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Research Paper

Advances in Targeting Drug Delivery to Glomerular Mesangial Cells by Long Circulating Cationic Liposomes for the Treatment of Glomerulonephritis

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Purpose. Newly designed polyethylene glycol (PEG)-modified cationic liposomes, containing a novel cationic lipid TRX-20 (3,5-dipentadecyloxybenzamidine hydrochloride), bind specifically to cultured human mesangial cells, and not to endothelial cells. In this study, we investigated targeting the delivery of PEG-modified liposomes containing TRX-20 (TRX-liposomes) to mesangial cells and evaluated their pharmacokinetic behavior in a rat experimental glomerulonephritis model, using prednisolone phosphate (PSLP) as a model drug.

Material and Methods. TRX-liposomes were injected intravenously into experimental glomerulonephritic rats and normal rats to compare its pharmacokinetic behavior with that of non-cationic liposomes (PEG-liposomes). Rhodamine-labeled liposomes were used to evaluate the accumulation in inflamed kidneys. Pharmacological effects of three formulations of PSLP (i.e., a single injection of two liposomal formulations and daily injections of PSLP in saline solution) were estimated in terms of suppressing glomerular cell proliferation in the rat nephritis model.

Results. TRX-liposomes markedly accumulated in the glomeruli of inflamed kidneys, but did not accumulate in the glomeruli of normal kidneys. Although the PEG-liposomes also accumulated in the glomeruli of the inflamed kidneys, their pharmacological behavior was quite different from that of the TRX-liposomes, which were internalized by the target cells. In a comparison among the three formulations of PSLP, the dose of TRX-liposomes required for significant suppression of glomerular cell proliferation was much less (dose of 0.032 mg/kg and above) than that required for the same effect by the PSLP saline solution (3.2 mg/kg daily; 12.8 mg/kg total) and PEG-liposomes (0.32 mg/kg). Interestingly, significant suppression of mesangial cell activation, as assessed by the expression of α-smooth muscle actin, was observed in nephritic rats treated with TRX-liposomes, but not in the other two treatment groups.

Conclusions. The pharmaceutical properties of TRX-liposomes due to their preferential binding to mesangial cells and long circulation time make this a likely candidate system for targeted drug delivery to the inflamed glomeruli of glomerulonephritis.

KEY WORDS: active targeting; cationic liposomes; glomerulonephritis; polyethylene glycol; prednisolone phosphate.

INTRODUCTION

The development of new drug delivery systems (DDSs) has focused on improving the therapeutic effects of drugs while reducing their harmful side effects. The formulation of liposomes first reported by Bangham *et al.* has been actively studied as a potential pharmaceutical carrier for the targeted delivery of drugs (1). There were, however, several limitations to this system when it was introduced, including insufficient pharmaceutical stability, the lack of a suitable method for mass production, and suboptimal pharmacokinetic properties. The liposomes' short half-life in blood, a critical problem, was resolved by modifying the liposomal surface with hydrophilic polymers such as polyethylene glycol (PEG) (2). The PEG-modified liposomes (PEG-liposomes) had a size of around 100 nm, and their prolonged blood-circulation time allowed them to accumulate in tissues with increased vascular permeability, such as those that are found at tumor sites or caused by infection or inflammation (3,4). The advantages of these liposomes led to their being marketed worldwide, starting in 1995, as passive targeting and doxorubicin-encapsulating PEG-liposomes (liposomal doxorubicin; Doxil®). However, PEG-liposomes are limited by their insufficient binding to target cells at the accumulation sites.

We sought to develop a novel cell-specific ligand for the liposomal surface and found that 3,5-dipentadecyloxybenzamidine hydrochloride (TRX-20) specifically binds to chondroitin sulfate proteoglycan. PEG-liposomes with TRX-20

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added to the surface (TRX-liposomes) showed a positive zeta-potential and a high affinity for cultured human subendothelial cells, such as mesangial cells and smooth muscle cells, but not vascular endothelial cells (5). Furthermore, the adjustment to a liposomal diameter of around 100 nm, conferred on TRX-liposomes a long blood-circulation time (half-life of 14 h) (6). These characteristics suggested TRX-liposomes might be useful for the targeted delivery of drugs to subendothelial cells at sites of inflammation only, where the permeable endothelium could be penetrated by the TRX-liposomes.

Mesangial cell proliferation and matrix expansion are central features of various glomerular diseases, such as IgA nephropathy and lupus nephritis (7–9), which are followed by the development of glomerular sclerosis and renal failure. Therefore, the successful control of mesangial cell activation and proliferation should significantly improve the therapy of glomerular diseases. In a previous report, we showed that glucocorticoid encapsulated in TRX-liposomes effectively lowers glomerular IgA and C3 depositions in ddY mice as a spontaneous animal model of the IgA nephropathy (10). In the present study, to clarify the pharmaceutical properties of TRX-liposomes, we evaluated biodistribution and efficacy on glomerular nephritic animals in detail using rat anti-Thy-1 nephritis model. This model of glomerulonephritis resembles the morphological features of human mesangial proliferative glomerulonephritis. It is characterized by an initial mesangiolysis followed by mesangial cell proliferation and matrix expansion, with a subsequent resolution and return to almost normal histology (11). Using this model, we examined TRX-liposomes for their pharmacokinetic behavior, their usefulness for targeting inflamed kidney, and their therapeutic effects; we used prednisolone phosphate (PSLP) as our model drug.

MATERIALS AND METHODS

Materials

PSLP was purchased from Diosynth Co. Ltd. (Oss, The Netherlands). Hydrogenated soybean phosphatidylcholine (HSPC), polyethylene glycol 5000-sn-glycrero-3-phosphatydilethanolamine (PEG5000-PE), and cholesterol were purchased from Lipoid (Ludwigshafen, Germany), NOF Corporation (Tokyo, Japan), and Merck & Co., Inc. (Darmstadt, Germany), respectively. The 3,5-dipentadecyloxybenzamidine hydrochloride (TRX-20) shown in Fig. 1 was synthesized by Terumo Corporation (Tokyo, Japan), as described previously (5). Rhodamine B 1, 2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine triethylammonium salt (rhodamine-DHPE), 8-Hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS), and p-Xylene-bis-pyridinium bromide (DPX) were obtained from Molecular Probes, Inc. (Eugene, OR). The other materials used in the study were of reagent grade.

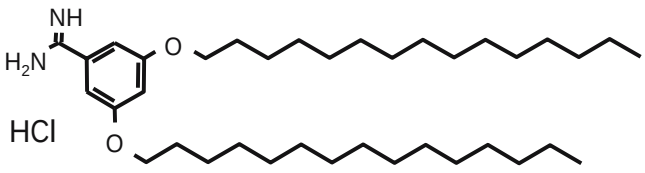

Fig. 1. Molecular structure of TRX-20 (3,5-dipentadecyloxybenzamidine hydrochloride).

Liposome Preparation

The preparation of liposomes was described previously (5,12), and we used a modified method. The lipid mixtures of HSPC:cholesterol=54:46 for PEG-liposomes or HSPC:cholesterol:TRX-20=50:42:8 for TRX-liposomes were well hydrated by sonication with phosphate-buffered solution (pH 7.2) containing PSLP and then extruded through two stacks of three Nucleopore filters (each pore size: 0.4, 0.2, and 0.1 μm) to obtain liposomes of around 100 nm. To modify the liposomal surface, PEG5000-PE solution was added at a ratio of 0.75 mol% of the total lipids of liposomal solution. The unencapsulated PSLP was then removed by gel filtration using Sepharose 4FF (Amersham Biosciences Co., Piscataway, NJ). Rhodamine-labeled TRX-liposomes and PEG-liposomes were prepared by basically the same methods as above, except that rhodamine-DHPE was dissolved in the lipid mixture solution at the ratio of 0.2 mol% of the total lipids. HSPC and PSLP concentrations in the liposomal formulation were respectively determined using a phospholipid determination kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and HPLC. The size of the liposomes was estimated using a dynamic light scattering system (Zetamaster-S; Malvern Instrument, Ltd., Malvern, UK). The average diameter of TRX-liposomes and PEG-liposomes was about 100 nm, and they contained approximately 0.1 mg PSLP per 1 mg HSPC.

The HPTS/DPX-encapsulating TRX-liposomes and PEG-liposomes were prepared as follows: the lipid mixtures were well hydrated with HEPES-buffered solution (pH 7.4) containing 35 mM HPTS and 50 mM DPX. PEG5000-PE solution was added at a ratio of 0.75 mol% of the total lipids of liposomal solution, and unencapsulated HPTS and DPX were removed by gel filtration. HPTS is a water-soluble fluorescent dye and is well known to be quenched with DPX (13,14), so the fluorescence intensity of free HPTS was approximately 30-fold stronger than that of liposomal HPTS/DPX.

Assay of Liposomes in Binding and Uptake Studies

Glomeruli were isolated from male Sprague–Dawley rats (6 weeks old; Charles River Japan, Kanagawa, Japan) by the sieving method and cultured in RPMI 1640 medium containing 20% fetal bovine serum, 1% Insulin-Transferrin-Selenium Supplement, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B. Cultured glomerular cells were identified as mesangial cells by morphological and immunocytochemical staining. The cells were spindle-shaped and expressed Thy 1.1 antigen and α-smooth muscle actin (α-SMA), but were negative for RECA-1 and von Willebrand factor. In addition, epithelial cells were excluded by visual examination. After 10–12 passages, the cells were seeded (1×10^4 cells per cm^2) into 12-well plates. Mesangial cells in subconfluent monolayers were rinsed twice with phosphate-buffed saline (PBS) and then incubated at 37°C for 24 h with rhodamine-labeled TRX-liposomes or PEG-liposomes in the same medium described above at an

HSPC concentration of 50 or 100 μg/ml. After the incubation, the cells were washed twice with ice-cold PBS and lysed by adding RIPA buffer (pH 7.4) containing 20 mM Tris-HCl, 0.1% SDS, 1% Triton X100, and 1% sodium deoxycholate. The fluorescence intensity was determined at excitation and emission wavelengths of 544 and 590 nm, respectively.

Preparation of Anti-Thy-1.1 Antibody

OX-7, a monoclonal anti-Thy-1.1 antibody, was prepared as described previously (15) and used to initiate rat experimental glomerulonephritis. The OX-7-producing hybridoma line was a gift from the Cell Resource Center for Biomedical Research, Tohoku University. Female BALB/c mice (7 weeks old weighing 25 to 30 g; Charles River Japan, Kanagawa, Japan) received an intraperitoneal injection of Freund's Incomplete Adjuvant (FIA) (DIFCO Labs, Detroit, MI) at 0.5 ml per body. The FIA-primed mice received an intraperitoneal injection of 2.0×10^7 OX-7-producing hybridomas 1 week later, and ascitic fluid was obtained from the mice on days 10 to 14 after the injection. OX-7 was purified from the ascitic fluid using HiTrap protein G Columns (Amersham Biosciences Co., Piscataway, NJ) and analyzed by enzyme-linked immunosorbent assay.

Induction of Experimental Glomerulonephritis

Male Sprague-Dawley rats (6 weeks old weighing 170–210 g; Charles River Japan, Kanagawa, Japan) were used in this study. All experiments were performed according to Terumo internal ethical guideline for animal experiments. The rat experimental glomerulonephritis model was prepared by injecting the anti-rat Thy 1.1 mouse monoclonal antibody (50 μg/rat) into the rats. Urine samples were collected from the rats in metabolic cages for 24 h on day 3 after the injection. The urinary protein concentration was determined using a Tonein-TP kit (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), and the total amount of urinary protein produced in 24 h was calculated.

Difference in the Biodistribution of TRX-liposomes Between Normal and Anti-Thy-1 Nephritic Rats

Rhodamine-labeled, PSLP-containing TRX-liposomes were injected into normal rats and anti-Thy-1 nephritic rats (0.32 mg prednisolone [PSL] per kg) via the tail vein on day 4 after the injection of anti-Thy-1.1 antibody. Blood samples were collected 24 h later, and the rats were sacrificed by perfusion with heparinized saline under anesthesia. Liver, spleen, kidneys, lung, heart, and brain were obtained, weighed, and homogenized. To extract rhodamine-DHPE, to 1 ml of homogenate or blood sample was added 2.4 ml of methanol, 2.5 ml of chloroform, and 1.25 ml of saturated aqueous sodium chloride, which formed a layer under the rest of the mixture. This mixture was shaken and allowed to form an aqueous and a lipid layer. The rhodamine-DHPE was in the under layer, which we removed. The accumulation of liposomes was estimated by determining the fluorescence intensity at excitation and emission wavelengths of 544 and 590 nm, respectively.

Pieces of kidney were frozen in O.C.T. compound (Miles Inc., Elkhart, IN) without fixation, sectioned, and the cryostat sections (approximately 6-μm thick) were mounted on glass slides. After the sections were air dried, any rhodamine fluorescence attributable to the liposomes that had accumulated in the glomeruli was photographed using a fluorescence microscope equipped with a camera. The glass slides were then immersed in 10% buffered formalin, and the fixed sections were subjected to periodic acid-Schiff (PAS) staining. The histopathological findings were examined by light microscopy to determine their correspondence to the fluorescence micrographs.

Difference of Biodistribution Between TRX-liposomes and PEG-liposomes in Blood and Kidney in Anti-Thy-1 Nephritic Rats

Rhodamine-labeled, PSLP-containing TRX-liposomes or PEG-liposomes were injected into anti-Thy-1 nephritic rats (0.32 mg PSL/kg) via the tail vein on day 4 after the injection of anti-Thy-1.1 antibody. Blood samples and kidneys were collected at 1, 3, 6, 24, and 48 h after the injection, and at each point five rats were sacrificed by perfusion with heparinized saline under anesthesia. The cortex and medulla of the kidney were obtained, weighed, and homogenized. The accumulation of liposomes was estimated by determining the fluorescence intensity of the extracted rhodamine-DHPE as described above.

Localization of TRX-liposomes in the Glomeruli of Anti-Thy-1 Nephritic Rats

HPTS/DPX-encapsulating TRX-liposomes or PEG-liposomes were injected into anti-Thy-1 nephritic rats (6 μmol total lipids/kg as an equivalent dose of 0.32 PSL/kg) via the tail vein on day 4 after the injection of anti-Thy-1.1 antibody. Anesthetized rats were sacrificed by perfusion with heparinized saline at 6 and 24 h after the injection. The kidneys were collected, and pieces were frozen in O.C.T. compound without fixation and sectioned. The cryostat sections with a thickness of approximately 6-μm were mounted on glass slides, the sections were air-dried, and the accumulation of HPTS in the kidney was then observed by fluorescence microscopy (380- to 425-nm band-pass excitation filter and 450-nm long-pass emission filter). In this condition, autofluorescence was present in many areas of kidney, but not in glomeruli. The fluorescence of liposomal HPTS/DPX is much weaker than that of free HPTS, because HPTS was quenched with DPX in these liposomes. Therefore, the observed fluorescence in the kidney may be based on free HPTS released from liposomes in the cells.

Different Effects Between Two Liposomal Formulations of PSLP on Glomerular Cell Proliferation in Experimental Glomerulonephritis

The pharmacological effects of PSLP delivered by three vehicles, TRX-liposomes, PEG-liposomes, and saline solution, were evaluated using anti-Thy-1 nephritic rats. We measured the increase in urinary protein for each rat on day 3 after the injection of anti-Thy-1.1 antibody. Using this information, we divided the rats into groups (n=6–8) so that the health of the rats, as measured by increased

urinary protein, was uniform across the groups. TRX-liposomes or PEG-liposomes at a dose of 0.032, 0.1, 0.32, or 1 mg PSL/kg were given intravenously to the anti-Thy-1 nephritic rats on day 4 after the injection of anti-Thy-1.1 antibody. The PSLP saline solution was used as the reference agent, and was injected into the anti-Thy-1 nephritic rats at a dose of 0.32, 1, or 3.2 mg PSL/kg every day from days 4 to 7. On day 8 after the anti-Thy-1.1 antibody injection, the rats were anesthetized and sacrificed by perfusion with heparinized saline, and the kidneys harvested. Pieces of kidney fixed in 10% buffered formalin were embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin-eosin (HE). For each specimen, 60 glomeruli were sequentially examined, and the number of nuclei per glomerular cross section was counted.

Different pieces of kidney were used for indirect immunoperoxidase staining. These pieces were fixed in methyl Carnoy's solution, embedded in paraffin, and sectioned at 3 μm. The sections were labeled with a mouse monoclonal antibody to α-SMA, antibody 1A4 (Sigma, St. Louis, MO). After blocking the endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol for 30 min at ambient temperature, the sections were pre-incubated with 1% bovine serum albumin in PBS for 1 h and then with the primary antibodies for more than 16 h at 4°C. The sections were carefully washed three times in PBS and exposed to biotinylated secondary antibodies for 1 h. The samples were washed again with PBS and incubated with peroxidase-conjugated streptavidin for 1 h. Peroxidase in the sections was visualized using diaminobenzidine (Sigma, St. Louis, MO), and the sections were counterstained with hematoxylin.

Statistics

Values are expressed as the means ± standard error. The significance of a difference between two groups was evaluated using Student's unpaired *t* test. One-way analysis of variance (ANOVA) was used for multiple comparisons. When one-way ANOVA showed significant differences, post-hoc analysis was performed with a Dunnett-type test. Values of $p<0.05$ were considered statistically significant.

RESULTS

Assay of Liposomes in Binding and Uptake Studies

The binding affinity of TRX-liposomes for rat mesangial cells as the sum of cell binding and intracellular uptake was determined based on the fluorescence intensity due to rhodamine. As shown in Fig. 2, these results indicated that rhodamine-labeled TRX-liposomes bound to rat mesangial cells, and rhodamine-labeled PEG-liposomes did not.

Biodistribution of TRX-liposomes Differs Between Normal and Anti-Thy-1 Nephritic Rats

The blood level of rhodamine-labeled TRX-liposomes was over 5 μg HSPC/ml in both normal and anti-Thy-1 nephritic rats, even 24 h after the injections, indicating that approximately 15% of the injected liposomes were still circulating in the blood. On the other hand, the biodistribution of these liposomes was the same for the nephritic and normal rats, except in the kidney cortex (Table I), where there was significantly

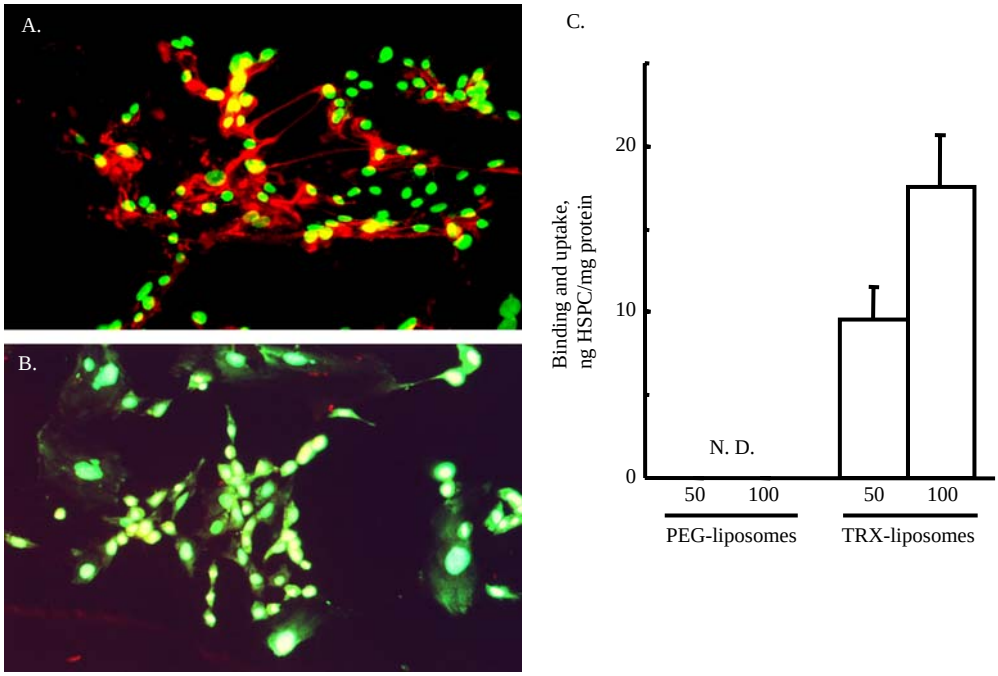

Fig. 2. Fluorescence micrographs (**A**) and (**B**) show rat mesangial cells incubated with rhodamine-labeled liposomes (100 μg HSPC). All cells were stained with SYTO 24 green fluorescent nucleic acid staining reagent: (**A**) TRX-liposomes; (**B**) PEG-liposomes. Chart (**C**) shows the interaction of TRX-liposomes and PEG-liposomes with rat mesangial cells.

Table I. Biodistribution of TRX-liposomes at 24 h Post-injection in Normal and Anti-Thy-1 Nephritic Rats

	Concentration of HSPC, μg/g Tissue	
	Normal Rats	Anti-Thy-1 Nephritic Rats
Blood	5.13±0.13	5.26±0.31
Liver	0.90±0.23	1.04±0.27
Kidney cortex	1.50±0.46	5.07±0.97*
medulla	1.46±0.22	2.52±0.42
Spleen	4.04±0.30	5.05±0.41
Lung	0.74±0.12	0.74±0.05
Heart	0.72±0.09	0.56±0.09
Brain	0.32±0.14	0.40±0.12

Each value represents the mean±S.E. (n=5).
Abbreviation: *HSPC*, hydrogenated soy bean phosphatidylcholine.
* indicates significant differences from the normal rats at $p<0.05$.

higher accumulation in the anti-Thy-1 nephritic rats than in normal rats. Fluorescence microscopy also indicated the accumulation of liposomes in the glomeruli of the anti-Thy-1 nephritic rats, but not in the glomeruli of normal rats (Fig. 3).

Biodistribution of TRX-Liposomes and PEG-Liposomes in the Blood and Kidney of Anti-Thy-1 Nephritic Rats

The levels of TRX-liposomes and PEG-liposomes in the blood and kidney of nephritic rats after injection of the liposomes are shown in Fig. 4. Both the TRX-liposomes and PEG-liposomes disappeared from the blood with a half-life of around 12 h, indicating that despite their different surface properties, both types of liposomes were long-circulating. Approximately six times more TRX-liposomes accumulated in the kidney cortex, where the glomeruli reside, than in the medulla. About equal amounts of TRX-liposomes and PEG-liposomes accumulated in the kidney cortex.

When HPTS/DPX-encapsulating TRX-liposomes or PEG-liposomes were injected into anti-Thy-1 nephritic rats, marked HPTS fluorescence was observed in the glomeruli of the kidney cortex of rats given TRX-liposomes, but not of those given PEG-liposomes (Fig. 5). Thus, although both the TRX-liposomes and PEG-liposomes labeled by rhodamine accumulated in nephritic kidney cortex at the same level, the HPTS/DPX experiment showed that only the TRX-liposomes were localized to the glomerular cells.

Effects of the Two Liposomal Formulations of PSLP on Glomerular Cell Proliferation in Experimental Glomerulonephritis

Three days after the anti-Thy-1.1 antibody injection, the mesangial cells began to proliferate; proliferation peaked between days 4 and 8 (data not shown). Therefore, to investigate the effects of the three formulations of PSLP on glomerular cell proliferation in the rat experimental glomerulonephritis model, TRX-liposomes, PEG-liposomes, or PSLP saline solution was first given on day 4 after the antibody injection. The effects of a single injection of the liposomal formulations on glomerular cell proliferation were compared with those of daily injections of the PSLP saline solution. As shown in Figs. 6A and 7A, the PSLP-containing TRX-liposomes significantly reduced the number of total

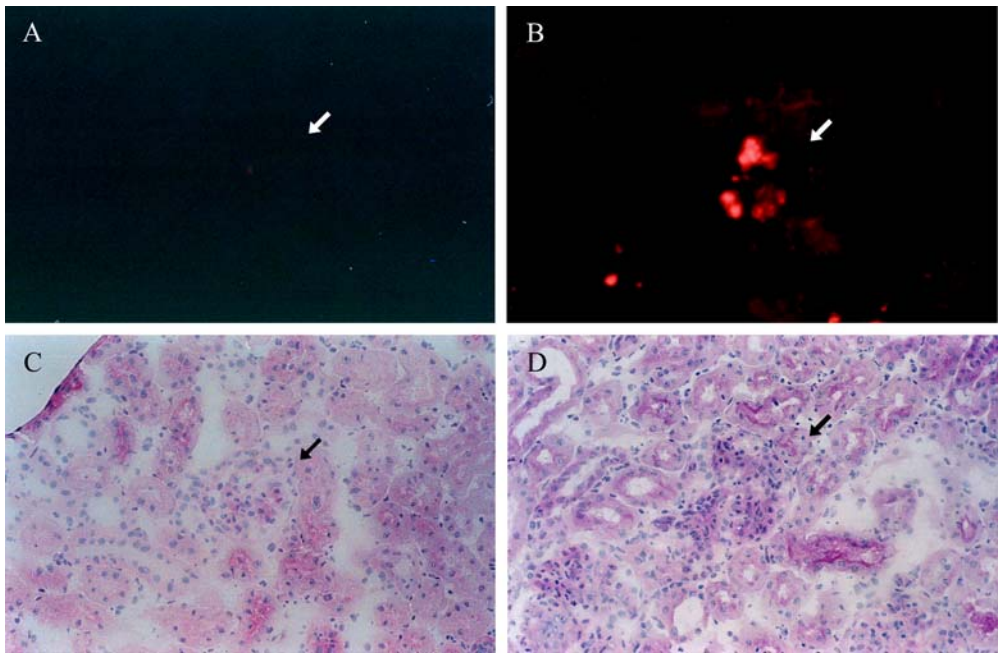

Fig. 3. Fluorescence micrographs of the glomerular localization of rhodamine-labeled TRX-liposomes in normal rats (**A**) and anti-Thy-1 nephritic rats (**B**). The light-microscopic findings (periodic acid-Schiff staining) of the glomeruli corresponding to the ones in the fluorescent micrographs in normal rats (**C**) and anti-Thy-1 nephritic rats (**D**). Note the glomerulus (*white and black arrows*).

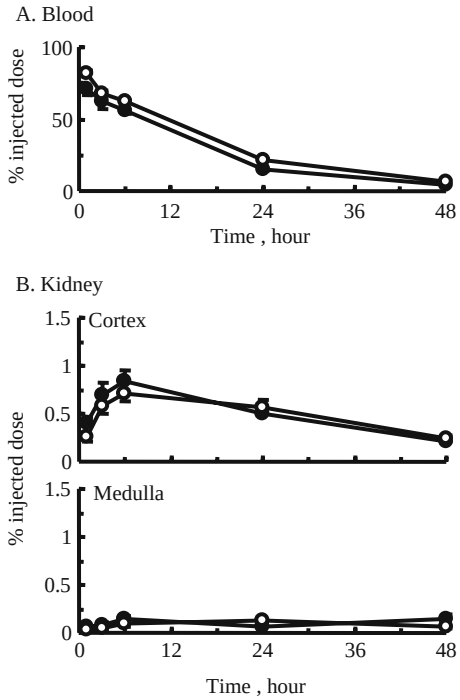

Fig. 4. Time course for the levels of TRX-liposomes and PEG-liposomes in the blood (**A**) and kidney (**B**) of the rat experimental glomerulonephritis model. Each value represents the mean±S.E. (*n*=5). Symbols: (closed circle) TRX-liposomes; (open circle) PEG-liposomes.

glomerular cells in a dose-dependent manner, at 0.032 mg PSL/kg and above. The number of total glomerular cells in normal rats was approximately 60 cells per glomerulus cross section. TRX-liposomes suppressed more than 50% of the cell proliferation, because the increase in total glomerular cells was already observed on day 4. In contrast, PEG-liposomes and PSLP saline solution also reduced the number of total glomerular cells, but the doses required were greater (more than 0.32 mg PSL/kg for PEG-liposomes and a total amount of 12.8 mg PSL/kg for the saline solution), showing that the TRX-liposomes provided more efficient delivery of the drug. In addition, neither a single injection of PSLP saline solution (3.2 mg PSL/kg) nor one of empty TRX-liposomes (60 μmol total lipids/kg as an equivalent dose of 3.2 PSL/kg) on day 4 suppressed the glomerular cell proliferation (data not shown).

Furthermore, although all three formulations of PSLP inhibited the increase in total glomerular cells, only the TRX-liposomes, at 0.32 mg PSL/kg and above, significantly reduced the number of α-SMA positive cells (Figs. 6B and 7B).

DISCUSSION

In general, macromolecules and polymeric drugs can preferentially accumulate in tumor tissues because of their enhanced vascular permeability compared with normal tissue (16). This phenomenon is known as the Enhanced Permeability and Retention effect (EPR effect). So far, only PEG-liposomes with their 100-nm size have been reported to accumulate in inflammatory lesions and tumor sites due to the EPR effect, also called "passive targeting" (3,4). Liposomal size and prolonged circulation times are critical to the success of this targeting system. The problem with this system, however, is that the PEG-liposomes do not bind well to target cells at the accumulation sites. To achieve ideal targeting for the most specific therapeutic effects, three factors, the accumulation of liposomes at disease sites, target cell-specific binding, and the delivery of drugs into target cells, must all be in play (17).

TRX-20, a cationic lipid, has been developed to overcome this problem and create a liposomal formulation with an active targeting function. Our previous study revealed that TRX-20-modified PEG-liposomes (TRX-liposomes) bind specifically to mesangial cells and smooth muscle cells, but not to endothelial cells (5). In this study,

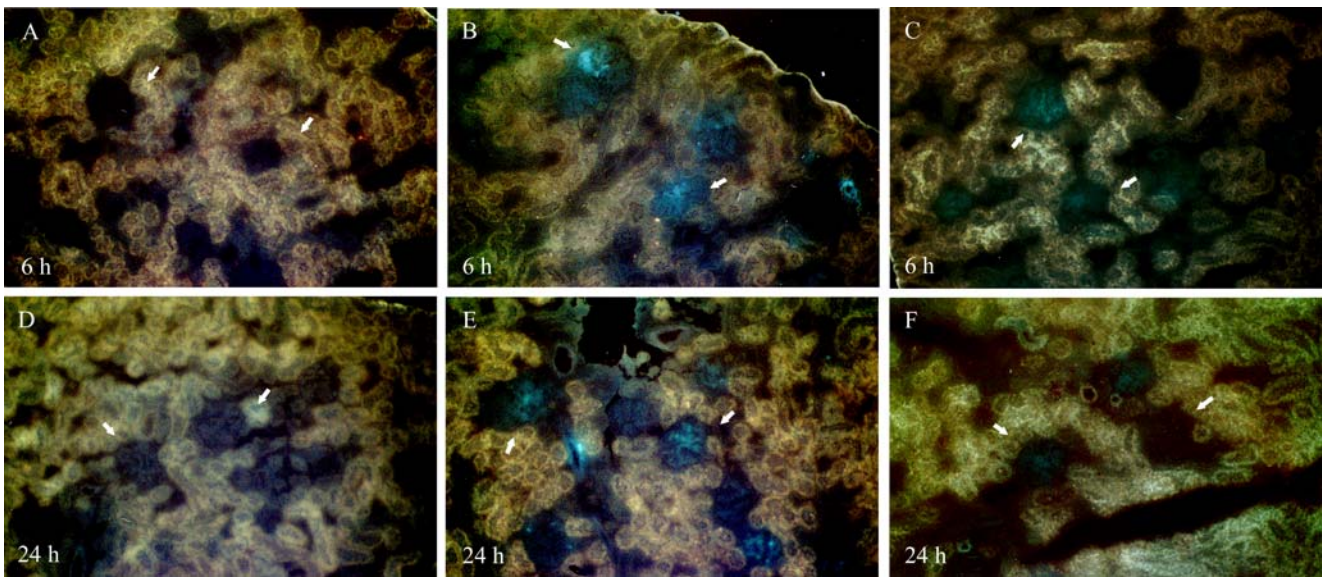

Fig. 5. Fluorescent micrographs of kidneys from anti-Thy-1 nephritic rats treated with HPTS/DPX-encapsulating TRX-liposomes (**B**, **E**) and PEG-liposomes (**C**, **F**). The HPTS dye provides a means of detecting the binding of and endocytosis of its conjugates by the glomerular cells. Micrographs (**A**) and (**D**) were obtained from untreated nephritic rats. Note the glomerulus (*white arrows*).

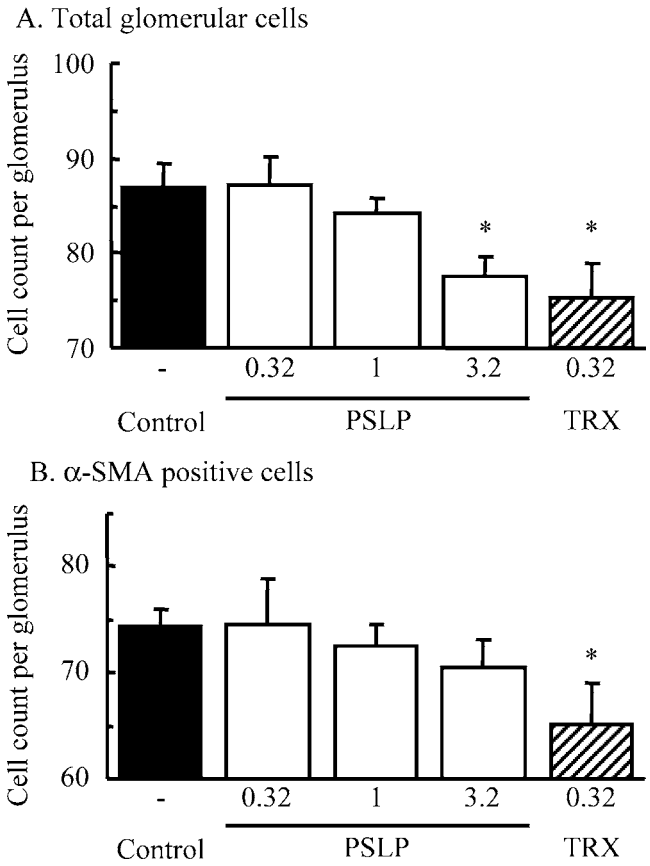

Fig. 6. Effects of PSLP saline solution on glomerular cell proliferation in the rat experimental glomerulonephritis model. The cell counts in normal rats were 58.1±1.9 and 0.3±0.2 cells per glomerulus for total glomerular cells and *α-SMA* positive cells, respectively. Each value represents the mean±S.E. (n=6). * indicates significant differences from the nephritic control at $p<0.05$ and $p<0.01$, respectively. Abbreviations are: *PSLP*, prednisolone phosphate; *α-SMA*, α-smooth muscle actin; *TRX*, TRX-liposomes.

we found that TRX-liposomes have a prolonged circulation time in the blood (half-life of 12 h), even though their surface (zeta potential: 3.9 mV) is quite different from that of PEG-liposomes (zeta potential: −7.5 mV), which have a comparable half-life. The affinity of TRX-liposomes for subendothelial cells is consistent with their long-circulating property, which is probably also closely related to their lack of binding with vascular endothelial cells. The mechanism of passive targeting suggests that TRX-liposomes will accumulate in markedly permeable tissues such as inflamed lesions and tumor sites.

In our comparison between experimental glomerulonephritic rats and normal rats, there was a significant difference in the accumulation of TRX-liposomes only at the kidney cortex, as shown in Table I, where the TRX-liposomes accumulated at a level 3.4-fold higher in nephritic rats than in normal rats. In particular, intense fluorescence due to rhodamine-labeled TRX-liposomes was observed in the glomeruli of the nephritic kidney cortex but not in the glomeruli of the normal kidney, and the accumulation of TRX-liposomes was similar to that of PEG-liposomes. The accumulation of TRX-liposomes in nephritic kidney is probably not based on the effect of TRX-20, but on that of passive targeting. The data shown in Fig. 4 indicate that the accumulation of liposomes in the cortex of the inflamed kidney 6 h after the injection was approximately sixfold higher than in the medulla, where there are no glomeruli. These results indicate that this passive targeting system will be useful for targeting the delivery of drugs to the glomerulus in glomerular disorders.

In contrast with the results of the accumulation experiments, intense fluorescence from HPTS/DPX-encapsulating TRX-liposomes was observed in the glomeruli of the experimental glomerulonephritic rats, but this fluorescence was very weak in nephritic rats that received HPTS/DPX-encapsulating PEG-liposomes. As explained in the Results section, marked fluorescence due to HPTS is observed only when HPTS is released from liposomes. In addition, the hydrophilicity of HPTS largely traps it within cell membranes, suggesting that the fluorescence observed in this study was due to HPTS that had been delivered into cells by the liposomes. Therefore, we conclude that

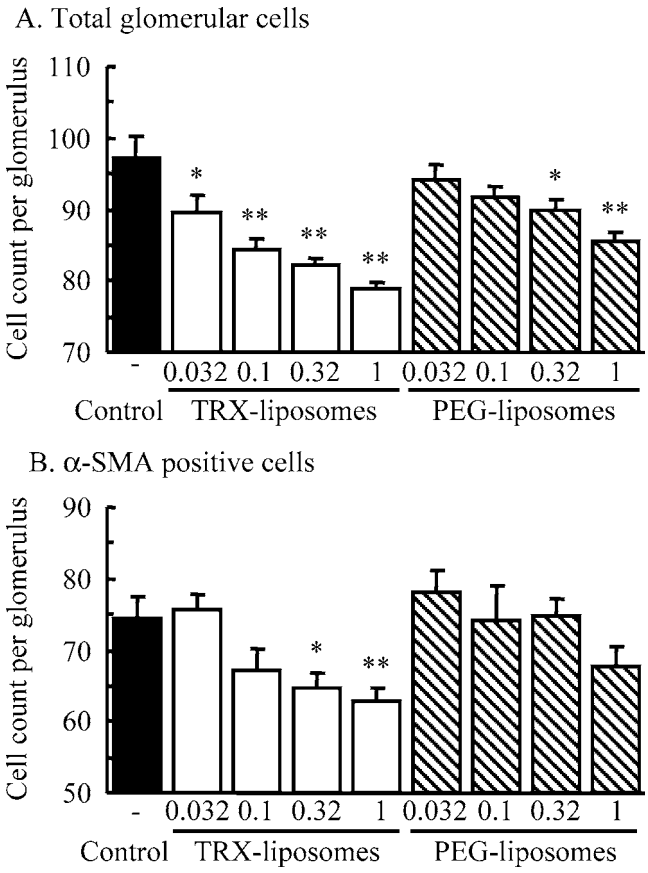

Fig. 7. Effects of the TRX-liposomal formulation of *PSLP* on glomerular cell proliferation in the rat experimental glomerulonephritis model compared with the PEG-liposomal formulation. The cell counts in normal rats were 63.3±1.8 and 0.1±0.1 cells per glomerulus for total glomerular cells and *α-SMA* positive cells, respectively. Each value represents the mean±S.E. (n=8). * and ** indicate significant differences from the nephritic control at $p<0.05$ and $p<0.01$, respectively. Abbreviations are: *PSLP*, prednisolone phosphate; *α-SMA*, α-smooth muscle actin.

the TRX-liposomes were internalized into glomerular cells and then HPTS was released. In contrast, rhodamine-labeled PEG-liposomes accumulated at the glomeruli of the inflamed kidney, but the HPTS/DPX-encapsulating PEG-liposomes appeared not to be internalized into the glomerular cells.

When studied *in vitro*, TRX-liposomes bound human mesangial cells through chondroitin sulfate proteoglycans, mainly recognizing chondroitin sulfate E, D, and B (5,12), whereas no binding to hyaluronic acid, heparan sulfate or keratan sulfate was observed. There are three kinds of cells in the glomerular tuft: mesangial cells, endothelial cells, and podocytes. Most proteoglycans in the glomerulus are supplied by mesangial cells, which produce the chondroitin sulfate proteoglycan versican and the chondroitin sulfate/dermatan sulfate proteoglycans decorin and biglycan (18,19). In particular, an overexpression of decorin and biglycan is reported in the glomerular mesangial area of anti-Thy-1 nephritic rats (20). Accordingly, the difference observed between TRX-liposomes and PEG-liposomes in the present study could be explained by the preferential binding to mesangial cells by the TRX-liposomes, which is not displayed by PEG-liposomes.

Whether this specific binding of TRX-liposomes is reflected in pharmacological effectiveness or not was investigated using experimental glomerulonephritic rats, and the results clearly indicated the usefulness of the targeting function on the suppression of glomerular hypercellularity, as shown in Figs. 6 and 7. Notably, the TRX-liposomes were effective at a dose that was less than one hundredth of the total dose of PSLP required using the traditional formulation. In detail, a significant suppression by TRX-liposomes of glomerular cell proliferation was attained at doses greater than 0.032 mg/kg, whereas the PSLP saline solution and PEG-liposomes required doses of 3.2 mg/kg (total amount of 12.8 mg/kg) and 0.32 mg/kg, respectively, to achieve a significant effect. These results mean that adverse effects often observed in glucocorticoid therapy could possibly be avoided by using a liposomal formulation of PSLP, especially the TRX-liposomal formulation.

Phenotypic changes in mesangial cells are reported in experimental and human glomerulonephritis and are considered to be an index of the functional activation and proliferation of the cells (21,22). In addition, α-smooth muscle actin (α-SMA) is expressed on these phenotypically altered mesangial cells. In this experimental model, the number of α-SMA positive cells was counted to evaluate mesangial cell activation, and only the PSLP delivered by TRX-liposomes significantly reduced the number of these cells, compared with delivery by saline solution and PEG-liposomes. This result suggests that PSLP delivered by TRX-liposomes has distinct pharmacological effects compared with PSLP delivered by saline solution or PEG-liposomes, and this difference is likely due to the preferential binding of TRX-liposomes to the glomerular cells.

Consequently, the results obtained in the present study suggest that the passive targeting and preferential binding affinity of TRX-20 make optimal use of the EPR effect, and indicate that the TRX-liposome system is a promising candidate for drug delivery targeting the treatment of glomerular diseases.

CONCLUSION

TRX-liposomes actively targeted glomeruli in the kidney cortex of the rat experimental glomerulonephritis model through preferential binding to chondroitin proteoglycans. In particular, TRX-liposomes had a significant effect at a much lower dose of PSLP than was required for the conventional injection and PEG-liposomal formulations. The distinct pharmacological effects observed in this study support the idea that TRX-liposomes will be useful as a drug delivery system for targeting the mesangial cells of inflamed glomeruli in glomerular diseases.

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