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Review Article

Pharmaceutical Strategies Utilizing Recombinant Human Serum Albumin

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Gene manipulation techniques open up the possibility of making recombinant human serum albumin (rHSA) or mutants with desirable therapeutic properties and for protein fusion products. rHSA can serve as a carrier in synthetic heme protein, thus reversibly carrying oxygen. Myristoylation of insulin results in a prolonged half-life because of self aggregation and increased albumin binding. Preferential albumin uptake by tumor cells serves as the basis for albumin-anticancer drug conjugate formulation. Furthermore, drug targeting can be achieved by incorporating drugs into albumin microspheres whereas liver targeting can be achieved by conjugating drug with galactosylated or mannosylated albumin. Microspheres and nanoparticles of different sizes can, with or without drugs and/or radioisotopes, be used for drug delivery or diagnostic purposes. *In vivo* implantation of albumin fusion protein expressing cells encapsulated in HSA-alginate coated beads showed promising results compared to organoids in rats. Chimeric peptide strategy with cationized albumin as the transport can deliver drugs via receptor mediated transcytosis through the blood brain barrier. Gene bearing, albumin microbubbles containing ultrasound contrast agents can non-invasively deliver gene after destruction by ultrasound. Various site-directed mutants of HSA can be tailor made depending on the application required.

KEY WORDS: recombinant albumin; drug targeting; albumin fusion; gene delivery; site-directed mutant; pharmaceutical applications.

INTRODUCTION

Human serum albumin (HSA) is the most well studied plasma protein and an attractive macromolecular carrier due to its availability in pure form and its biodegradability, nontoxicity and nonimmunogenicity (1) (Fig. 1). It is widely used as a stabilizing component in pharmaceutical and biologic products, such as vaccines, recombinant therapies and coatings for medical devices. Drug formulation using albumin as a carrier promises to acquire additive beneficial affects physiologically due to its known antioxidant properties (2,3). However, albumin, as it is currently obtained by conventional techniques involving the fractionation of plasma obtained from blood donors is with the risk of transmitting possible viral/prion contaminants. In this respect, recombinant DNA technology offers an important new source of biomaterials that are suitable for use as pharmaceuticals without any need for pooled human blood products. The expression of rHSA has been examined in *Eschericia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluyveromyces lactis*, plants and transgenic animals. Three expression systems that are in current use by the pharmaceutical companies for rHSA production are *S. cerevisiae*, *K. lactis* and *P. pastoris*. It appears that the biotechnology industry has conducted the necessary initial research and development for the production of recombinant albumin but this technologic expertise is currently proprietary.

Aside from the major usage of blood volume expanders, rHSA could also be used for various pharmaceutical designs such as an excipient or stabilizer in other recombinant and biotechnology based products, a component in serum-free cell culture media, a component for imaging agents and, possibly for the therapeutic apheresis where plasma exchange might be desirable (4). Aventis Behring recently announced the successful results of its large pivotal phase I clinical trial of Recombumin® 20%, a rHSA product, on its tolerability as a stabilizing component in pharmaceutical and biologic products. In addition, rHSA could also be utilized as a synthetic heme protein carrier or as a depot protein for long acting acylated insulin, as will be described later. Thus, in this review, we will introduce aspects of the pharmaceutical utility of rHSA (Fig. 2).

Recombinant Human Serum Albumin

S. cerevisiae was among the first of yeast selected for the production of heterologous eucaryotic proteins since an immense amount of data is available concerning its genetics and physiology (5). PGK, GUT2 and GAL7 promoters have been reported for use in the recombinant human serum albumin

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ABBREVIATIONS: HSA, human serum albumin; rHSA, recombinant human serum albumin; BBB, blood brain barrier; FeP, tetraphenylporphinatoiron (II) derivative; DDS, drug delivery systems.

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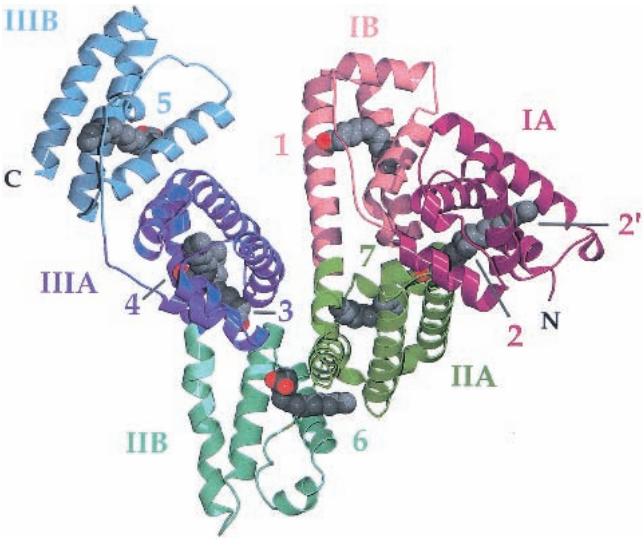

Fig. 1. Crystal structure of rHSA complexed with myristate. The subdivision of the protein into domains (I-III) and subdomains (A and B) is indicated with color codes; N and C represents the N- and C-terminus, respectively. Bound fatty acid is shown in a space-filling representation and colored by atom type (carbon, grey; oxygen, red). Eight molecules of bound myristate are shown (numbers 1–7 plus number 2′). The tail of the myristate molecule binding to site 2′ binds in an overlapping and anti-parallel fashion with the fatty acid anion binding to site 2, whereas the carboxylate head group (not shown) probably extends into the surrounding solvent. Because of lack of density, the position of the carboxylate group of myristate number 7 could not be given either. The figure was made with Molscript on the basis of the atomic coordinates 1e7g available at the Brookhaven Protein Data Bank.

(rHSA) expression systems using *S. cerevisiae* (6–8). A reduced degradation of the secreted rHSA was observed when the aspartyl protease gene (YAP3) was disrupted (9).

K. lactis, an aerobic yeast which is able to grow on lactose as the sole carbon source, has been successfully used as an alternative host to *S. cerevisiae* for heterologous protein expression. pKD1-based expression vectors have been used for the efficient secretion of rHSA by industrial strains of Kluyveromyces yeasts (10). In addition to the PGK and LAC4 promoters, rHSA can also be expressed in *K. lactis* by utilizing the KlADH4 gene (11). The insensitivity of this promoter to glucose repression, combined with the specificity of ethanol as the activator, permits the expression of cloned genes after the addition of the inducer, independently of the carbon source present in the medium. A recent study has shown that the efficiency of rHSA secretion with *K. lactis* is increased with duplication of either the KlPDI1 or KlUBI4 gene (12). It is noteworthy that the overexpression of the KlPDI1 gene in particular, which codes for an endoplasmic reticulum chaperone protein disulfide isomerase, resulted in a striking more than 15-fold increase in the amount of secreted rHSA compared to the parental strain. This suggests that correct protein folding may be a major limiting factor in the secretion process of rHSA, which has a highly disulfide-bonded structure.

The methylotropic eukaryotic yeast, *P. pastoris*, has been developed as an outstanding host for the production of rHSA as well as other plasma proteins (13,14). *P. pastoris* is able to utilize methanol as a carbon source in the absence of glucose. Although *P. pastoris* can assimilate glucose and glycerol, modification of the alcohol oxidase 2 (AOX2) promoter and the alcohol oxidase 1 (AOX1) terminator enables the expression of rHSA to be strictly regulated by methanol via the AOX2 promoter (15). In a recent report, another strain of methylotrophic yeast, *Hansenula polymorpha* DL-1 has been utilized to optimize the secretory expression of rHSA under the control of the methanol oxidase (MOX) promoter. It has been proposed that this *H. polymorpha* expression system might be suitable for the large-scale production of rHSA (16).

Structural and Functional Properties of rHSA

For the time being, *P. pastoris* (strain GS115) is the most widely used expression system for the production of rHSA (17–23). By this system, rHSA can be produced on a large scale and afterwards purified to such an extent, that the content of yeast derived contaminants amount to less than 1ng/250 mg of rHSA (19). The results from structural analyses such as SDS/PAGE gel electrophoresis (17,18,20,22), far-UV and near-UV CD spectra (20,22) and intrinsic fluorescence spectra (21) propose identical structures of rHSA and HSA, which also have comparable contents of free cysteine residue at position 34 (19,22). No immunologic differences have been found for the two types of albumin (17,19,20). Furthermore, the resistance of rHSA and HSA against denaturation by heat (21,22) or guanidine hydrochloride (20,22) is similar. Finally, the crystal structures of the two albumins obtained at a 2.5 Å resolution are identical (23).

No functional differences between rHSA and HSA have been found either. For example, the albumins bind diazepam and ketoprofen equally well (20). Furthermore, the stereoselective binding properties of rHSA for warfarin, phenprocoumon, pranoprofen and ibuprofen are the same as those of HSA (22). In addition to its unique ligand binding properties, albumin possesses an esterase-like activity, and studies with *p*-nitrophenyl acetate as a substrate revealed no differences in the enzymatic properties of rHSA and HSA (20).

rHSA and HSA labeled with ^{125}I have been given intravenously to rats, and no significant differences between the two proteins were found with respect to blood half-life, urinary and fecal excretion or tissue distribution (22). The efficacy and safety of rHSA have been tested in humans as Phase I, II and III clinical trials, and high efficacy with little or no adverse reaction was observed (19). Thus, rHSA from *P. pastoris* seems to be clinically safe and structurally and functionally identical to albumin isolated from human plasma.

Artificial Blood Substitutes

During hemorrhagic hypovolemia shock, a blood transfusion is the only effective treatment. When the plasma volume is dramatically reduced, albumin can restore the losses quickly in most cases. The administration of 100 mL of a 25% solution of albumin draws 350 mL of interstitial fluid, yielding an intravascular volume of approximately 450 mL of whole blood over a 30–60 m period. If blood loss is severe, a transfusion of whole blood or packed red cells may also be needed to restore hematocrit and improve oxygen delivery. However, a major problem is to obtain a reliably sufficient blood supply with the correct blood type for such emergency clinical situ-

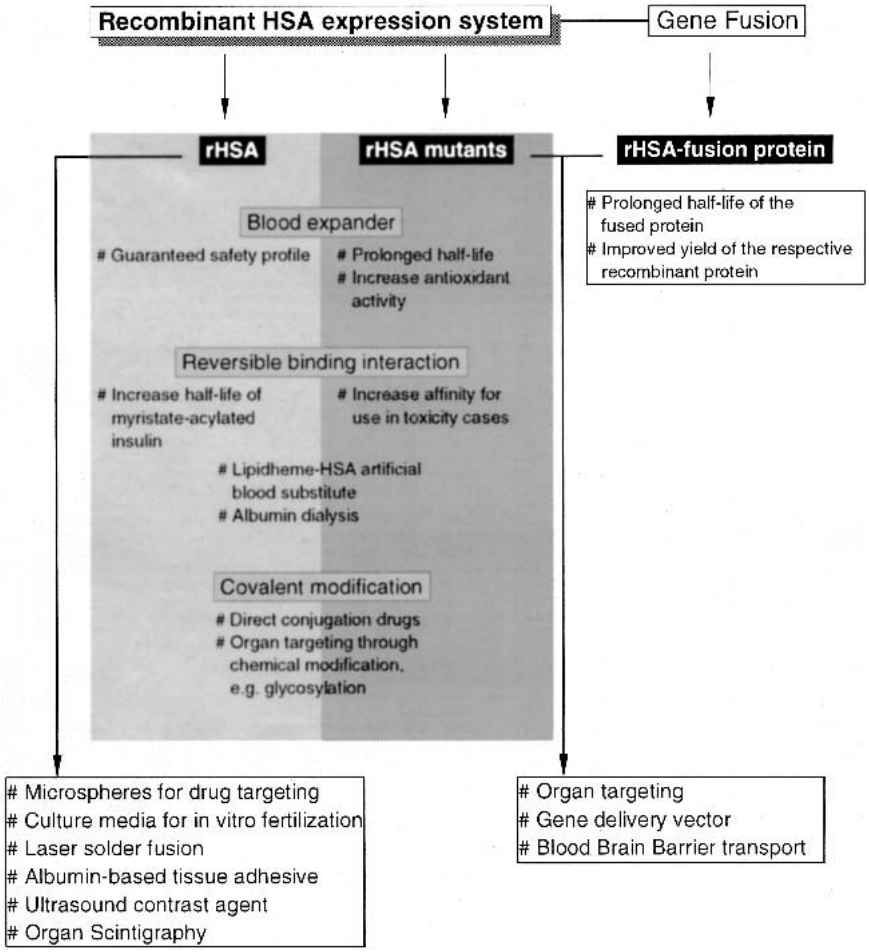

Fig. 2. Outline of the main pharmaceutical applications of rHSA.

ations. The problem is compounded by the fact that laborious screening must be done to ensure that the donated blood is not infectious. Therefore, one possible solution would to be to produce a synthetic blood substitute and albumin appears to be a good candidate in view of its volume expander properties (24).

Based on the binding properties of porphyrin and serum albumin, Tsuchida *et al.* successfully incorporated a tetraphenylporphinatoiron (II) derivative (FeP) into HSA or rHSA (25,26). The maximum number of FeP molecules that can be incorporated into one molecule of HSA was determined to be eight, with binding constants in the range from 1.3×10^4 to 1.2×10^6 M^{-1} (27). The interaction was concluded to be mainly hydrophobic in nature, with no alteration in the surface charge distribution (28). Furthermore, the incorporation of FeP had no apparent effects on the structure or essential biologic roles of the protein. Mixing rHSA-FeP with an equal volume of human blood showed good compatibility and the homogeneous morphology of the red cells was not affected.

The rHSA-FeP hybrid can bind and release oxygen reversibly under physiological conditions similar to hemoglobin and myoglobin, as has been confirmed in pre-clinical experiments using rats. The oxygen transporting efficiency of rHSA-FeP between the lungs and muscle tissue was 22% at 37°C, which is comparable to that of red cells (23%) (25). Physiologic responses to exchange transfusion with HSA-FeP solution into rats after hemodilution and hemorrhage have been evaluated. The decreased mean arterial pressure and blood flow after a 70% exchange with HSA and a further 40% loss of blood were significantly recovered up to about 90% of the initial values as a result of an injection of HSA-FeP. Furthermore, renal cortical O_2-tensions and skeletal tissue O_2-tensions were increased, indicating the *in vivo* O_2-delivery of HSA-FeP. The HSA-FeP treated rats survived for more than 12 h until sacrifice, and a significant difference in survival time was observed compared with the group treated with HSA alone (29). HSA incorporated with synthetic heme therefore has great potential as a resuscitant in hemorrhagic conditions.

Infusions with HSA-FeP solutions should be without immunologic problems. Although the complex is expected to fulfill functions such as oxygen delivery, ligand binding and re-establishing the colloidal osmotic pressure, the infusions are not a complete mimic of blood transfusions. For example, the blood clotting property is lacking. In addition, the oxygen-transporting efficiency of HSA-FeP, when the HSA concentration was adjusted to 0.75 mM, was only about 60% of that of human blood (24). Furthermore, since FeP interacts reversibly with HSA, care must be taken if a strong albumin-bound drug is co-administered. Thus, further detailed examination of the safety profile, including the *in vivo* stability of the

complex, is needed before use in clinical conditions other than hemorrhagic hypovolemia.

Prolongation of Plasma Half-Life of Insulin by Increased Binding to Albumin

The plasma half-lives of therapeutic agents can be substantially extended through reversible or covalent binding to HSA. The high affinity and high capacity fatty acid binding properties of serum albumin and its abundance in extracellular fluids have been utilized in a strategy to optimize the pharmacokinetics of insulin (30). An insulin analogue that had been acylated with myristate at Lys^{B29}, NN304, has been shown to bind HSA with high affinity. NN304 exists in the presence of zinc and phenol, such as native insulin, predominantly in the hexameric state. The fatty acid side-chain causes aggregation of hexamers, thus delaying the dissociation and absorption processes. On the other hand, monomeric NN304 binds primarily to domain IIIA of HSA with $K_a = 2.4 \times 10^5$ M^{-1} through the myristate side-chain. The ensuing slow dissociation of the analogue from albumin further prolongs the blood glucose lowering action of the molecule (31).

One of the barriers for optimal glycaemic control in patients with type 1 diabetes is that low and relatively constant basal insulin levels between meals and at night are difficult to obtain with the basal insulin formulations currently available such as Neutral Protamine Hagedorn (NPH) insulin. Unlike NPH-insulin, NN304 is readily soluble, thus ensuring a homogeneous concentration, with no need for agitation before administration. Albumin binding increases the half-life in circulation, and decreases transendothelial transport, clearance in the kidney and liver (32). Clinical trials with healthy subjects suggest that NN304 has a less pronounced peak of action and lower intrasubject variation, in terms of pharmacokinetic parameters compared with NPH-insulin but was equally effective when administered at a higher molar dose (33). The acylated analogue is suitable for basal insulin delivery because both properties, albumin binding and aggregation of hexamers, contribute to retardation of absorption after subcutaneous injection. This results in steady concentrations in the blood, enabling a closer approximation to normal glucose regulation (34).

Drug Targeting through Microspheronization and Chemical Modification

Intravenous injection can provide for the efficient targeting of albumin microspheres to either the lung or the liver, depending strictly on the size range of the microspheres. Microspheres 15–30 μm or larger in size will pass through the heart and then deposit with 99% efficiency into the capillary bed of the lungs. Microspheres of about an order of magnitude smaller (e.g., 1–3 μm) will pass into the reticuloendothelial system and deposit with about a 90% efficiency in the liver. Magnetically responsive microspheres could be used to restrict drug activity to a specific body region, thus allowing for the administration of small doses and therefore decreased side effects.

Albumin microspheres can be labeled with various radioisotopes to produce diagnostic agents that, on intravenous injection, are particularly useful for assessing the vascular integrity or detecting the presence of thrombi in organs such as the lung, liver, and spleen or for detecting the presence of tumors in these organs. Radiolabeled albumin microspheres are also useful as an indicator for Kupffer cells phagocytosis following drug uptake by these cells (35). In some cases, radiolabeled microspheres can be utilized for therapeutic purposes such as tumor treatment (36,37). rHSA microcapsules have been used in scintigraphic studies as 99mTc-labeled rHSA with the potential for lung, liver and cardiovascular imaging (38). Lymphoscintigraphy using 99mTc-HSA is useful in conditions such as chyluria (39), sentinel lymph node biopsy (40) and sentinel node detection in breast cancer (41), protein-losing enteropathy (42) and leg edema (43).

Recognition by receptors present on the surface of cells should offer another effective way for molecular manipulation to achieve cell-specific delivery of proteins. In addition, it is well known that glycoproteins are rapidly taken up by hepatocytes via D-galactosyl receptor-mediated endocytosis on removal of their sialic acid residues (44). Glycoproteins with mannose residues are also taken up by Kupffer and endothelial cells in the liver through recognition by the mannose receptor (45,46). Therefore, the introduction of sugar moieties to proteins would be expected to result in a specific delivery to the cells that express these receptors. This strategy has been employed in liver targeting of anticancer drugs such as methotrexate (47) or antifibrotic drug (48). Aside from sugar moieties, albumin modified with cyclic peptides that recognize the collagen type VI receptor has been successfully targeted to rat hepatic stellate cells (49). These modifications demonstrate the applicability of modified albumins as drug carriers to cells in cirrhotic livers (50). Liver scintigraphy with Technetium-99m-diethylenetriaminepentaacetic acid-galactosyl-HSA, which is a hepatocyte asialoglycoprotein receptor specific binding agent, can be applied to various liver conditions such as cirrhosis (51,52).

Direct Conjugation with Therapeutic Agents

Albumin-based drug carrier systems have been developed, particularly in the field of chemotherapy to improve the passive tumor targeting properties of anti-cancer drugs (53,54). Linking chemotherapeutic drugs to a macromolecular carrier system may enhance tumor targeting, reduce toxicity and overcome drug resistance mechanisms. The rationale of this drug design is because plasma proteins constitute the major transportable nitrogen reserve in vertebrates (55,56). Proliferating tumor cells utilize albumin and other plasma proteins for their nutrition and take up albumin by fluid-phase endocytosis at a greater rate than normal tissues. After lysosomal digestion, the derived amino acids serve as a source for nitrogen and energy in the tumor cells. These favorable properties provide a stimulus for the choice of albumin as a drug carrier where the conjugates enjoy the same favorable tumor targeting properties as albumin, e.g. high tumor uptake rates, low liver uptake rates and a very long biologic half-life.

Albumin conjugates with methotrexate at an equimolar drug carrier ratio (1:1), in contrast to those with multiple methotrexate molecules, resulted in a favorable distribution pattern with a high tumor-uptake rate comparable to native albumin (57). The phase I study of methotrexate-HSA in cancer patients reported a partial and a minor response in two patients with renal-cell carcinoma and a minor response in a patient with pleural mesothelioma (58). The antitumor activ-

ity of an acid-sensitive doxorubicin-albumin conjugate has been shown superior to that of the free doxorubicin in tumor bearing mice (59). The *in vitro* activity of the doxorubicin-HSA microcapsule appeared to overcome p-glycoprotein mediated doxorubicin resistance (60). In a novel drug targeting approach, the protease activity of matrix metalloproteinases has been exploited to release the anticancer agent doxorubicin from a doxorubicin-spacer peptide-albumin conjugate (61). Chlorambucil, when bound to thiolated HSA through an acetaldehyde carboxylic hydrazone bond was as or more active than chlorambucil in both MCF7 mamma carcinoma and MOLT4 leukemia cell lines. Preliminary toxicity studies in mice showed that this conjugate could be administered at higher doses in comparison to unbound chlorambucil (62). Other albumin conjugates include the surface-immobilized albumin-heparin conjugate which forms a suitable substrate for the seeding of endothelial cells in low density (63), dexamethasone-HSA for nonparenchymal cell targeting, to improve the efficacy and safety of corticosteroid treatment of liver fibrosis (64), and naproxen-HSA. Naproxen-HSA is efficiently targeted to endothelial and Kupffer cells of the liver and may offer a new therapeutic approach in the treatment of liver disease associated with inflammatory processes (65).

Meijer and coworkers found that succinylation or aconitylation of HSA caused the negatively charged HSA to acquire anti-HIV activity *in vitro*. This was made possible because the modified protein is able to bind to, and thereby block, a positively charged loop of the viral receptor (66,67). In addition, these negatively charged albumins were distributed to lymph much more rapidly than albumin, depending on the total net negative charge added to the protein (68). Due to the potent anti-HIV properties and the versatile polypeptide backbone, the modified albumins have potential as intrinsic active carriers for antiviral drugs such as AZT, a concept named by the author as "dual targeting" (69). Such preparations are expected to interfere with HIV replication at various points. These combined activities can lead to synergistic effects and could limit the development of drug resistance.

Organ specific drug targeting can also be carried out using the rHSA mutant with different tissue distribution characteristics from that of the native counterpart. Manipulation of the total surface charge of albumin, which could be achieved genetically, could result in a different tissue accumulation of the protein. Conjugation of drugs with these tissue-specific albumin mutants is expected to achieve better targeting efficiency through a reduction in chemical maneuvers required and an increased purity of the conjugates. Therefore, functionally modified or improved albumin developed by genetic engineering is key to the better usage of albumin. In this respect, we are currently investigating the molecular design of antioxidant albumin and organ-directed mutants (21,70).

Albumin Fusion Protein

Most biopharmaceuticals are injected, to avoid rapid degradation in the digestive tract. However, most injected proteins are also rapidly cleared from the blood. Therefore, relatively high doses and/or frequent injections are typically required to maintain therapeutic blood levels, resulting in potentially increased side effects and increased treatment costs, as well as significant patient inconvenience and non-compliance. Albumin fusion may allow the re-engineering of existing recombinant protein therapeutics to address the above shortcoming in the existing therapies. The albumin fusion consists of fusing the albumin gene with the gene for a therapeutic protein or peptide. This fused gene then signals to produce the albumin fusion protein, using suitable expression systems.

Nomura *et al.* found that when the human Apolipoprotein E (hApoE) gene was fused to truncated or whole HSA encoding sequences and expressed under the control of the GAL7 promoter in *S. cerevisiae*, the amount of the whole HSA-hApoE protein secreted was the highest. Since hApoE was known to be very unstable and secreted with great difficulty from *S. cerevisiae*, HSA may serve as a "signal sequence" for the extracellular export of the fused hApoE, thus protecting it from proteolysis by host cell proteases (8).

A genetic conjugate of the HSA-CD4 hybrid aimed at specifically blocking the entry of HIV into $CD4^+$ cells has been designed and successfully expressed by Yeh *et al.* using Kluyveromyces yeast (71). The HSA-CD4 hybrid secretion and anti-HIV infection activities have also been tested in immunodeficient or immunocompetent mice by *in vivo* implantation of the transfected xenogeneic 293 cells as organoids as well as encapsulated in HSA alginate-coated beads (72). The plasma concentrations are dramatically depended on the number of secreting cells and the half-life of the recombinant protein as well. HSA-CD4 showed a plasma concentration of 125 μg/ml, which was considerably higher than the monomeric CD4 counterpart. In addition, it was concluded that allogeneic cells encapsulated in HSA coated alginate beads represented an efficient route to the secretion of recombinant therapeutic proteins.

Syed *et al.* (73) sought to extend the plasma half-life while maintaining the potent antithrombin activity of hirudin, a medicinal leech salivary gland protein comprised of about 66 amino acid residues, via genetic fusion with rabbit serum albumin. The genetic conjugate was expressed in COS-1 cells, thus taking advantage of the optimal folding and disulfide bond formation afforded by passage through the mammalian cell secretory pathway. Hirudin, when linked via its C-terminus to albumin inhibited both the amidolytic and fibrinogenolytic activities of thrombin, without compromising the dye-binding characteristics of rabbit serum albumin. Pharmacokinetic analysis of the fusion protein confirmed an extension of hirudin half-life *in vivo* of greater than two orders of magnitude while gel analysis of the recovered fusion protein from rabbits showed that it circulated in an intact form.

Principia Pharmaceutical Corporation, a wholly owned subsidiary of Human Genome Sciences, Inc. (HGS), has developed an albumin-fusion version of human interferon, Albuferon. Albuferon will initially be evaluated in the treatment of hepatitis C, a viral inflammation of the liver. It is expected that Albuferon may improve patient benefit by providing a sustained source of interferon in the body, thus reducing side effects and the need for frequent injections. Meanwhile, Albutropin™, a long-acting recombinant growth hormone created by fusing the gene of human growth hormone to the albumin gene, has been announced by HGS to undergo Phase I clinical trial recently. Pre-clinical data suggest that Albutropin is eliminated from the body 50 times more slowly than regular growth hormone and is detectable in the blood for at

least a week after its administration. Therefore, Albutropin may offer patients a more convenient administration schedule when compared to the currently used existing short-acting therapies.

Blood Brain Barrier Transport and Non-Invasive Gene Delivery Vectors

Peptide and protein therapeutics are generally excluded from transport from the blood to brain, owing to the negligible permeability of these drugs to the blood-brain barrier (BBB) *in vivo*. However, peptide or protein therapeutics may be delivered to the brain with the use of the chimeric peptide strategy for peptide drug delivery. Chimeric peptides are formed when a non-transportable peptide therapeutic is coupled to BBB drug transport vectors such as cationized HSA, which undergo absorptive-mediated and receptor-mediated transcytosis through the BBB, respectively (74). The amino groups of cationized HSA can be conjugated with various compounds or modified for tissue or cell targeting. Through electrostatic interactions with the glycocalyx of the BBB endothelium, a chimeric peptide of cationized albumin-β-endorphin undergoes absorptive-mediated endocytosis into the brain (75).

A prospective brain targeting strategy would be expected to be based on the present knowledge of the cationic albumin receptor and gene fusion techniques. Theoretically, recombinant HSA mutant with increased cationic amino acid residues or decreased anionic amino acid residues can be genetically fused to the non-transportable peptide drug. This approach has actually been executed by Shin *et al.* (76) and Penichet *et al.* (77), who demonstrated that the fusion proteins are effective as a transport vector. However, the feasibility of recombinant cationic HSA fusion proteins as a BBB transport vector awaits future confirmation attempts.

There is an intense interest in developing drug-delivery systems that deliver drugs and genes to diseased tissues and organs without producing detrimental side effects in healthy tissues and organs. However, many intravascular drug- and gene-delivery vehicles are limited by the endothelial barrier. Ultrasound contrast agents has been used to develop new vectors for gene delivery. The bioeffect of ultrasound application to thin-shelled microbubbles flowing through small microvessels produces vessel wall ruptures *in vivo* that are sufficiently large to permit the extravasation of red blood cells, yet cell and tissue damage are limited to the rupture site itself (78). By taking advantage of the physical properties of microbubbles and coating materials, genetic drugs can be incorporated into ultrasound contrast agents. These gene-bearing microbubbles can be injected intravenously and as the microbubbles enter the region of insonation, ultrasound energy can be applied to the target region whereby the threshold for cavitation by ultrasound energy is lowered by the ultrasound contrast agents. The microbubbles then cavitate, locally releasing DNA. Cavitation likely causes a local shockwave that improves the cellular uptake of DNA. In the cardiovascular system, gene therapy has potential for improving myocardial vascularization and ameliorate congestive heart failure. With transthoracic ultrasound, using a commercially available diagnostic ultrasound system and an IV injection of gene-bearing microbubbles, high levels of transgene expression are observed in the insonated region of the myocardium (79). This new technology using microbubbles and ultrasound for gene delivery, which could also be used for local drug delivery, angiogenesis, and vascular remodeling, or for tumor destruction, merits further study and development (80).

HSA has been found to enhance DNA transfection by cationic liposome-DNA complexes, lipoplexes, which are used as gene delivery vehicles. The interaction of highly positive charged lipoplexes with biologic macromolecules in blood and tissues is one of the drawbacks of this system (81). Coating cationic liposomes with HSA could generate negatively charged HSA-lipoplexes complexes that can facilitate the efficient transfection of cultured cells, and the ternary complexes may overcome some of the problems associated with the use of highly positive charged complexes for gene delivery *in vivo*. On the other hand, cationized albumin could serve as a potential non-viral vector system for gene delivery. In a recent study, cationized albumin was shown to undergo spontaneous self-assembly with DNA, with low reporter gene expression in the presence of chloroquine shock treatment (82). Furthermore, an attempt has been made using macroaggregated polyethyleneimine-albumin conjugates loaded with plasmid for genetic immunization, with the goal of eliciting mucosal immunity in the lung. Both pulmonary mucosal and systemic immune responses have been elicited, indicating that this method may have utility for genetic immunization via intravascular delivery to the lung and, potentially, to other organs and tissues (83).

Site-Directed HSA Mutant with Antidote Activities

The major treatment for jaundice in newborns and type I Crigler-Najjar syndrome is phototherapy. Phototherapy combined with albumin infusion has been shown effective in immediately reducing serum unbound bilirubin values, a potentially neurotoxic fraction (84). Maintaining the concentration of unbound bilirubin at <20 nmol/L and the total bilirubin concentration at a level lower than the binding capacity of serum albumin is important for preventing neurologic deficits in Crigler-Najjar syndrome (85). Phototherapy converts the bilirubin bound to HSA into more soluble nontoxic structural isomers that are easily eliminated from the circulatory system. The rate of formation of isomers and the isomer composition of the reaction products have been shown to be highly dependent on the conformation and electronic environment of bilirubin bound to HSA. Hence, an rHSA mutant that produces soluble bilirubin photoproducts at a greater rate than wild type HSA on illumination would be expected to increase the efficiency of phototherapy. The administration of such a mutant to jaundiced newborns could potentially result in a more efficient phototherapy in certain patients, especially premature infants with low levels of HSA (86). Similarly, appropriate rHSA mutants that bind digoxin with a higher affinity have the potential for use in the treatment of digoxin toxicity (87). rHSA mutants with antidote activities such as these are attractive therapeutic agents especially for pregnant patients because the protein will not cross the placental barrier and affect the fetus. In addition, these mutants can also be employed as an extracorporeal detoxifier in albumin dialysis (88–90).

CONCLUSION

Albumin is in widespread use as a stabilizing agent in protein or peptide drugs or as a carrier in drug delivery sys-

tems. In other words, albumin can be treated as a useful pharmaceutical material. The pharmaceutical applications of albumin have been evaluated intensely during the eighties. However, due to a variety of technical problems, only a handful of products were actually successful in reaching the market. Since the X-ray crystallographic structure of albumin is now available, the chemical reactivity as well as structural stability aspects of the protein can be clarified more readily. This suggests that the previously available albumin DDS formulations should be reevaluated.

In addition, advances achieved in current DNA recombinant and synthetic technologies enable polymeric albumin or albumin fragments to be easily produced. These recombinant albumin products are of high purity and quality. Furthermore, having similar functions as native HSA, rHSA is superior to other DDS materials with respect to ligand binding capabilities. Covalent interaction of ligands with, for example, nitro, sulfhydryl and/or carboxyl moieties is also feasible. Because there is no concern regarding contamination by invasive pathogens, recombinant albumin products are suitable for use as DDS materials, and can be tailor made according to the therapeutic agents.

On the other hand, rHSA without any modification may not be as competent as other DDS materials for use in organ specific targeting of drugs. In this respect, though it is still preliminary results, we have found that certain rHSA mutants produced in our laboratory exhibit organ specific disposition properties. However, the immunogenecity as well as the safety profile of these mutants must be examined before further application as drug carrier. A major challenge in the synthetic production of rHSA is scaling up to achieve mass production where the efficiency of expression and purification are particularly crucial (91). At the same time, for extensive use in pharmaceutical designs, the cost of rHSA must be reduced to a reasonable level.

Hence, the "Old but with new functions protein" albumin is entering a new horizon in protein drug development, where the answers for the old puzzles will soon be obtained in the present proteomic era.

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