergic lesions. This problem may be overcome by using lactosaminated homologous (i.e., of same species) albumin as hepatotropic carrier of ara-A. Some years ago it was suggested [48,49] that a protein may be made to penetrate into a cell which it can not normally enter alone by coupling it with a smaller molecule for which a specific binding site exists on the cell membrane. This hypothesis received experimental support from independent experiments which showed that proteins, after coupling to small sugar molecules, penetrate selectively into cells which possess surface receptors for the coupled carbohydrates [26,30,50,51]. In this context it was found that galactosylated proteins, e.g., lactosaminated serum albumins (L-SA), enter specifically into hepatocytes after binding to the receptor for galactosyl-terminating glycoproteins [26,52]. To ascertain whether L-SA can substitute AF for liver targeting of ara-A, this drug was linked to rabbit and to human L-SA by carbodiimide coupling and the experiments described for ara-A–AF were repeated with these conjugates [32,53]. Various doses of free ara-A, free ara-AMP and L-SA conjugates were injected in *Ectromelia* virus-infected mice and their effect on thymidine incorporation into DNA was determined in liver, intestine [32,53] and in some experiments in bone marrow [32]. In animals injected with free drugs, according to the different doses administered, the inhibitions of DNA synthesis ranged from 25–50% in liver, from 31–62% in intestine and from 0–43% in bone marrow. In animals injected with the coupled drug the percentages of inhibition in the same organs were 21–58%, 0–23% and 0–2%, respectively. The amounts of ara-A required to inhibit DNA synthesis in liver were about 10-times smaller when the compound was conjugated with L-SA than when it was administered as a free drug [53]. These results indicate that, after injection of L-SA conjugates, ara-A is concentrated in an active form into hepatocytes. On the contrary to conjugates prepared with AF or with heterologous lac-

tosaminated albumin, ara-A conjugates prepared with homologous lactosaminated albumin are completely devoid of humoral and cellular immunogenicity in mice [47]. Provided that the same immunological tolerance is true in man, human lactosaminated albumin appears to be the appropriate carrier to obtain liver targeting of ara-A in the treatment of chronic hepatitis B. In contrast to the naturally occurring glycoproteins of human blood, which after desialylation are also taken up by hepatocytes [24], human lactosaminated albumin can be easily obtained in the amounts required for clinical use of the conjugates. L-SA conjugates of ara-A do not display acute toxic effects, at least in mice. Administration of doses 11-times higher (the maximum tested) than those which produce a 50% inhibition of virus DNA synthesis in liver of *Ectromelia* virus-infected mice, did not cause any recognizable signs of toxicity in mice [47]. Chronic toxicity with these conjugates has not yet been studied. In subsequent experiments [53] the hepatic rate of uptake of two L-(human)SA (L-HSA) preparations with 22 and 40 galactosyl residues, respectively, was measured in mice. It was found that the maximal quantities of L_{22}-HSA and of L_{40}-HSA which can enter into mouse liver are 0.24 and 0.26 μg.g body $wt^{-1}.min^{-1}$, respectively. At plasma concentrations saturating the hepatic receptor, L_{22}-HSA penetrates almost exclusively into the liver, only very small quantities being taken up by the cells of intestine, spleen and kidney. Uptake by other organs was not studied. In the case of L_{40}-HSA, a non-negligible amount of glycoconjugate is also taken up by the kidney. This finding indicates that L_{22}-HSA is a better carrier than L_{40}-HSA for liver-targeting of drugs.

For the treatment of chronic hepatitis B [34–37] as well as other infections caused by DNA viruses [43,44], free ara-A is given by continuous infusion at a dose usually of 15 $mg.kg^{-1}.12\ h^{-1}$ which corresponds to 21 $ng.g^{-1}.min^{-1}$. Since the amounts of ara-A required to inhibit DNA synthesis in liver are about 10-times smaller when the drug is coupled to L-HSA (see above), the dose of conjugated ara-A which should be given to patients with chronic hepatitis B is 2.1 ng.g body $wt^{-1}.min^{-1}$. In a conjugate with a molar ratio of ara-A to L_{22}-HSA of 11 (as in the conjugates usually prepared [53]), 2.1 ng ara-A is bound to 0.055 μg L_{22}-HSA. According to the data reported above, L_{22}-HSA enters into mouse liver at a rate of 0.24 μg.g body $wt^{-1}.min^{-1}$. Therefore, provided that human hepatocytes infected by hepatitis B virus have a similar capacity for L_{22}-HSA uptake as mouse hepatocytes, this carrier should be able to transport to such cells the dose of ara-A required to exert antiviral activity.

5. CONCLUSIONS

The experiments reported above provide evidence that antiviral agents can be selectively delivered into infected cells by conjugation with protein carriers. As far as potential applications of conjugates for the treatment of viral diseases in man are concerned, the experiments on ara-A conjugates in *Ectromelia* virus infected mice lend support to the hope of increasing the chemotherapeutic index of this drug in the treatment of chronic hepatitis B by its coupling to lactosaminated albumin.

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