Lactosaminated human serum albumin as hepatotropic drug carrier

Rate of uptake by mouse liver

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1. INTRODUCTION

In chronic hepatitis B infection, adenine-9-β-D-arabinofuranoside (ara-A) reduces, in a dose dependent fashion, the serum levels of virus DNA-polymerase [1–4] with an improvement in liver function and histology [5] and a loss of infectivity in some patients [4]. However this drug produces dose-dependent side effects [6–8] which might be avoided or reduced by selectively delivering ara-A to hepatocytes. In mice, liver targeting of ara-A has been obtained by conjugation with asialofetuin (AF) [9] or with lactosaminated serum albumin (L-SA) [10], two galactosyl-terminating glycoproteins which penetrate by a receptor-mediated endocytosis only in hepatocytes where they are digested in lysosomes [11–16]. AF–ara-A and L-SA–ara-A conjugates, administered to mice with Ectromelia virus-hepatitis, inhibited virus DNA synthesis in liver without producing significant inhibition of cellular DNA synthesis in intestine and bone marrow [9,10]. L-SA has a definitive advantage over AF as hepatotropic carrier of ara-A since conjugates prepared with lactosaminated homologous albumin are not immunogenic, at least in mice, to the contrary of AF conjugates which are strong antibody inducers [17]. Moreover as opposed to naturally occurring glycoproteins of human blood, which after desialylation are also taken up by hepatocytes [12], L-(human)SA (L-HSA) can easily be obtained in the amounts required for clinical purposes.

By receptor-mediated endocytosis proteins enter into cells only in limited amounts; the hepatic uptake of AF in rats does not exceed 0.18 $\mu g \cdot g$ body $wt^{-1} \cdot min^{-1}$ [18]. In the present experiments we have calculated the maximal rate of hepatic uptake in mice of two L-HSA preparations with 22 and 40 galactosyl residues, respectively. Moreover, we have determined the minimal doses of free or conjugated ara-A which inhibit DNA synthesis in liver of Ectromelia virus-infected mice. These experiments were undertaken in order to calculate whether the amounts of ara-A needed to exert antiviral activity in chronic hepatitis B can be transported to hepatocytes by L-HSA.

2. MATERIALS AND METHODS

2.1. *Tissue distribution of L-[^{14}C]HSA*

Lactose was coupled to the ε-NH_2 of lysine residues of HSA by reductive amination with cyanoborhydride [19,20]. The albumin concentration was determined as in [21]. The amount of sugar conjugated to protein was established by the phenol/sulfuric acid method [22] calibrated against galactose. In different preparations of L-HSA increasing amounts of lactose were coupled as a function of time of reaction [20,14]. Labelling of L_{22}-HSA (2×10^6 dpm/mg), L_{40}-HSA (3.7×10^6 dpm/mg) and of non-desialylated fetuin (3×10^6 dpm/mg) was performed with iodo[^{14}C]acetic acid (Amersham, 54 mCi/mmol) as in [14], but using a higher concentration of iodo[^{14}C]acetic acid. Female Swiss mice (25–26 g), fasted overnight, were injected i.v. with L_{22}-[^{14}C]HSA or L_{40}-[^{14}C]HSA administered in saline (10 μl/g body wt) at the indicated doses. After 7 min, when hydrolysis of L-HSA, and consequently losses of radioactivity from the liver, are negligible (see table 2),

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the animals were bled from the retro-orbital plexus under ether anaesthesia, and liver, kidneys, spleen and a tract of the intestine (8 cm long starting from pylorus) were rapidly removed and homogenized in 4 vol (wt/vol) of water. The total radioactivity of plasma and homogenates as well as the radioactivity of their acid soluble fraction were measured. The radioactive contribution given by the plasma trapped in the organs was calculated and subtracted. To assess the amounts of trapped plasma, 5 female Swiss mice (25–26 g), fasted overnight, were i.v. injected with ^{14}C-labelled non-desialylated fetuin (10 μg/g body wt in 5 μl saline). After 2 min, the animals were killed and the radioactivity of plasma and organs was measured. Since plasma clearance of fetuin is very slow (unpublished experiments), it was assumed that all the radioactivity contained in the organs at 2 min was due to trapped plasma. It was thus calculated that 1 mg of liver, intestine, spleen and kidney contains respectively 0.1060 ± 0.006, 0.0192 ± 0.006, 0.0532 ± 0.007 and 0.1160 ± 0.007 μl of plasma.

2.2. *L-HSA–ara-A conjugates*

Ara-A monophosphate (ara-AMP) was coupled to L-HSA by the use of 1-ethyl-3-(dimethylaminopropyl)carbodiimide [10]. Knowing that the amount of galactosyl residues bound to albumin decreases during ara-AMP coupling [10], we obtained 22 and 43 galactosyl residue conjugates when starting from L_{34}-HSA and L_{57}-HSA respectively. After gel chromatography on a Sephadex G-100 column, equilibrated and eluted with 0.05% NaCl solution, the fractions corresponding to the monomeric form of the conjugate (about 70% of the whole conjugate preparation) were pooled, dialyzed against water and lyophilized. Unconjugated ara-AMP was recovered, as will be described elsewhere. The molar ratio sugar/albumin in L-HSA–ara-A conjugates was measured taking into consideration the contribution given by arabinose in the phenol/sulphur reaction [22]. The molar ratio ara-A/albumin was determined spectrophotometrically.

2.3. *[methyl-^{3}H]thymidine incorporation into DNA in liver and intestine of Ectromelia virus-infected mice*

Mice were killed 46 h after infection with Ectromelia virus (Hampstead mouse strain) (2×10^5 p.f.u./g body wt) [23,24]. The experiments were performed as previously described [10] with the difference that 9-erythro-(2-hydroxyl-3-nonyl)-adenine (ENHA), ara-A (free or coupled to L-HSA) and [*methyl*-^{3}H]thymidine were administered 45, 30 and 15 min, respectively, before animals were killed.

3. RESULTS AND DISCUSSION

L_{22}-HSA and L_{40}-HSA interact with the hepatic receptor for asialoglycoproteins; as shown in table 1, they competitively inhibited the blood clearance of [^{14}C]AF in mice. L_{40}-HSA exerted a higher inhibition than L_{22}-HSA, in agreement with the finding that the affinity in vitro of galactosylated albumins to the hepatocyte receptor increases with increase in the number of coupled sugar molecules [25].

Table 2 shows that doses of 2.5 μg/g body wt of both L_{22}-[^{14}C]HSA and L_{40}-[^{14}C]HSA are enough to saturate the hepatic receptor for a 7 min period: by increasing the dose to 4 μg, the liver uptake of the two L-[^{14}C]HSAs does not increase. At these doses L_{22}-HSA penetrates almost exclusively in the liver, only very small quantities being taken up by the cells of intestine, spleen and kidney. In the case of L_{40}-[^{14}C]HSA a non-negligible amount of glycoconjugate is taken up also by the kidney. This finding (confirmed by unpublished experiments) indicates that L_{22}-HSA is a better carrier than L_{40}-

Table 1

Effect of L_{22}-HSA and L_{40}-HSA on plasma clearance of [^{14}C]AF

Exp. no.	Compounds injected with [^{14}C]/AF	dpm/ml plasma
1	None	19140 ± 212
2	AF	25490 ± 1407
3	L_{22}-HSA	25120 ± 2142
4	L_{40}-HSA	29510 ± 3450

Swiss female mice (25–26 g) were injected i.v. with 2 μg/g body wt of [^{14}C]AF (4.4×10^5 dpm/mg) prepared according to [29]. Unlabelled AF or L-HSA were administered simultaneously with [^{14}C]AF at 4 μg/g body wt. After 5 min animals were killed and the radioactivity of plasma was measured. Each entry represents the mean value (± SD) of results from 2 animals

Table 2

Tissue distribution of L-[^{14}C]HSA 7 min after injection

Compound injected	Dose (μg/g body wt)	L-HSA in tissues (ng/mg)				L-HSA in plasma	Wet weight
		Liver	Intestine	Spleen	Kidney	(μg/ml)	of liver (g)
L_{22}-[^{14}C]HSA	1.25	19.6 ± 6.48(1.3)[a]	0.4 ± 0	0.6 ± 0.30	0.6 ± 0.22	3.4 ± 0.20	1.35 ± 0.20
	2.5	32.8 ± 2.18(1.6)	0.5 ± 0.10	0.7 ± 0.20	0.5 ± 0.23	20.7 ± 4.98	1.15 ± 0.09
	4.0	35.0 ± 6.38(1.4)	0.2 ± 0.23	0.9 ± 0.23	0.2 ± 0.23	49.5 ± 5.80	1.12 ± 0.02
L_{40}-[^{14}C]HSA	1.25	24.8 ± 3.43(1.7)	0.3 ± 0	0.4 ± 0.14	1.5 ± 0.28	3.2 ± 2.64	1.12 ± 0.08
	2.5	36.2 ± 3.01(1.7)	0.4 ± 0.10	0.4 ± 0.25	2.4 ± 0.30	12.9 ± 1.17	1.29 ± 0.05
	4.0	33.3 ± 9.66(2.2)	0.6 ± 0.35	1.4 ± 0.44	3.4 ± 1.45	51.8 ± 2.62	1.13 ± 0.07

[a] In parenthesis the percentage of acid soluble radioactivity over total radioactivity.

Each entry represents the mean value (± SD) of results from 4 animals

Table 3

Inhibition of DNA synthesis in liver and intestine of Ectromelia virus-infected mice after injection of free or conjugated ara-A

Exp. no.	Compound injected	ara-A admin. (μg/g body wt)	Inhibition of DNA synthesis (%)	
			Liver	Intestine
1	ara-A	1	25(NS)[b]	31(NS)
2	ara-A	3.5	41(S)	62(S)
3	ara-A	5	44(S)	61(S)
4	L_{22}-HSA–ara-A_{11}	0.15(4)[a]	32(S)	8(NS)
5	L_{22}-HSA–ara-A_{11}	0.3(8)	37(S)	4(NS)
6	L_{22}-HSA–ara-A_{11}	0.3(8)	44(S)	8(NS)
7	L_{43}-HSA–ara-A_{10}	0.25(8)	41(S)	6(NS)

[a] In parentheses the amount of conjugate injected (in μg/g body wt)

[b] Results were statistically evaluated by means of Student's t-test. The difference was considered statistically significant (S) or not significant (NS) for $P <$ or > 0.05, respectively

In each experiment 14 animals were used (7 controls; 7 injected with free or conjugated ara-A)

HSA for liver targeting of drugs. Taking into account the hepatic concentration of L-[^{14}C]HSA at 7 min, the weight of liver and that of mice after a 15 h fast (22–23 g), it can be calculated that the maximum amounts of L_{22}-HSA and of L_{40}-HSA which can enter into mouse liver are 0.24 ± 0.01 and 0.26 ± 0.04 $\mu g \cdot g$ body $wt^{-1} \cdot min^{-1}$, respectively. On an equimolar basis these values fit well with that of the maximal hepatic uptake of AF in rat (0.18 $\mu g \cdot g$ body $wt^{-1} \cdot min^{-1}$) [18] (M_r of AF, L_{22}-HSA and L_{40}-HSA: 45 500, 76 500 and 82 500, respectively). An electron microscope autoradiographic study [15] has shown that [^{125}I]AF, administered to rats at a dose of about 4 μg/g body wt, was associated almost exclusively with hepatocytes after 15 min. This finding together with the observation that albumin containing 25 galactosyl residues binds in vitro to the liver asialoglycoprotein receptor with an affinity similar to that of asialoorosomucoid [25], (which is twice as high as that of AF [12]) suggests that in our experiments the uptake of L-[^{14}C]HSA by the liver took place mainly in hepatocytes. The hepatic receptor for galactosyl-terminating glycoproteins is not destroyed after

internalization following ligand binding and unoccupied receptor molecules are promptly returned to the cell surface from an internal pool [26]. Consequently the hepatic uptake of galactosyl-terminating glycoproteins remains constant with time [18].

Table 3 shows the effects of free and coupled ara-A on [*methyl*-^{3}H]thymidine incorporation in liver and intestinal DNA of mice which had been injected with Ectromelia virus 46 h earlier. At this time of infection the largest amount of thymidine incorporation into liver DNA is due to virus DNA synthesis [27,10], most of which occurs in hepatocytes [10]. Free ara-A produced a greater inhibition of DNA synthesis in the intestine than in the liver; on the contrary, and in agreement with previous results [10], inhibition of DNA synthesis by ara-A coupled to L_{22}-HSA or to L_{43}-HSA was higher in liver than in the intestine. Doses of conjugated ara-A about 10 times lower than those of the free drug produced a comparable inhibition of DNA synthesis in liver.

Free ara-A is given by continuous infusion in the treatment of chronic hepatitis B [1,2,4] as well as in infections caused by other DNA viruses [28], the dose usually being 15 $mg \cdot kg^{-1} \cdot 12\ h^{-1}$ which corresponds to 21 $ng \cdot g^{-1} \cdot min^{-1}$. Since the amounts of ara-A required to inhibit virus DNA synthesis in liver are approximately 10 times smaller when the drug is coupled to L-HSA (table 3), 2.1 $ng \cdot g$ body $wt^{-1} \cdot min^{-1}$ is the dose of conjugated ara-A which should be infused to patients with chronic hepatitis B. In the conjugate L_{22}-HSA–ara-A_{11}, 2.1 ng ara-A is bound to 0.055 μg L_{22}-HSA. According to the data of the present experiments L_{22}-HSA enters into mouse liver at a rate of 0.24 $\mu g \cdot g$ body wt^{-1}/min. Therefore provided that human hepatocytes infected by hepatitis B virus have a similar capacity of taking up L_{22}-HSA as do mouse hepatocytes, this carrier should be able to transport to such hepatocytes the dose of ara-A required to exert antiviral activity.

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