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A receptor for formaldehyde-treated serum albumin on human placental brush-border membrane

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Formaldehyde-treated serum albumin (f-Alb) is known to be taken up and degraded by sinusoidal liver cells via receptor-mediated endocytosis. We report that ^{125}I-labeled f-Alb (^{125}I-f-Alb) binding to human placental brush-border membranes also occurs. This binding reached equilibrium within 40 min at 37°C. Kinetic studies demonstrated the presence of saturable binding with an apparent K_d of 2.1 μg of f-Alb/ml and a maximal binding of 2.3 μg/mg of membrane protein at pH 7.5. Maximal binding was observed at between pH 7.5 and 8.0. ^{125}I-f-Alb binding to the membranes was little inhibited by a 1000-fold molar excess of ovalbumin, human apo-transferrin and native bovine serum albumin. No binding was observed with membranes which had been pretreated with proteinase or trypsin. This f-Alb receptor was extremely heat-stable, since the binding was not abolished even by pretreatment of the membranes at 78°C for 30 min. EDTA, Ca^{2+} and Mg^{2+} had no effect on ^{125}I-f-Alb binding, so the binding was independent of divalent cations. These data suggest that a receptor specific for f-Alb exists on human placental brush-border membranes of syncytial trophoblasts.

Introduction

Stemming from a report that serum albumin treated in vitro with formaldehyde (f-Alb) is rapidly cleared from the bloodstream after injection into mice [1], and subsequent studies showing that sinusoidal liver cells were able to take up f-Alb in vitro and were largely responsible for in vivo uptake also [2], the membrane-associated receptor for f-Alb has been characterized and purified from rat sinusoidal liver cells [3,4]. However, the physiological role of this receptor remains unclear, since the conditions necessary for f-Alb formation are unlikely to occur in vivo, and thus the natural ligand is unknown. Since f-Alb is also effectively taken up by macrophages via a receptor-mediated pathway [5,6], it has been suggested that this receptor may belong to a family of receptors with a scavenging function present on macrophages or macrophage-derived cells and recognizing a variety of natural and artificial ligands [4,7]. Lately, it has been reported that at least two distinct scavenger receptor exist on the plasma membranes of sinusoidal cells and peritoneal macrophages, one specific for negatively charged proteins and the other for aldehyde-modified proteins [7].

Sinusoidal liver cells comprise the major intravascular scavenger cells, in vivo, of chemically

Abbreviations: f-Alb, formaldehyde-treated bovine serum albumin; BSA, bovine serum albumin; PBS, phosphate-buffered saline; LDL, low-density lipoprotein.

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modified proteins such as acetylated low-density lipoprotein (LDL), in addition to formaldehyde- or nitroguanidine-treated albumin [8], suggesting the existence of a family of receptors for these ligands on macrophages and other cells of reticuloendothelial origin. However, the fundamental argument about the physiological role of these receptors remains, fueled by the fact that the ligands are of a non-physiological nature. To obtain a clearer picture of the function of the f-Alb receptor, we present evidence of such a receptor on human placental brush-border membranes of syncytial trophoblasts, which, in contrast to sinusoidal liver cells, do not belong to the reticuloendothelial system.

Materials and Methods

Preparation of human placental brush-border membranes

Fresh placentas from uncomplicated term pregnancies were obtained within 60 min of delivery. Brush-border membranes were prepared by a modification [9] of the method reported by Smith et al. [10]. All preparative procedures were performed at 4°C. Phosphate-buffered saline (0.15 M NaCl/0.01 M sodium phosphate buffer (pH 7.1)) was used in all preparative steps. In brief, pieces of placenta were cut down from the chorionic plate. To remove excess blood, pieces of a whole placenta (about 500 g) were rinsed in ice-cold PBS (approx. 1 liter). After slight stirring with a spatula, the PBS solution was discarded. This procedure was repeated two more times. The washed pieces of placenta were cut into small pieces (about 3 cm^3 cubes) with scissors and rinsed in 1 liter of ice-cold PBS. This suspension was stirred with a large stirring bar for 45 min at 4°C, and then filtered through four layers of gauze. The filtrate was centrifuged at $10000 \times g$ for 20 min and then the supernatant solution was further centrifuged at $100000 \times g$ for 30 min to bring down the cream-colored membrane pellet. The resulting pellet was washed twice with 100 ml ice-cold PBS by centrifugation at $100000 \times g$ for 30 min. Resuspension of the pellet was carried out by ten strokes with a Teflon homogenizer. The purity of the placental brush-border membrane preparation and contamination with other organelles were analyzed by electron microscopy and marker enzyme determinations employing 5′-nucleotidase [11], alkaline phosphatase [12], cytochrome-*c* oxidase [13] and arylsulfatase [14]. This membrane pellet was stored at −80°C until use. Prior to use, this pellet was resuspended with the incubation buffer used in the binding assay, as described below.

Preparation of ligand and iodination

Formaldehyde-treated bovine serum albumin (f-Alb) was prepared by a modification of the method reported [1,2]. Briefly, 0.4 g of bovine serum albumin was dissolved at room temperature in 5.2 ml of 0.45 M sodium carbonate buffer (pH 10.0). To this solution were added 5.4 ml of formaldehyde solution (37% w/v), dropwise under stirring, to make a final concentration of 20% (w/v). After incubation at 37°C for 1 h, the solution was extensively dialyzed against PBS. Following removal of insoluble materials by filtration with a 0.2 μm Dispo syringe holder (Spectrum Medical Industries), the resulting filtrate was stored at −80°C before use. The f-Alb thus prepared was radiolabeled to a specific activity of 4000–6000 cpm/ng with $Na^{125}I$ and Iodo beads. In brief, the reaction mixture containing 100 μg f-Alb and 1 mCi of $Na^{125}I$ and two Iodo beads in 500 μl 0.5 M sodium phosphate buffer (pH 7.1) was incubated at room temperature for 20 min and free iodine was removed by gel-filtration on a Sephadex G-25 column pre-equilibrated with PBS containing 0.02% ovalbumin.

Binding assay of ^{125}I-f-Alb to human placental brush-border membranes

In order to separate ligand-receptor complexes from free ligand, we used a filtration method through Whatman GF/C glass filters, as described below. The binding assay was carried out in disposable 10 × 75 mm glass tubes. The incubation mixture contained, in a final volume of 400 μl, the following: incubation buffer (0.05 M Tris-HCl (pH 7.5)/2% BSA/0.15 M NaCl/0.5 mM $CaCl_2$), ^{125}I-f-Alb in the presence or absence of a 1000-fold excess of unlabeled f-Alb, and placental membrane fraction. The reaction mixture was incubated for 30 min at 37°C with shaking and then the suspension was filtered through a Whatman GF/C glass filter, under reduced pressure,

using a Millipore 1225 Sample Manifold. The incubation tube was washed twice with 2 ml ice-cold PBS. The filter disc was removed and counted in a Berthord gamma counter. All binding values stated represent specific binding, as determined by subtraction of nonspecific binding obtained in the presence of unlabeled f-Alb from the total binding obtained in the absence of unlabeled f-Alb. The filter was rinsed in PBS containing 2% BSA (w/v) for 60 min prior to use. Each value in the figures and tables represents the mean value of duplicate assays. The deviation from the mean was within 10%. Protein concentrations were determined by the method of Lowry et al. [15], with BSA as the standard.

Chemicals

$Na^{125}I$ was purchased from Amersham; Bovine serum albumin (BSA), concanavalin A, methyl α-D-glucoside, human apo-transferrin, horse ferric ferritin, dextran sulfate (M_r approx. 5000), poly (L-glutamic acid) (M_r 13000), proteinase from Tritirachium album, trypsin, and ovalbumin (Grade III) were from Sigma; Sephadex G-25 and Sepharose 6B were from Pharmacia. Iodo beads were from Pierce. Other reagents were of analytical grade obtained commercially.

Results

Chemical modification of albumin by formaldehyde produces both polymeric and monomeric molecules [16]. Although the precise chemical nature of the modification by formaldehyde remains unclear, Horiuchi et al. have suggested that the protein-aldehyde adduct might be not of a Schiff-base type but of a carbinolamine type [7]. To examine the degree of polymerization of albumin following our formaldehyde treatment, the ^{125}I-f-Alb prepared as described in Materials and Methods was applied onto a Sepharose 6B column. ^{125}I-f-Alb was eluted between horse ferric ferritin (M_r 450000) and BSA (M_r 67000) (Fig. 1), indicating that polymerization had occurred. However, from Fig. 1, it is difficult to determine the mean size of the polymers, and there appeared to be a considerable amount of unpolymerized ^{125}I-f-Alb. The biological activity of our prepared ^{125}I-f-Alb was briefly examined by administration

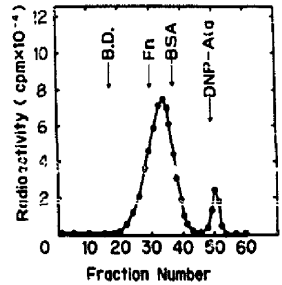

Fig. 1. Gel-filtration of ^{125}I-f-Alb. The solution (500 μl) containing blue dextran (B.D.), horse spleen ferritin (Fn), native bovine serum albumin (BSA), and DNP-alanine (DNP-Ala) was applied onto a Sepharose 6B column (1×45 cm) pre-equilibrated with PBS and eluted at the rate of 0.1 ml/min. Arrows indicate elution peaks of the marker proteins.

of a small amount (0.4 μg/100 g body weight) into rat, whereupon 70% of the radioactivity was taken up by the liver within 5 min. This uptake was inhibited to about 40% by administration in the presence of a 1000-fold excess of unlabeled f-Alb.

We then examined the binding of our prepared ligand to human placental brush-border membranes. Fig. 2 shows the time-course of binding of ^{125}I-f-Alb to the membranes, and the binding reaction appeared to reach equilibrium within 40 min at 37°C. At a fixed concentration of ^{125}I-f-Alb, the amounts of ^{125}I-f-Alb bound to the mem-

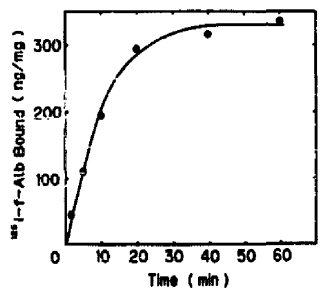

Fig. 2. Time-course of binding of ^{125}I-f-Alb to brush-border membranes. Each tube, in a total volume of 400 μl, containing 29 μg of membrane proteins, 0.28 μg of ^{125}I-f-Alb, and incubation buffer in the presence or absence of a 1000-fold excess of unlabeled f-Alb was incubated for the indicated times at 37°C. The binding assay was performed as described under Materials and Methods and the values of ^{125}I-f-Alb binding are represented as specific binding.

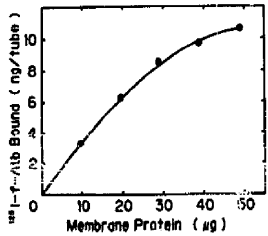

Fig. 3. ^{125}I-f-Alb binding as a function of amount of brush-border membranes. Each tube, in a total volume of 400 μl, containing 0.28 μg of ^{125}I-f-Alb, increasing membrane proteins (0 to 48.5 μg) and incubation buffer in the presence or absence of a 100-fold excess of unlabeled f-Alb, was incubated for 40 min at 37 °C. The values of ^{125}I-f-Alb binding are represented as specific binding.

branes were approximately proportional to the amount of membrane present in the assay mixture up to 20 μg of the membrane protein (Fig. 3). Fig. 4 shows the effect of pH on ^{125}I-f-Alb binding to the membranes. Within the range of pH 5.0 to 9.0, maximal binding was observed between pH 7.5 and 8.0. In order to examine the specificity of ^{125}I-f-Alb binding to the membranes, binding studies were performed with increasing concentrations of human apo-transferrin, ovalbumin, BSA

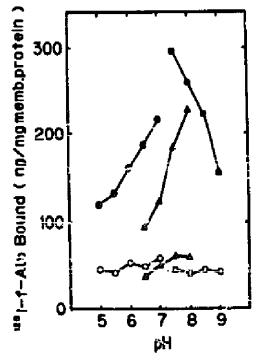

Fig. 4. Effect of pH on ^{125}I-f-Alb binding to brush-border membranes. Each tube, in a total volume of 400 μl, containing 0.14 μg of ^{125}I-f-Alb, 43.9 μg of membrane proteins, and incubation buffer comprising 2% BSA, 0.15 M NaCl, 0.5 mM $CaCl_2$, and 0.05 M buffer described below in the presence (□, △, ○), or absence (■, ▲, ●) of a 1000-fold excess of unlabeled f-Alb was incubated for 40 min at 37 °C. Tris-HCl buffer (0.05 M), sodium phosphate buffer (0.05 M), and cacodylate buffer (0.05 M) were used to adjust the pH from pH 7.5 to 9.0 (□, ■), pH 6.5 to 8.0 (△, ▲), and pH 5.0 to 7.0 (○, ●), respectively.

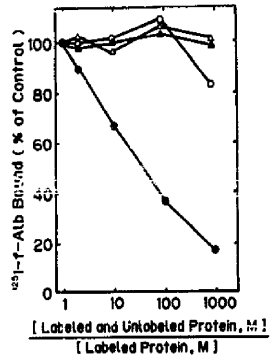

Fig. 5. Specificity of ^{125}I-f-Alb binding to brush-border membranes. Each tube, in a total volume of 400 μl, containing 0.28 μg of ^{125}I-f-Alb, 87.8 μg of membrane proteins, and incubation buffer in the presence of increasing amounts of f-Alb (●), ovalbumin (▲), human apo-transferrin (△), and native bovine serum albumin (○) was incubated for 40 min at 37 °C. After incubation, the amount of the binding to the membranes was determined as described under Materials and Methods. Molecular weight of ^{125}I-f-Alb was regarded as 67000 the same as that of monomeric albumin. In the case of the inhibition assay by native bovine serum albumin, 2% ovalbumin was used in the incubation buffer instead of 2% native bovine serum albumin.

or unlabeled f-Alb in the reaction mixture (Fig. 5). The binding of ^{125}I-f-Alb was unaffected by the presence of a 1000-fold molar excess of human apo-transferrin or ovalbumin. The binding was inhibited to 85% of control by the presence of a 1000-fold molar excess of BSA and to 18% by the presence of a 1000-fold molar excess of f-Alb. Fig. 6A shows the amount of bound ^{125}I-f-Alb as a function of the concentration of ^{125}I-f-Alb in the reaction mixture at 37 °C and pH 7.5. A Scatchard plot of these data (Fig. 6, B) indicates that ^{125}I-f-Alb binding is saturable, with an apparent K_d = 2.1 μg of ^{125}I-f-Alb/ml and that the maximal ^{125}I-f-Alb binding is 2.3 μg/mg of membrane protein.

To characterize further the f-Alb receptor on placental membranes, firstly, the divalent cation dependence of binding was examined (Table I). However, EDTA, Ca^{2+} and Mg^{2+} had no substantial effect on ^{125}I-f-Alb binding. Then the effect of concanavalin A on binding was studied (Table II), and binding was not inhibited by 100 μg/ml of concanavalin A. The effect of polyan-

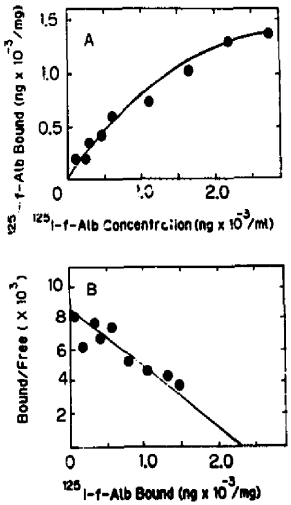

Fig. 6. Effect of ^{125}I-f-Alb concentration on the binding to brush-border membranes. (A) Each tube, in a total volume of 400 µl, containing 2.9 µg of membrane proteins, increasing amounts of ^{125}I-f-Alb, and incubation buffer in the presence or absence of a 1000-fold excess of unlabeled f-Alb was incubated for 40 min at 37°C. The values of ^{125}I-f-Alb binding are represented as specific binding. (B) Scatchard plots calculated from the data of (A).

ions on binding was tested, but while dextran sulfate (50 µg/ml) inhibited ^{125}I-f-Alb binding to 15%, with poly(L-glutamic acid) there was no inhibition, even at 500 µg/ml (Fig. 7). The protein nature of the receptor was demonstrated by pretreatment of membranes with trypsin or proteinase (20 µg/ml) at 37°C for 10 min (Table III), both of which abolished binding activity. Heat stability of the receptor was examined by pretreatment of the membranes for various periods at 37, 50, or 78°C. As shown in Fig. 8, the f-Alb receptor on human placental brush-border membranes is extremely heat-stable; in fact, short

TABLE I

EFFECT OF DIVALENT CATIONS ON BINDING OF ^{125}f-Alb TO THE MEMBRANES

Each tube, in a total volume of 400 µl, containing 29 µg of membrane proteins, 0.28 µg of ^{125}I-f-Alb, incubation buffer in the presence or absence of a 1000-fold excess of unlabeled f-Alb, and the indicated amounts of EDTA or EDTA in the presence of divalent cation (1 mM or 10 mM), was incubated for 40 min at 37°C.

Treatment	Specific binding		Control
	cpm	ng	(%)
None	34175	13.7	100
EDTA (1 mM)	37064	14.8	108
EDTA (1 mM)			
+ Ca^{2+} (1 mM)	37231	14.9	109
+ Ca^{2+} (10 mM)	33197	13.3	97
+ Mg^{2+} (1 mM)	36707	14.7	107
+ Mg^{2+} (10 mM)	27560	11.0	81

TABLE II

EFFECT OF CONCANAVALIN A ON BINDING OF ^{125}I-f-Alb TO THE MEMBRANES

Each tube, in a total volume of 400 µl, containing 29 µg of membrane proteins, 0.28 µg of ^{125}I-f-Alb, incubation buffer in the presence or absence of a 1000-fold excess of unlabeled f-Alb, and the indicated amounts of concanavalin A or methyl α-D-glucoside, was incubated for 40 min at 37°C.

Treatment	Specific binding		Control
	cpm	ng	(%)
None	40600	16.2	100
Concanavalin A (50 µg/ml)	47157	18.9	116
Concanavalin A (100 µg/m)	47046	18.8	116
+ methyl α-D-glucoside (0.1 M)	41205	16.5	101
Methyl α-D-glucoside (0.1 M)	40869	16.3	101

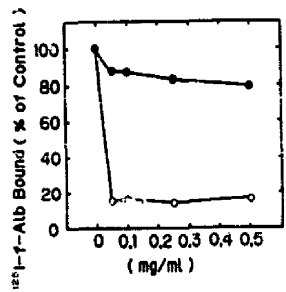

Fig. 7. Effect of polyanions on ^{125}I-f-Alb binding to brush-border membranes. Each tube, in a total volume of 400 µl, containing 0.28 µg of ^{125}I-f-Alb, 87.8 µg of membrane proteins, incubation buffer, and increasing amounts of dextran sulfate (○) or poly(L-glutamic acid) (●), was incubated for 40 min at 37°C. After incubation, amounts of ^{125}I-f-Alb binding was determined as described under Materials and Methods.

TABLE III

EFFECT OF PROTEINASE AND TRYPSIN TREATMENT ON BINDING OF ^{125}I-f-Alb TO THE MEMBRANES

Brush-border membranes (1.5 mg) was suspended in 500 μl of PBS containing 5 μg or 10 μg of proteinase or trypsin, respectively, in Eppendorf tube and incubated for 10 min or 20 min at 37°C. To remove proteinase or trypsin, the sample tube was centrifuged after the indicated incubation period and the resultant membrane pellet was washed twice with 500 μl of PBS. The washed membranes were resuspended with PBS and the f-Alb binding was carried out as described under Materials and Methods. As the control, membranes were incubated for 10 min at 37°C without proteinase or trypsin, and then treated with the same procedure as mentioned above.

Treatment	Specific binding		Control
	cpm	ng	(%)
None	33774	7.7	100
Proteinase (10 min)	1311	0.3	3.8
(20 min)	1302	0.3	3.8
Trypsin (10 min)	109	0.02	0.3
(20 min)	185	0.04	0.5

periods of heat treatment increased ^{125}I-f-Alb binding 3-fold.

Discussion

The receptor for f-Alb on liver cells belonging to the reticuloendothelial system has been well characterized [3,4,7,8]. In the present study, we report that the f-Alb receptor also occurs on human placental brush-border membranes of syncytial trophoblast cells not belonging to the reticuloendothelial system.

The f-Alb binding to the membranes was demonstrated to be saturable – with an apparent K_d of 2.1 μg of f-Alb/ml and a maximal binding of 2.3 μg/mg of membrane protein – and specific, since binding was not competed out with human apo-transferrin, ovalbumin, although slightly by native BSA. Perhaps native albumin is able to bind weakly to the f-Alb receptor in placental membrane. Concanavalin A did not affect binding, which suggests that a mannose core of oligosaccharides is not involved in f-Alb binding in the placenta. Binding activity was demonstrated to be protein-mediated and extremely heat-stable, in fact, increasing up to 3-fold after heat pretreatment of membranes. In contrast to the f-Alb receptor of rat liver, which is not heat-stable, the f-Alb receptor of human placental brush-border membrane was extremely heat-stable. At the moment, we have no explanation for the heat-stability of the f-Alb receptor of human placenta. After purification of the placental f-Alb receptor, the structural nature of the ligand-binding site(s) will be examined. We also do not know the reason for the increase in f-Alb binding after heat treatment, but the appearance of f-Alb receptors previously sequestered within membrane vesicles may be responsible.

Scavenger receptors for chemically modified proteins (formaldehyde treated and maleylated albumin, malondialdehyde treated and acetylated LDL, etc.) have been reported to exist on macrophages and cells belonging to the reticuloendothelial system, i.e., endothelial cells. However, the membrane fraction prepared by us from human placenta has been previously shown by marker enzyme analysis to be 13.7- and 12.3-fold enriched in the membrane markers 5′-nucleotidase and alkaline phosphatase, respectively, and by electron microscopy to consist predominantly of smooth membrane and microvillus structures containing longitudinally oriented microfilament [9].

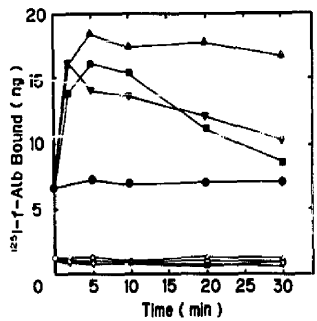

Fig. 8. Effect of the membrane pretreatment at higher temperature on ^{125}I-f-Alb binding. After 1.18 mg/ml of membrane proteins was pretreated for the indicated times at 37 (○, ●), 50 (△, ▲), 60 (▽, ▼), and 78°C (□, ■), the membranes were rapidly immersed in a bath at 37°C. Each tube, in a total volume of 400 μl, containing 0.24 μ of ^{125}I-f-Alb, 118 μg of the pretreated membrane proteins, and incubation buffer in the presence (○, △, ▽, □) or absence (●, ▲, ▼, ■) of a 1000-fold excess of unlabeled f-Alb, was then incubated for 40 min at 37°C. After incubation, the ^{125}I-f-Alb binding was determined as described under Materials and Methods.

Even though contamination of endothelial cells may be present, their amount would be too low to explain the binding capacity for f-Alb detected in brush-border membranes. Further evidence that the binding of f-Alb cannot be accounted for by endothelial cells in our placental membrane preparation is lacking at this time, but a supporting point is that tissue homogenization, which would be expected to release endothelial cells, is not a step in our membrane preparation method. The availability of antibodies against this receptor should allow immunohistochemical characterization to clarify this point.

Although the assay system employed in this paper differs from that used in characterization of the major f-Alb receptor in rat liver, we believe that several qualitative differences between the rat liver and placental receptor described within this paper are valid. These differences include: glycosylation (concanavalin A blocks f-Alb binding in liver [3], but not in placenta), the effect of polyanions (both dextran sulfate and poly (L-glutamic acid) block f-Alb binding in liver [8], but only dextran sulfate blocks binding in placenta), and heat stability (the liver receptor is unstable [3], whereas the placental receptor is heat stable). Thus, the f-Alb receptor in placenta may be different from that in liver.

Considering that syncytial trophoblasts are bathed in maternal blood and in a position to mediate many important process between the mother and the fetus, such as transfer of metabolites, the presence of f-Alb receptors on these cells highlights their physiological significance, albeit unknown at this time. Binding of another scavenger molecule, acetylated LDL, to syncytial trophoblast membranes has been previously reported, where a role in supplying cholesterol needs of the placenta was proposed [17]. The liver and macrophage f-Alb receptors are considered to play mainly a scavenger role, clearing modified or denatured proteins from the bloodstream. The placental receptor may also play such a scavenging, protective role. Determination of the physiological ligand(s) for this receptor may clarify this issue. Evidence presented in this paper suggest that the liver and placental f-Alb receptors may be different.

In conclusion – the human placenta may be a useful source of f-Alb receptor for further studies of the molecular basis of f-Alb uptake. Such studies might complement the findings about the major in vivo receptor, present on sinusoidal liver cells, and hasten our understanding of the physiological role of the f-Alb receptor.

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