Preferential Binding of Polyethylene Glycol-Coated Liposomes Containing a Novel Cationic Lipid, TRX-20, to Human Subendthelial Cells via Chondroitin Sulfate

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Purpose. To design novel cationic liposomes, polyethylene glycol (PEG)-coated cationic liposomes containing a newly synthesized cationic lipid, 3,5-dipentadecyloxybenzamidine hydrochloride (TRX-20) were formulated and their cellular binding and uptake investigated *in vitro* in the following cells: human subendothelial cells (aortic smooth muscle cells and mesangial cells) and human endothelial cells.

Methods. Three different PEG-coated cationic liposomes were prepared by the extrusion method, and their mean particle size and zeta potential were determined. Rhodamine-labeled PEG-coated cationic liposomes were incubated with smooth muscle cells, mesangial cells, and endothelial cells at 37°C for 24 h. The amounts of cellular binding and uptake of liposomes were estimated by measuring the cell-associated fluorescence intensity of rhodamine. To investigate the binding property of the liposomes, the changes of the binding to the cells pretreated by various kinds of glycosaminoglycan lyases were examined. Fluorescence microscopy is used to seek localization of liposomes in the cells.

Results. The cellular binding and uptake of PEG-coated cationic liposomes to smooth muscle cells was depended strongly on the chemical species of cationic lipids in these liposomes. Smooth muscle cells bound higher amount of PEG-coated TRX-20 liposomes than other cationic liposomes containing *N*-(1-(2,3-dioleoyloxy) propyl)-*N, N, N*-trimethylammonium salts or *N*-(α-(trimethylammonio)acetyl)-D-glutamate chloride. Despite of the higher affinity of PEG-coated TRX-20 liposomes for subendothelial cells, their binding to endothelial cells was very small. The binding to subendothelial cells was inhibited when cells were pretreated by certain kinds of chondroitinase, but not by heparitinase. These results suggest that PEG-coated TRX-20 liposomes have strong and selective binding property to subendothelial cells by interacting with certain kinds of chondroitin sulfate proteoglycans (not with heparan sulfate proteoglycans) on the cell surface and in the extracellular matrix of the cells. This binding feature was different from that reported for other cationic liposomes.

Conclusions. PEG-coated TRX-20 liposomes can strongly and selectively bind to subendothelial cells via certain kinds of chondroitin sulfate proteoglycans and would have an advantage to use as a specific drug delivery system.

KEY WORDS: cationic liposomes; polyethylene glycol; subendothelial cells; chondroitin sulfate proteoglycan.

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INTRODUCTION

Liposome formulations are currently being investigated as tools for the targeting delivery of drugs (1). Several liposomes, such as Doxil (2), have already been launched in the worldwide market. The surface of these liposomes has been modified with polyethylene glycol (PEG) to improve their stability in the blood (3), and as a result, better clinical results have been obtained through the modified liposome's long circulation in the blood followed by the enhanced permeability and retention (EPR) effect (2). However, the ideal liposome formulation should contain the following features: stability in the blood, controlled circulation lifetime, disease site localization, and target cell-specific binding and delivery (4). In particular, the latter two features appear to be most important and difficult features in the development of liposomes. It is well known that the EPR effect of long circulating liposomes increases the accumulation of liposomes in tissues with enhanced vascular permeability, by endothelial damage, inflammation, or tumorigenesis. After penetration across the endothelium, liposomes enter into the subendothelial area of these tissues. Therefore, the binding affinity to the subendothelial cells, such as smooth muscle cells (SMCs) and mesangial cells (MCs), appears to be an important factor for the retention of liposomes in the diseased tissues.

In the last decade, cationic liposomes have been studied closely because of their high binding affinity to cells. The positively charged surface of cationic liposomes, however, is associated with the binding to the negatively charged surfaces of serum protein, blood cells, and vessel endothelium, and via these binding the liposomes are rapidly cleared from the blood circulation (5,6). With that knowledge, for achieving the practical application, it seems necessary to develop cationic liposomes that are stable in the circulation. For this reason, cationic liposomes that will bind to target cells in diseased tissues but not bind to blood cells or vascular endothelial cells will become a useful tool for the targeting delivery of drugs.

It has been reported that the interaction of cationic liposomes with cell surface proteoglycans, in particular by heparan sulfate proteoglycans (HSPGs), is essential in the case of cationic liposome-mediated gene transfection (7). However another type of proteoglycan, chondroitin sulfate proteoglycans (CSPGs), also exists on cell surfaces, and the distribution of these two types of proteoglycans differs in various cell species. Endothelial cells are considered to have HSPGs mainly, but SMCs are reported to have a lot of chondroitin sulfate proteoglycans on their surface (8,9). However, only a few studies are reported on the interaction of cationic liposomes with CSPGs (10,11). From this viewpoint, we investigated PEG-coated cationic liposomes as a novel drug delivery system, and liposomes containing a newly synthesized cationic lipid, 3,5-dipentadecyloxybenzamidine hydrochloride (TRX-20) were developed. The binding features of PEG-coated TRX-20 liposomes were investigated using human endothelial cells, SMCs, and MCs under *in vitro* conditions. The binding properties are discussed, focusing on the interaction of PEG-coated TRX-20 liposomes with proteoglycans in particular.

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MATERIALS AND METHODS

Materials

The materials used in the study were as follows: *N*-(1-(2,3-dioleoyloxy) propyl)-*N,N,N*-trimethylammonium salts (DOTAP) from Sigma-Aldrich Co. (St. Louis, MO), *N*-(α-(trimethylammonio)acetyl)-D-glutamate chloride (TMAG) from Sogo Pharmaceutical Co. (Tokyo, Japan), HSPC from Lipoid (Ludwigshafen, Germany), cholesterol from Merck (Darmstadt, Germany), distearoylphosphatidylethanolamine-polyethylene glycol (M_r = 5000) (PEG-DSPE) from NOF Co. (Tokyo), and Lissamine rhodamine B-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (rhodamine) from Molecular Probes, Inc. (Eugene, OR). DOTAP (ESCORT®) and TMAG (Gene Transfer®) are commercially available cationic lipids used as gene transfection reagents.

Tissue culture plastics were obtained from Falcon labware (Becton Dickinson, Franklin Lakes, NJ). Dulbecco's modified eagle medium: nutrient mixture F-12 (DMEM-F12) 1:1(v/v) mixture and Hanks' balanced salt solution (HBSS) were obtained from Life Technologies, Inc. (Rockville, MD). Gentamicin and amphotericin B were purchased from ICN biomedicals, Inc. (Costa Mesa, CA). Fetal bovine serum (FBS) was purchased from Asahi techno-glass Co. (Tokyo, Japan). Acetylated low-density lipoprotein labeled with 1,1'-dioctadecyl-3,3,3',3'-tetrametylindo-carbocyanine perchlorate (DiI-Ac-LDL) was obtained from Biomedical Technologies, Inc. (Stoughton, MA). Glycosaminoglycan (GAG) lyase: chondroitinase ABC protease free (chondroitinase ABC), chondroitinase ACII arthro (chondroitinase ACII), and chondroitinase B, heparitinase, heparitinaseI, heparitinase II, and hyaluronidase from *Streptomyces hyalurolytics*) were purchased from Seikagaku Kogyo Co. (Tokyo). Micro BCA protein assay reagent kit was obtained from Pierce (Rockford, IL). Other agents used in the study are of reagent grade.

Synthesis of TRX-20

TRX-20 was synthesized as follows (12). The reaction of 3,5-dihydroxybenzonitrile (20.27 g) with 1-bromopentadecane (89.73 g) by means of potassium carbonate (42.57 g) in *N,N*-dimethylformamide (500 mL) at 60°C yielded 3,5-dipentadecyloxybenzonitrile (51.45 g, yield 62%). The treatment of 3,5-dipentadecyloxybenzonitrile (51.45 g) with hydrogen chloride (100 g) in methanol (150 mL) and chloroform (1350 mL) at room temperature followed by ammonia (100 g) in methanol (300 mL) and chloroform (1200 mL) by refluxing gave rise to 3,5-dipentadecyloxybenzamidine hydrochloride as colorless crystals (48.52 g, yield 86%, mp. 153-154°C). The chemical structure of TRX-20 is shown in Fig.1.

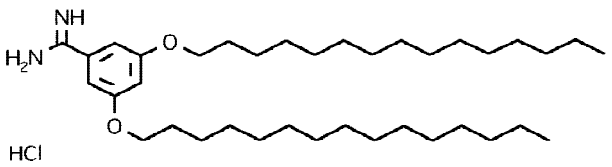

Fig. 1. Molecular structure of TRX-20 (3,5-dipentadecyloxybenzamidine hydrochloride).

Preparation of Cationic Liposomes

Rhodamine-labeled (0.2 mole%) liposomes composed of HSPC, cholesterol, and cationic lipid (TRX-20, DOTAP, or TMAG) (molar ratio 50:42.4:7.6) were prepared by the following process: All components were dissolved in t-butyl alcohol, lyophilized, and then hydrated in physiologic saline. Liposomes were prepared by vigorous vortexing and then sonicated for 5 min at 55°C in a bath-type sonicator. Then they were extruded through double-stacked polycarbonate membranes (Nucleopore, Corning Costar Co., Acton, MA) with pore sizes of 400, 200, and 100 nm to obtain particles of approximately 100 nm in size using an extruder device from Lipex Biomembranes (Vancouver, BC, Canada). Liposomes were incubated in various concentrations of PEG solution to incorporate PEG-DSPE (0.25, 0.50, 0.75, or 1.00 mole%) into the outer membrane. Liposome concentrations were determined using a phospholipid determination kit (Wako Pure Chemical Industries, Osaka). The particle diameter and zeta potential of the liposomes were determined by using Zeta Master S ZEM5002 (Malvern Instruments, Malvern, UK).

Human Cells and Media

Human aortic endothelial cells (ECs) (donor: 27-year-old female), SMCs (donor: 2-year-old male infant), and MCs (donor: 19-week female fetus) were purchased from Clonetics Co. (Walkersville, MD). The cells were grown in recommended media (EGM2 Bullet Kit, SMGM2 Bullet Kit, and MsGM Bullet Kit, Clonetics Co.). Glomerular microvascular endothelial cells (GMVECs) (lot: RI-128, pooled cells from several donors) were purchased from Cell Systems Co. (Kirkland, WA) and grown in CSC medium (Cell Systems Co.). All cells were maintained at 37°C in a humidified atmosphere (95% air, 5% CO_2). In all the experiments, cells from the 4-8th passage were used.

Binding and Uptake Assay of Liposomes

Cells were seeded (1×10^4 cells per cm^2) into 12-well plates. At subconfluent monolayers, cells were rinsed twice with incubation medium (DMEM-F12 supplemented with 10% FBS, 0.05 mg/mL gentamicin, and 0.05 μg/mL amphotericin B), and then incubated with 50 μg phosphatidylcholine (PC)/mL of rhodamine-labeled cationic liposomes in the incubation medium at 37°C for 24 h (13,14). After incubation, the cells were washed twice with ice-cold PBS and lysed by adding RIPA buffer (20 mM Tris-HCl (pH 7.4)/0.1% SDS/1% Triton X100/1% sodium deoxycholate). Then, each lysate (200 μL) was transferred using a Gilson (Middleton, WI) pipette into white opaque tissue 96-well culture plate (Becton Dickinson, NJ), and fluorescence was measured using a Labosystems Fluoroskan II (Dainippon Pharmaceutical Co., Osaka) equipped with a 544-nm excitation filter and a 590-nm emission filter.

Treatment of Cells with GAG Lyases

Cells grown at subconfluent monolayers in 12-well plates were rinsed twice with HBSS and then incubated in a solution containing 20 mU/mL of a GAG lyase (chondroitinase ABC, chondroitinase ACII, chondroitinase B, heparitinase, heparitinase I or heparitinase II), or 0.02 TRU/mL of hyaluroni-

dase for 60 min at 37°C except for cells treated with chondroitinase B, which were incubated at 30°C (7,13,15,16). The incubation solutions were HBSS (pH 7.4) for chondroitinase ABC, chondroitinase ACII, chondroitinase B, heparitinase I, and heparitinase II, HBSS (pH 6) for hyaluronidase, and HBSS: HBSS (−) (without $CaCl_2$, $MgCl_2$, $MgSO_4$) 1:99 mixture (final calcium concentration was 1.26×10^{-5} M) (pH 7.4) for heparitinase, and all of them contained 50 mM CH_3COONa. After removal of surface GAGs, the cells were incubated with 50 μg PC/mL of rhodamine-labeled PEG-coated TRX-20 liposomes in the incubation medium, shaken gently 10 times, and lysed as described above. To estimate the uptake of liposomes by cells, cells grown in 12-well plates were incubated with 50 μg PC/mL of rhodamine-labeled PEG-coated liposomes in the incubation medium for 24 h at 37°C (13), rinsed twice with HBSS, and then treated with 20 mU/mL chondroitinase ABC in incubation solution for 60 min. Fluorescence was measured as described above.

For fluorescence microscopy, SMCs and ECs grown to subconfluence in the 8-well Lab-Tek chamber, Permanox slide (Nunc Inc., Naperville, IL), were incubated with 100 μg PC/mL of rhodamine-labeled liposomes for 24 h, rinsed twice with DMEM-F12 medium, and treated with 0.1 U/mL chondroitinase ABC containing 50 mM CH_3COONa in DMEM-F12 medium for 30 min.The images were obtained as described below.

Fluorescence Microscopy

Cells were seeded (~1×10^4 cells per cm^2) into 8-well chambers. After 2–3 days, they were rinsed with incubation medium and then incubated with 100 μg PC/mL of rhodamine-labeled liposomes or 10 μg/mL of DiI-Ac-LDL in the incubation medium for 4 or 24 h at 37°C, and at the end of the last 30 min, 1-5 μM SYTO 24 green fluorescent nucleic acid stain reagent (Molecular Probes, Inc.) was added. The cells were washed three times with PBS, fixed in 10% phosphate-buffered formalin for 20 min, and mounted with GEL/Mount (Biomeda Co., Foster City, CA). Fluorescence images were obtained using a Leica DMRB fluorescence microscope (Wetzlar, Germany) equipped with N2.1 and L4 filter blocks (Leica).

RESULTS AND DISCUSSION

Physical Properties of Cationic Liposomes

Particle size and zeta potential of cationic liposomes (HSPC:cholesterol:cationic lipids:PEG-DSPE = 50.0:42.4:7.6:0.25, 0.50, 0.75, or 1.00) prepared in the study are shown in Table I. With an increase in amount of PEG-DSPE, liposome sizes increased and the zeta potential of liposomes decreased. There were only small differences in these physical properties among PEG-coated TRX-20 liposomes and liposomes containing other cationic lipids.

Binding and Uptake of Liposomes to Human Aortic SMCs

The interaction of these liposomes with cultured SMCs was estimated as the total binding efficiency (sum of cell binding and intracellular uptake) of the liposomes by measuring fluorescence intensities of the cells. The results were shown in Fig. 2 in which the total binding efficiency of liposomes was calculated as the amount of PC per unit protein from fluorescence intensity. The binding of various cationic liposomes was plotted against the PEG content in these liposomes. As clearly shown, increasing the PEG content, the total binding efficiency of all liposomes decreased. Although there were only small differences in physical properties of the three kinds of cationic liposomes, the total binding efficiency was quite different. No binding and uptake of PEG-coated TMAG liposomes was observed when the mole percent of PEG in the liposomes was more than 0.25. On the contrary, PEG-coated TRX-20 liposomes showed the largest total binding efficiency among the three cationic liposomes, and yet the binding remained at the PEG content of 1.0 mole percent. These results suggested that TRX-20 would be a valuable cationic component for a cell-binding property of liposome formulations.

It is well known that PEG modification forms the fixed water layer, and it decreases the zeta potential of the liposomes (17). The affinity of cationic liposomes to the anionic surface of cells is decreased by the decreased zeta potential of liposomes. The affinity of cationic liposomes to the anionic surface of cells is decreased by the decreased zeta potential of liposomes. However, it could not be explained by such simple charge interactions why the total binding efficiency of liposomes varied so greatly among liposomes containing three different cationic agents, even though the zeta potentials of these liposomes are almost the same. As Kuhl *et al.* (18) and Torchilin *et al.* (19) suggested, the PEG chain of liposome surfaces gives a steric-hindrance effect on the interaction of the liposomes with various molecules, and this effect is also considered to reduce the interaction of liposomes with cells. This steric-hindrance depends on the surface content of the PEG chain and also on the character of the interacting molecules themselves, e.g., structure, molecular size, charge, and

Table I. Physical Properties of Liposomes Containing Three Different Cationic Lipid

mole % PEG	Particle size (nm)[a]			Zeta potential (mV)[b]		
	TRX	DOTAP	TMAG	TRX	DOTAP	TMAG
0.00	105.0 ± 0.8	106.6 ± 0.1	93.9 ± 0.7	59.2 ± 0.4	56.5 ± 0.7	53.5 ± 0.2
0.25	110.0 ± 1.1	112.4 ± 0.3	100.7 ± 1.4	36.6 ± 0.2	31.7 ± 0.4	38.7 ± 0.7
0.50	113.6 ± 1.2	115.5 ± 0.9	104.1 ± 2.2	28.2 ± 0.7	23.6 ± 0.6	27.3 ± 0.6
0.75	115.0 ± 1.6	117.3 ± 0.7	105.4 ± 1.2	19.5 ± 0.7	19.7 ± 0.7	23.2 ± 0.5
1.00	115.1 ± 1.6	118.8 ± 1.5	106.8 ± 0.6	18.8 ± 0.8	16.2 ± 0.2	20.2 ± 0.3

The data are given as the mean ± SD of three experiments for each sample.
[a] Data obtained using the dynamic light scattering method.
[b] Data obtained using electrophoretic mobility based on the Helmholtz–Smoluchowski equation.

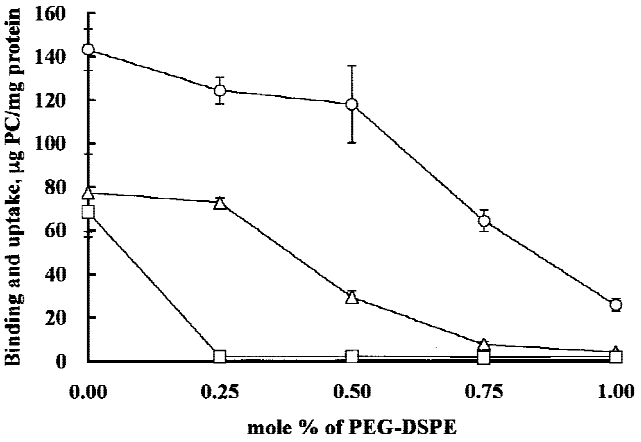

Fig. 2. The interactions of rhodamine-labeled PEG-coated cationic liposomes with SMCs. SMCs were incubated for 24 h with 50 μg PC/mL of liposomes containing three different cationic lipids and several amounts of PEG-DSPE in the incubation medium at 37°C. Liposomes composed of TRX-20 (○), DOTAP (△), or TMAG (□) were labeled with 0.2 mole% of rhodamine. The amount of rhodamine-labeled PEG-coated TRX-20 liposomes bound to SMCs, at the PEG-DSPE content of 0.5 mole%, was estimated as about 20% against total incubated liposomes. Data represent mean ± SD for the three experiments.

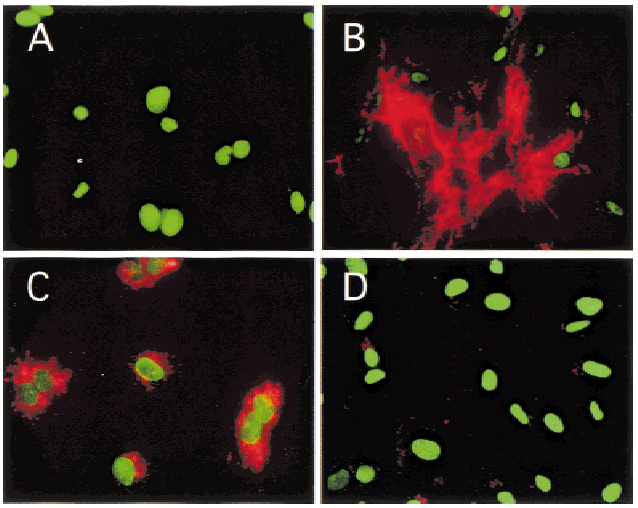

Fig. 3. Fluorescence micrographs of cells incubated with rhodamine-labeled PEG (0.5 mole%)-coated TRX-20 liposomes or DiI-Ac-LDL. EC (A and C) and SMC (B and D) were incubated with 100 μg PC/mL of rhodamine-labeled PEG-coated TRX-20 liposomes (A and B) or 10 μg/mL of DiI-Ac-LDL (C and D) in the incubation medium for 4 h. All cells were stained with SYTO 24 green fluorescent nucleic acid stain reagent.

the macroscopic structures (fibrous or globular) of molecules if the molecules are polymeric molecules. In this study, the interactions of liposomes to SMCs depended on the structures of cationic lipids—not merely on the zeta potentials of them—so it is important to clarify which molecules on the cell surfaces interact with cationic liposomes. The different affinity of cationic lipids to the molecules seems related to the various binding affinity of the liposomes.

Differences of Cell-Binding Properties of TRX-20 Liposomes to ECs from Those to SMCs or MCs

The binding properties of PEG-coated TRX-20 liposomes to ECs were compared with those to SMCs and MCs by fluorescence microscopy using DiI-Ac-LDL, which is commonly used as an EC marker (20), as a reference. Binding of DiI-Ac-LDL was observed on ECs (Fig. 3C) but was scarcely observed on SMCs (Fig.3D). On the other hand, when rhodamine-labeled PEG-coated TRX-20 liposomes were incubated with cells, strong rhodamine fluorescence was observed with SMC (Fig. 3B) but was hardly observed with ECs (Fig. 3A). Furthermore, there was a difference in the observed fluorescence patterns, that is, the fluorescence of the liposomes (Fig. 3B) extended over large numbers of cells whereas the fluorescence of DiI-Ac-LDL was restricted to individual cells (Fig. 3C). For quantitative evaluation, the binding amount of rhodamine-labeled PEG-coated TRX-20 liposomes was compared between endothelial cells (EC and GMVEC), and in subendothelial cells (SMC and MC). The total amounts of the liposomes bound to SMCs and MCs were 120 μg PC/mg protein and 60 μg PC/mg protein and were markedly higher than these bound to ECs (2 μg PC/mg protein) and GMVECs (11 μg PC/mg protein) (Fig. 4). These results suggest that the specific binding may be related to interactions with the components distributed differently on the cell surface and in the extracellular matrix of those cultured cells. In addition, the content of these components would be markedly lesser in ECs and GMVECs than in SMCs and MCs.

Effects of Enzymatic Digestion of GAGs on the Binding of TRX-20 Liposomes

As shown above, binding properties of PEG-coated TRX-20 liposomes depended strongly on the type of the cells used in the study. To investigate the cell surface molecules interacting with the liposomes, the effects of enzymatic diges-

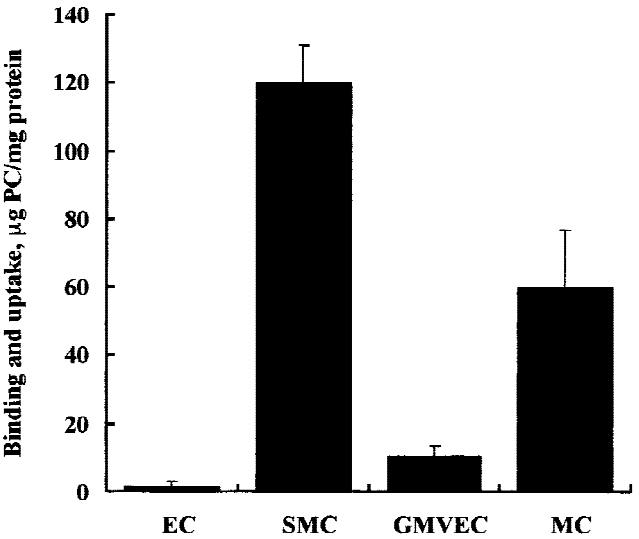

Fig. 4. Interactions of PEG (0.5 mole%)-coated TRX-20 liposomes with different cells. ECs, SMCs, GMVECs, and MCs in 12-well plates were incubated with 50 μg PC/mL of rhodamine-labeled PEG-coated TRX-20 liposomes in the presence of 10% of FBS at 37°C for 24 h. Data represent the mean ± SD of three experiments.

tion of GAGs were estimated by pretreating the cells with GAG lyases. GAGs are acidic polysaccharides that are widely distributed in many kinds of tissues and are expected to adsorb cationic liposomes because of their polyanionic properties. For example, interactions of cationic liposome DNA complex with HeLa cells have been investigated previously for their binding to GAGs, and binding to cell surface heparan sulfate was reported as one of the important steps for the internalization of cationic liposome DNA complex from the cell surface (7).

We investigated the inhibitory effects of pretreatment with GAG lyases on the binding of rhodamine-labeled PEG-coated TRX-20 liposomes to SMCs and MCs. Cultured SMCs and MCs were treated with sufficient amounts of various GAG lyases (chondroitinase ABC, chondroitinase ACII, chondroitinase B, heparitinase, heparitinase I or heparitinase II, and hyaluronidase) and then the total binding of the liposomes to treated cells were estimated (Fig. 5) (7,13,15). The activity of GAG lyases was confirmed by detecting digested GAGs by measuring the absorption at 232 nm (16). The total amount of the liposomes bound to both cells was not reduced by pretreatment with heparitinase, heparitinase I, or heparitinase II. However, the binding to SMCs and MCs was reduced to 11% and 33%, respectively, by pretreatment with chondroitinase ABC. The binding of the liposomes to SMCs was also reduced to 43% and 73% by pretreatment with chondroitinase ACII and chondroitinase B, respectively. On the other hand, these reduction rates of MCs were 52% and 40%, respectively. Pretreatment with hyaluronidase had different effects on SMCs and MCs, that is, it did not effectively reduce the binding to SMCs but reduced the binding to MCs to 34%. These results indicated that the binding of PEG-coated TRX-20 liposomes is mediated mainly through the binding of the liposomes to certain kinds of CSPGs distributed on the cell surface and in the extracellular matrix of cultured SMCs and MCs. The different effect of hyaluronidase on MCs may be related to the fact that MCs have been reported to synthesize a hyaluronan-binding type of CSPGs, such as versican and CD44, in culture (21–23). This may be one possible explanation of our results. In this case, binding may vary among various types of cells depending on the kind, amount, and ratio of CSPGs produced by cells. Generally, HSPGs such as syndecan and glypican are major proteoglycans found on the surface of ECs (24–26). It may be one possible explanation for the reduced binding efficiency of PEG-coated TRX-20 liposomes to ECs. As for the structural characteristics of heparan sulfate and chondroitin sulfate, it has been demonstrated that the total content of sulfate in both GAGs is almost the same, but the constitutional sugar moiety, the sulfate substitution position of the sugar residues, and the molecular size of both GAGs are considerably different (25). Because even the total sulfate content of both GAGs is similar, the smaller molecular size and, hence, the lower overall charge of HSPG appear to be partly responsible for its low affinity with PEG-coated TRX-20 liposomes. Similar observations have been reported previously concerning the interaction between low-density lipoprotein and these GAGs (27).

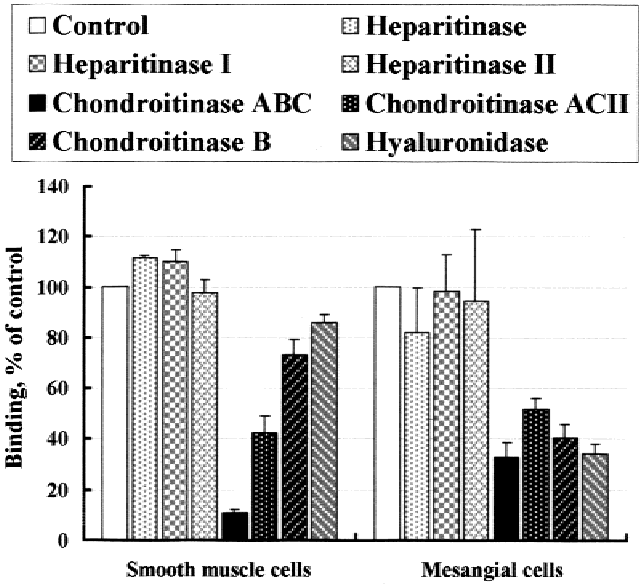

Fig. 5. Effects of enzymatic digestion of glycosaminoglycans on the binding of rhodamine-labeled PEG (0.5 mole%)-coated TRX-20 liposomes. SMCs and MCs were treated with sufficient amounts of GAG lyases for 60 min. After the removal of surface GAGs, cells were incubated with 50 μg PC of rhodamine-labeled PEG-coated TRX-20 liposomes in the incubation medium, shaken gently ten times, and lysed as material and methods. The data represent the mean ± SD of the three experiments. The results are presented as a percentage of binding relative to cells not treated with GAG lyases.

Cellular Uptake of TRX-20 Liposomes

Although the PEG-coated TRX-20 liposomes were found to have a strong binding ability to the cells, for estimation of the usefulness of the liposomes, it should be evaluated whether the liposomes can be internalized into cells or not. After incubation for 24 h with rhodamine-labeled PEG-coated TRX-20 liposomes, SMCs and MCs were washed with culture medium and then were incubated with chondroitinase ABC. The fluorescence in these cells was observed by fluorescence microscopy. The results are shown in Fig. 6a. After incubation with chondroitinase ABC, the fluorescence that had extended over the cells (Fig. 6a, A and C) was diminished, and the fluorescence was localized to the cytoplasmic area of cells (Fig. 6a, B and D). This fluorescence pattern is quite similar to that obtained when ECs were treated with DiI-Ac-LDL (Fig. 3C). Because DiI-Ac-LDL is used commonly as the EC marker since it can be easily taken up by EC, these results suggest that the remaining fluorescence in SMCs and MCs after treatment of chondroitinase ABC would be attributed to the liposomes incorporated by the cells after binding. Based on the remaining amount of fluorescence, the fraction incorporated into cells was calculated to be about 10% of the total amount of the liposomes bound to SMCs and MCs (Fig. 6b). To estimate the time-dependent uptake of PEG-coated TRX-20 and DOTAP liposomes, SMCs were treated by chondroitinase ABC after incubation for 0, 2, 4, 8, and 24 h with these liposomes, and the intensities of the fluorescence were plotted against the incubation periods. The results are shown in Fig. 6c. Both PEG-coated TRX-20 liposomes and PEG-coated DOTAP liposomes were incorporated by SMCs in a time-dependent manner, and the incorporated amount of PEG-coated TRX-20 liposomes was about three times greater than that of PEG-coated DOTAP liposomes after incubation for 24 h. The mechanism of the internalization of DiI-Ac-LDL to EC has been well described by

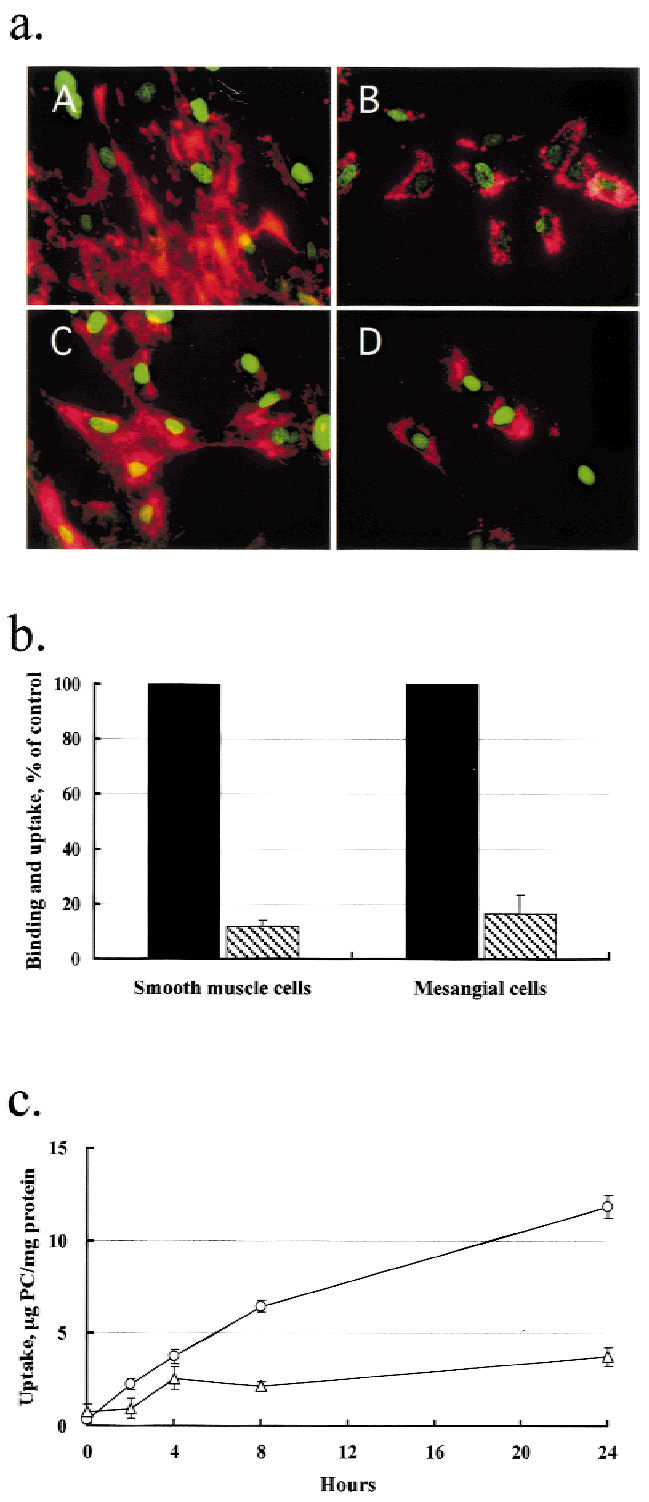

Fig. 6. Cellular uptake of rhodamine-labeled PEG (0.5 mole%)-coated TRX-20 liposomes. In (a), fluorescence micrographs of SMCs (A and B) and MCs (C and D) were incubated with rhodamine-labeled PEG-coated TRX-20 liposomes for 24 h (A and C) and then digested with 100 mU/mL chondroitinase ABC for 30 min (B and D). In (b), the fluorescence of cell lysates was measured. Lanes: closed box, control (no treatment); hatched box, cells treated with 20mU/mL of chondroitinase ABC for 60 min. In (c), SMCs were incubated for 0, 2, 4, 8, and 24 h with 50 μg PC of rhodamine-labeled PEG (0.5 mole%)-coated TRX-20 (○) or DOTAP (△) liposomes were treated with 20 mU/mL of chondroitinase ABC for 60 min and fluorescence was plotted against the incubation periods. The data represent the mean ± SD of the three experiments.

Voyta *et al.* (20); however, the uptake mechanism of PEG-coated TRX-20 liposomes observed here could not be clearly explained at this time. Further studies will clarify the binding and uptake behaviors of the cationic liposomes.

CONCLUSION

PEG-coated TRX-20 liposomes bound strongly to SMCs compared with other two PEG-coated cationic liposomes containing DOTAP or TMAG. This binding depends strongly on the PEG content in liposomes and the chemical species of cationic lipids. Furthermore, the binding of PEG-coated TRX-20 liposomes depends on cell species and the binding to ECs was markedly less than that to SMCs. The binding was inhibited when the cells were pretreated with chondroitinase but not with heparitinase, indicating that the binding of PEG-coated TRX-20 liposomes occurs via certain kinds of chondroitin sulfate proteoglycans, not heparan sulfate proteoglycans on the cell surfaces and in the extracellular matrix of the cells. These results suggest that the preferential and selective binding characters of PEG-coated TRX-20 liposomes will become useful tools for development of drug targeting system in the future.

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