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SPECIFIC UPTAKE OF SUCCINYLATED PROTEINS *VIA* A SCAVENGER RECEPTOR-MEDIATED MECHANISM IN CULTURED BRAIN MICROVESSEL ENDOTHELIAL CELLS

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Summary: Cellular uptake of succinylated catalase (Suc-CAT; Mw 227 kDa), bovine serum albumin (Suc-BSA; Mw 70 kDa), superoxide dismutase (Suc-SOD; Mw 34 kDa) and soybean trypsin inhibitor (Suc-STI; Mw 21 kDa) was studied using primary cultures of bovine brain microvessel endothelial cells (BMECs) developed as an *in vitro* model of the blood-brain barrier. Large succinylated proteins (Suc-CAT, Suc-BSA) were taken up by BMECs whereas significant uptake was not observed for native proteins and small succinylated proteins (Suc-SOD, Suc-STI). Uptake of Suc-BSA was significantly inhibited at 4°C and in the presence of endocytosis inhibitors. Large succinylated proteins, maleylated BSA and dextran sulfate also showed competitive inhibition against Suc-BSA uptake while small succinylated proteins and carboxymethyl dextran did not show any effect. These results indicate that microvessel endothelial cells obtained from the brain endocytose succinylated proteins via a scavenger receptor-mediated mechanism for polyanions, and in addition, the importance of molecular weight or total numbers of anionic charges per one molecule of proteins is suggested. Usefulness of direct succinylation of proteins for their delivery to the brain capillary endothelium is thus demonstrated.

The blood-brain barrier (BBB) which consists of brain microvessel endothelial cells (BMECs) restricts the brain uptake of water-soluble substances including peptides and proteins from blood circulation. However, several BBB transport systems have been identified for specific macromolecules and some attempts have been made to utilize these systems for drug delivery

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Abbreviations: BBB, blood-brain barrier; BMECs, brain microvessel endothelial cells; BSA, bovine serum albumin; Suc-BSA, succinylated BSA; CAT, catalase; SOD, superoxide dismutase; STI, soybean trypsin inhibitor; DiI-Ac-LDL, acetylated low density lipoprotein labeled with 1,1'dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate; HBSS, Hanks' balanced salt solution.

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to the brain (1). It has been reported that BMECs take up acetylated low density lipoprotein (Ac-LDL) *in vivo* (2,3), and Ac-LDL has been used as a marker to identify endothelial cells in culture (4,5). However, little information is available on cellular uptake mechanism of Ac-LDL including effect of its electric charge in BMECs.

On the other hand, Ac-LDL, an anionic macromolecule, has been known as a typical ligand for scavenger receptors on macrophages and sinusoidal liver cells (6-13). The scavenger receptors have unusually broad, but circumscribed, ligand binding specificity. For example, high affinity ligands for the scavenger receptors include various types of polyanions, such as Ac-LDL, polyinosinic acid, dextran sulfate, and bacterial lipopolysaccharides while many polyanions involving poly(D-glutamic) acid, polycytidylic acid, and chondroitin sulfate are not ligands (6,7). Regarding chemically modified albumins, maleylated BSA (Mal-BSA) (6-9,12) and formaldehyde-treated albumin (12-15) are ligands for scavenger receptors while acetylated albumin (6) is not. However, it has not been reported whether succinylated BSA (Suc-BSA) can be a ligand for scavenger receptors even in macrophages.

In an attempt to assess the feasibility of anionization of proteins for their brain delivery, we carried out cellular uptake studies of succinylated proteins using cultured bovine BMECs, which have been developed as an *in vitro* BBB model system (16-18). In this report, we demonstrate that succinylated proteins are taken up by BMECs via a scavenger receptor-mediated mechanism for polyanions.

MATERIALS AND METHODS

Chemicals. Catalase (CAT; bovine liver), bovine serum albumin (BSA; fraction V), soybean trypsin inhibitor (STI), and dextran sulfate (Mw 8 kDa and 500 kDa) were obtained from Sigma, St. Louis, MO. Recombinant Human SOD (111-Ser) was kindly supplied by Asahi Chemical Industry Co., Shizuoka, Japan. Acetylated low density lipoprotein labeled with 1,1'dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI-Ac-LDL) was obtained from Biomedical Technologies, Stoughton, MA. [^{111}In]Indium chloride was kindly supplied by Nihon Medi-physics, Takarazuka, Japan. [^{125}I]Iodine was obtained from New England Nuclear, Boston, MA. All other chemicals were reagent grade products obtained commercially.

Synthesis and Radiolabeling of Succinylated Proteins. Proteins were dissolved in 0.2 M Tris-HCl buffer (pH 8.65) and succinic anhydride dissolved in dimethyl sulfoxide was added slowly. The mixture was stirred for 1 hr at room temperature. The solution was washed, concentrated by ultrafiltration, and lyophilized. Mal-BSA was synthesized by the same procedure using maleic anhydride. Proteins were radiolabeled with ^{111}In using bifunctional chelating agent diethylenetriaminepentaacetic acid anhydride (19) or with ^{125}I by chloramine T method (20).

Isolation and Culture of Bovine BMECs and Mouse Peritoneal Macrophages. Microvessel endothelial cells were isolated from the cerebral gray matter of bovine brains as described by Audus and Borchardt (16,17). Isolated BMECs were suspended in culture medium (MEM/F-12 with 10 % horse serum), seeded in 12-well culture plates pretreated with rat tail collagen and fibronectin at a density of approximately 5 x 10^5 cells per well and grown to confluence (9-11 days old in culture). The in vitro model system has been shown to possess all the features of the BBB including tight intercellular junctions, the lack of membrane fenestrations, γ-glutamyl transpeptidase and alkaline phosphatase activities, factor VIII antigen, and amino acid and hexose transport systems (21-24).

Resident macrophages were collected from the peritoneal cavity of male ICR mice (20-25 g) with RPMI 1640 medium. After preincubation for 2 hr, the cell layer was washed three times with the medium to remove non-attached cells and the attached cells were incubated for 18 hr with RPMI 1640 medium containing 10 % fetal bovine serum.

Microscopy. Confluent BMECs and macrophages were incubated with culture media containing DiI-Ac-LDL (10 μg/ml) for 4 hr at 37°C (5). Labeled cells were rinsed five times with cold Hanks' balanced salt solution (HBSS), fixed with 3 % (w/v) formaldehyde in HBSS for 20 min, rinsed, and mounted in 90 % glycerol in HBSS. Fluorescence microscopic examination was carried out by using a Nikon Optiphot (Nikon, Tokyo, Japan).

Uptake Study in BMECs and Mouse Peritoneal Macrophages. Uptake studies were carried out using confluent cell monolayers of BMECs or cultured macrophages in 12-well culture plates. Cells were washed three times and incubated with HBSS for 20 min at 37°C. After the preincubation HBSS was removed and 0.7 ml of HBSS containing 0.1 % of BSA and ^{111}In- or ^{125}I-labeled protein was added. The cell monolayers were washed three times with 0.7 ml of ice-cold HBSS at appropriate time intervals and then solubilized with 0.7 ml of 0.3 M NaOH with 0.1 % Triton X-100 for 1 hr at 37°C. Aliquots were taken for determination of radioactivity and protein content. The radioactivity of ^{111}In or ^{125}I was measured by a well-type NaI scintillation counter (ARC-500, Aloka Co., Tokyo, Japan) and the protein content was determined by modified Lowry method (25).

RESULTS AND DISCUSSION

Prior to uptake studies of succinylated proteins, we confirmed the DiI-Ac-LDL uptake by the monolayer of BMECs (Fig. 1B) in comparison with cultured mouse peritoneal macrophages as a positive control (Fig. 1A). The DiI-Ac-LDL uptake by BMECs was apparently inhibited in the presence of Suc-BSA (Fig. 1C) and dextran sulfate (Mw 500 kDa) (Fig. 1D), suggesting that these compounds and Ac-LDL were taken up by a common mechanism in BMECs.

Fig. 2 shows the uptake time courses of ^{111}In-labeled native, succinylated and maleylated proteins in BMECs. Native BSA and SOD were not significantly taken up by BMECs during the experimental period (Fig. 2A). In contrast, succinylated proteins showed varied uptake patterns between derivatives of different proteins (Fig. 2B). Suc-BSA and Suc-CAT were gradually taken up by BMECs for 5 hr whereas Suc-SOD and Suc-STI showed minimal uptake by the cells. Mal-BSA, a typical ligand for scavenger receptors in macrophages (6-9), also showed a significant uptake in BMECs (Fig. 2C). Similar uptake profiles were observed for succinylated proteins in cultured mouse peritoneal macrophages, *i.e.*, [^{111}In]Suc-BSA was avidly taken up by the macrophages while [^{111}In]Suc-SOD was not (data not shown).

Table 1 summarizes the effects of low temperature and metabolic and endocytosis inhibitors on [^{111}In]Suc-BSA uptake by BMECs. The uptake was significantly inhibited at 4°C and in the presence of 2,4-dinitrophenol, dansylcadaverine and colchicine. These results indicated that Suc-BSA was internalized by receptor-mediated endocytosis.

Table 2 summarizes the effects of protein and dextran derivatives on [^{125}I]Suc-BSA uptake by BMECs, together with physicochemical properties of these macromolecules. Suc-BSA with relatively large molecular weights such as Suc-BSA, Suc-γ-globulin and Suc-CAT. Mal-BSA and dextran sulfates, ligands for scavenger receptors, also suppressed the Suc-BSA

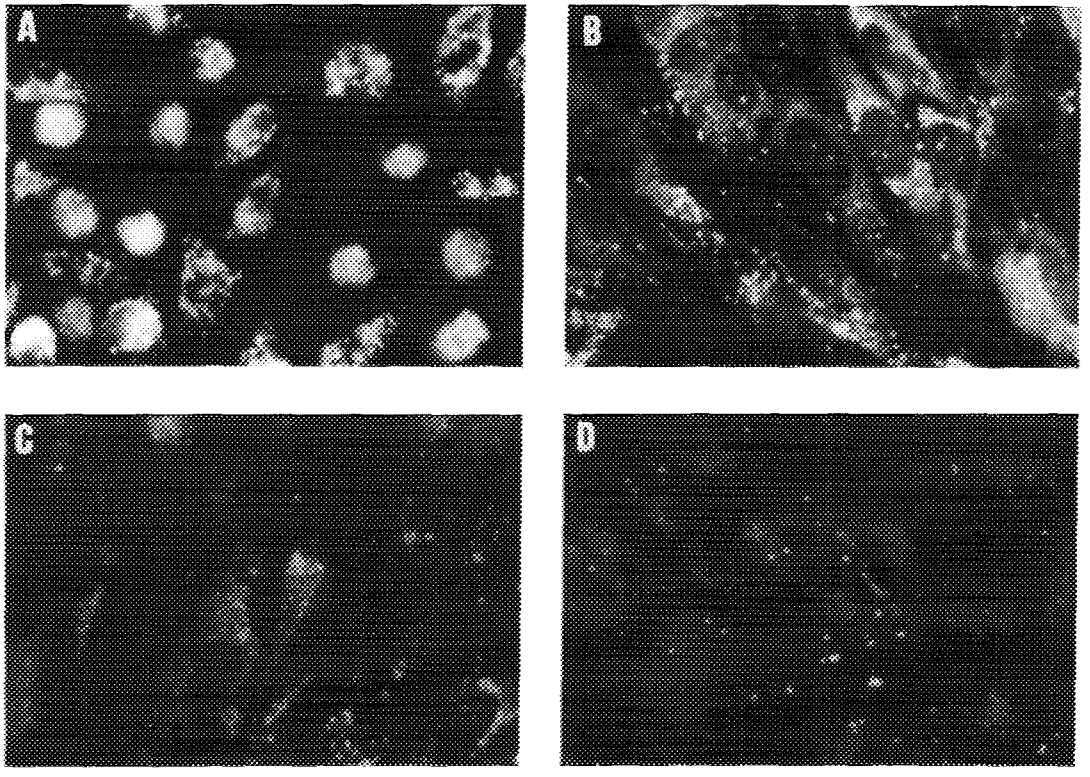

Fig. 1. DiI-Ac-LDL Uptake by BMECs and Mouse Peritoneal Macrophages
A. Mouse peritoneal macrophages (10 μg/ml DiI-Ac-LDL).
B. BMECs (10 μg/ml DiI-Ac-LDL).
C. BMECs (10 μg/ml DiI-Ac-LDL+1mg/ml Suc-BSA).
D. BMECs (10 μg/ml DiI-Ac-LDL+1mg/ml dextran sulfate (Mw 500 kDa)).

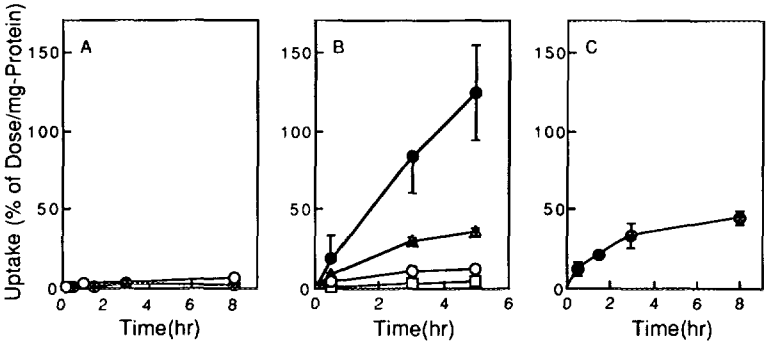

Fig. 2. Uptake Time Courses of ^{111}In-labeled Native (A), Succinylated (B) and Maleylated (C) Proteins in BMECs.
BMECs were incubated with ^{111}In-labeled proteins (1 μg/m) at 37°C. Key: CAT (▲), BSA (●), SOD (○), STI (□). Each point represents the mean ± S.D. values (n = 3).

Table 1. Effect of Low Temperature and Endocytosis Inhibitors on [^{111}In]Suc-BSA Uptake (1 μg/ml, 3 hr) by BMECs

Condition	Uptake (% of Control)	t-Test vs Control
4°C	47.0 ± 2.5	P < 0.01
+ 2,4-Dinitrophenol (1mM)	72.6 ± 3.6	P < 0.05
+ Dansylcadaverine (0.5mM)	74.9 ± 9.5	P < 0.05
+ Colchicine (50μg/ml)	77.7 ± 9.7	P < 0.05

Each value represents the mean ± S.D. (n = 3).

uptake by BMECs. On the other hand, small succinylated proteins (Suc-STI and Suc-SOD), dextran and carboxymethyl dextran failed to show significant inhibition.

Taken together, these results indicated that BMECs endocytosed succinylated proteins via a scavenger receptor-mediated mechanism for polyanions. So far, scavenger receptors on macrophages and sinusoidal liver cells have been identified and well-characterized (6-15). Recently, Schnitzer *et al.* have reported that membrane-associated glycoproteins in

Table 2. Effect of Protein (100 μg/ml) and Polysaccharide (100 μg/ml) Derivatives on [^{125}I]Suc-BSA Uptake (0.1 μg/ml, 3 hr) by BMECs at 37°C

Compound [a]	Molecular Weight (kDa)	Number of Amino Groups [b]	Isoelectric Point [c]	Uptake (% of Control) [d]	t-Test vs Control
Control				100 ± 12.1	
Suc-STI	21	4 (11)	< 4.0 (4.5-4.6)	99.6 ± 14.8	N.S.
Suc-SOD	34	2 (24)	< 4.0 (5.0-5.2)	89.1 ± 5.78	N.S.
Suc-BSA	70	20 (60)	< 4.0 (4.2-4.8)	60.6 ± 11.0	P < 0.02
Suc-BSA(H)	72	5 (60)	< 4.0 (4.2-4.8)	49.0 ± 15.6	P < 0.02
Suc-γ-Globulin	155	30 (86)	< 4.0 (6.3-7.3)	53.9 ± 1.51	P < 0.01
Suc-CAT	227	82 (162)	< 4.0 (5.4-5.8)	45.7 ± 5.28	P < 0.01
Mal-BSA	71	12 (60)	< 4.0 (4.2-4.8)	59.4 ± 11.5	P < 0.02
Control				100 ± 7.39	
Dextran	70	-	-	95.7 ± 8.24	N.S.
DS-8	8	-	-	71.0 ± 8.17	P < 0.02
DS-500	500	-	-	58.5 ± 5.15	P < 0.01
CMD	80	-	-	92.6 ± 12.6	N.S.

[a] Suc-BSA (H), Suc-BSA with higher succinylation ratio; DS, dextran sulfate; CMD, carboxymethyl dextran.
[b] Determined by TNBS method. The value in parenthesis is the number of amino groups of native proteins.
[c] Determined by chromatofocusing method using Polybuffer exchanger 94 resin and the Polybuffer 74 elusion buffer system (Pharmacia, Uppsala, Sweden). The values in parenthesis are the pI values of native proteins.
[d] Each value represents the mean ± S.D. (n = 3).
N.S., not significant.

microvascular endothelial cells obtained from rat epididymal fat pads act as scavenger receptors for conformationally modified albumins including BSA-gold complexes and Mal-BSA (26,27). To our knowledge, the present paper is the first report that capillary endothelial cells from the brain take up succinylated proteins through a scavenger-receptor mediated mechanism.

In addition, the present study suggested that several polyanions, such as succinylated proteins with relatively low molecular weights and carboxymethyl dextran, were not taken up by BMECs, which might involve structural requirements of polyanionic ligands in recognition by the receptor on BMECs. Similar characteristics have been shown for macrophage scavenger receptors for various polyanions (6,7).

It has been reported that Ac-LDL undergoes only endocytosis into BMECs but not transcytosis into the brain interstitial fluid (3). Further studies are required to clarify the fate of succinylated proteins after endocytosis by BMECs.

Thus, the present study has demonstrated that direct succinylation of protein drugs or application of succinylated proteins as a drug carrier may be a useful strategy for drug delivery to the brain capillary endothelium. Molecular weight or total numbers of anionic charges per one molecule of succinylated proteins is shown to be important for recognition by the receptor.

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