Application of liposomes incorporating doxorubicin
with sialyl Lewis X to prevent stenosis
after rat carotid artery injury

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ABSTRACT

Restenosis remains a serious complication that can occur after angioplasty. This study investigated the efficiency of an active targeting chemotherapy using liposomes, including doxorubicin, whose surface was decorated with sialyl Lewis X (SLX), (Dox-Lipo-SLX) to prevent stenosis after angioplasty. Its delivery was controlled via the affinity between SLX and E-selectin proteins, which are expressed on vessel walls with injury. *In vitro* experiments confirmed the accumulation of doxorubicin as a consequence of Dox-Lipo-SLX adhering to E-selectin-positive cells. Significant doxorubicin accumulation was observed on injured vessel walls in rats treated with Dox-Lipo-SLX. In contrast, there was little accumulation using free doxorubicin or a liposome containing doxorubicin (Dox-Lipo), but without SLX. Rats were assigned to one of four groups: Dox-Lipo-SLX, Dox-Lipo, free doxorubicin, or no treatment. Dox-Lipo-SLX, Dox-Lipo, and free doxorubicin, including a dose of 0.08 mg/kg doxorubicin, were intravenously administered three times in each group after angioplasty. The residual lumen area of rats in the group treated with Dox-Lipo-SLX was significantly larger than those in all other groups. These results demonstrate that an active targeting drug delivery system utilizing Dox-Lipo-SLX effectively prevents stenosis after angioplasty.

INTRODUCTION

Percutaneous transluminal angioplasty (PTA) and stenting are minimally invasive methods that are widely used to treat coronary, carotid, and peripheral arterial stenosis. Restenosis after angioplasty, however, remains a serious problem and occurs in 20-30% of coronary cases [1], in no less than 5% of carotid artery stenosis cases [2-4], and in 10-40% of intracranial artery stenosis cases [5-7]. This study investigated the possibility of chemotherapy using an active targeting drug delivery system (DDS) to prevent stenosis after vascular injury.

A liposome decorated with sugar chains was developed by our group [8-10] and used as an active targeting DDS for injured vessels. This study focused on the affinity between E-selectin protein, which facilitates leukocyte adherence to the vascular endothelium at the site of inflammation, and sialyl Lewis X (SLX) sugar chains, which bind E-selectin. E-selectin protein expression is increased in injured vessels walls after balloon angioplasty. Therefore, E-selectin protein might be a useful target for active targeting DDS. In the inflammatory regions, vascular endothelial cells and smooth muscle cells express E-selectin on the cell surface. Leukocytes presenting the SLX sugar chain on their surface could bind E-selectin and "roll" across vascular endothelial cells, resulting in their accumulation in the inflamed area. This inflammation may be associated with the restenotic region after angioplasty [11]. Based on this, we hypothesized that liposomes decorated with SLX would accumulate in the stenotic region after vascular injury in a manner similar to leukocytes. E-selectin protein expression was investigated after balloon angioplasty using a rat model of carotid artery

injury.　Furthermore, doxorubicin liposome accumulation was confirmed by observing the presence of doxorubicin fluorescence, and its efficiency for preventing stenosis was investigated.

MATERIALS AND METHODS

Preparation of Dox-Lipo-SLX and Dox-Lipo

As liposomes preparation has been described in detail in previous publications [8-10], we will briefly describe liposome preparation based on the improved cholate dialysis method. The lipids (Sigma Aldrich Co., St. Louis, MO) used in liposome preparation, including dipalmitoylphosphatidylcholine (16.8 mg), cholesterol (10.1 mg), dicetyl phosphate (1.8 mg), gangliosides (14.6 mg), dipalmitoylphosphatidylethanolamine (2.3 mg), and sodium cholate (46.9 mg), were mixed together. The mixtures were dissolved in 3 ml of a 1:1 (v/v) chloroform/methanol solution, and the solvent was evaporated by drying under a vacuum. The resulting lipid films were dissolved in 3 ml of TAPS buffer (pH 8.4), and a micelle suspension was obtained via sonication. Three milliliters of doxorubicin hydrochloride (3 mg/10 ml TAPS buffer, Wako Pure Chemical Industries Ltd., Osaka, Japan) was mixed with the micelle suspension, and the solution was filtered with TAPS using a PM10 membrane (Amicon; Millipore, Bedford, MA) to remove residual doxorubicin, yielding 10 ml of liposome solution. The solution was additionally filtered with sodium hydrogen carbonate buffer (CBS, pH 8.5) using an XM300 membrane (Amicon) in order to exchange the buffers. Ten milligrams of bis(sulfosuccinimidyl)suberate (BS_3, Pierce, Rockford, IL), a cross-linking agent, was added to 10 ml of the liposome solution and stirred at $20 - 25^{o}C$ for 2 hr, with additional stirring overnight at $4^{o}C$. In this manner, BS_3 was attached to the liposome surface. Thereafter, 40 mg of tris(hydroxymethyl)aminomethane (Tris) was added, and the solution was stirred at 20 –

25$^{\mathrm{o}}$C for 2 hr, with additional stirring overnight at 4$^{\mathrm{o}}$C to bind Tris to BS$_3$. This product was filtered with TAPS (pH 8.4) using an XM300 membrane to remove residual Tris. The coupling method was used to bind human serum albumin (HSA) to the liposome surface [8,9]. To oxidize the liposome surface, 10.8 mg of sodium periodate was added to 10 ml of liposome solution and stirred at 4$^{\mathrm{o}}$C overnight. To remove residual sodium periodate, the solution was filtered with phosphate saline buffer (PBS, pH 8.0). Twenty milligrams of HSA were added, and the mixture was stirred at 20 – 25$^{\mathrm{o}}$C for 2 hr. Subsequently, 3.13 mg of sodium cyanoborohydrate was added, and the solution was filtered with CBS (pH 8.5) to remove residual sodium cyanoborohydrate. Sugar chains were attached on the liposome surface using 3,3-dithiobis(sulfosuccinimidylpropionate) (DTSSP, Pierce) as the cross-linking reagent. Ten milligrams of DTSSP was added to 10 ml of the liposome solution and stirred at 20 – 25$^{\mathrm{o}}$C for 2 hr, with further stirring overnight at 4$^{\mathrm{o}}$C. To remove residual DTSSP, the solution was filtered with CBS (pH 8.5). The reducing group terminal to the sugar chain was aminated using the glycosyl amination reaction. Two milliliters of SLX (Calbiochem, Darmstadt, Germany) was dissolved in 0.5 ml of distilled water. Then, 0.25 g of NH_4HCO_3 was added, and the solution was stirred at 37$^{\mathrm{o}}$C for three days. Aminated SLX was added to a final concentration of 50 μg/ml, and the resulting solution was stirred at 25$^{\mathrm{o}}$C for 2 hr. Tris was then added to a final concentration of 132 mg/ml, and the solution was stirred overnight at 4$^{\mathrm{o}}$C. To remove residual SLX and Tris, the solution was filtered with n-(2-hydroxyethyl)piperazine-n'-(2-ethanesulfonic acid) (Hepes, pH 7.2). For liposomes without SLX (Dox-Lipo), the preparatory steps were similar to those utilized for

Dox-Lipo-SLX, except for the SLX binding process. Dox-Lipo-SLX or Dox-Lipo, prepared according to the above methods, demonstrated a particle size of 100 - 210 nm and zetapotential of –16.6 mV, measured with Zetasizer Nano-ZS (Malvern Instruments Ltd. UK). To measure the amount of doxorubicin incorporated into the liposomes, Triton X (final concentration 0.1%) and 1 vol of butanol were added. The mixture was centrifuged after vortexing, and doxorubicin present in the butanol layer was measured at an absorbance of 450 nm [12]. The doxorubicin concentration in the liposome solution was 24-30 μg/ml. As the final volume was adjusted to 50 ml, the total dose of doxorubicin in the solution was 1.2-1.5 mg.

Expression of E-selectin and Doxorubicin Uptake in HSMCs

In rat carotid artery injury models, stenosis is known to be induced in response to medial smooth cell growth [13]. Therefore, human aortic smooth muscle cells (HSMCs, Cell Application, Inc., San Diego, CA) were incubated in Modified Eagle's medium (MEM; GIBCO-BRL, Grand Island, NY) supplemented with 10% heat-inactivated fetal calf serum (FCS; GIBCO-BRL) in a humidified atmosphere with 5% CO_2 at 37^oC. HSMCs were seeded in chamber slides (2x4.5 cm) or 24-well culture plates at a concentration of $2x10^5$ cells per well (10^5 cells/ml, 2 ml) or $5x10^4$ cells per well (10^5 cells/ml, 0.5 ml) and cultured overnight. HSMCs were incubated with lipopolysaccharide (LPS; 0.5 μg/ml) for 5 hr to stimulate E-selectin expression, which was investigated by staining with mouse anti-human CD62E monoclonal antibody (10 μg/ml, clone# 68-5H11, BD Pharmingen, Franklin Lakes, NJ) and FITC-conjugated

goat anti-mouse IgG/IgM secondary antibody (1 μg/ml, BD Pharmingen). After three washes with PBS, the HSMCs were fixed with 2-propanone. Cells were observed by fluorescence microscopy (490 nm excitation, 520 nm emission).

HSMCs in chamber slides were incubated with LPS for 5 hr to induce E-selectin expression. After washing with PBS, the cells were immediately incubated for 15 min with Dox-Lipo-SLX, Dox-Lipo, or free doxorubicin, for which doxorubicin was dissolved in saline and adjusted to a final concentration of 5 μg/ml. As a control, the same volume of saline was added. To block E-selectin, mouse anti-human CD62E monoclonal antibody (50 μg/ml; BD Pharmingen) was added concomitantly with LPS. After three washes with PBS, the HSMCs were fixed with 2-propanone. Doxorubicin incorporated into HSMCs was examined by fluorescence microscopy (540 nm excitation, 605 nm emission).

The dose of doxorubicin taken up by HSMCs in 24-well culture plates was measured by the acid alcohol extraction method [14]. After LPS treatment, the cells were incubated with Dox-Lipo-SLX, Dox-Lipo, free doxorubicin, or saline for 15 min. After two washes with PBS, one ml of acid alcohol (0.3N HCl, 70% EtOH) was added, and doxorubicin extraction was performed for 24 hr at 4°C. The dose of extracted doxorubicin was quantified with a spectrofluorophotometer (RF-5300PC, Shimadzu, Kyoto, Japan; 470 nm excitation, 550 nm emission).

Rat Carotid Artery Post-angioplasty Stenosis Model

Preparation of rat carotid artery injury model has been described previously [13].

Male inbred Wistar rats were fed a normal diet until nine weeks of age, and then anesthetized with isoflurane by mask ventilation (air 400-500 ml/min. isoflurane 1.5-2.0%) (Univentor 400 Anesthesia Unit; Univentor Ltd., Zejtun, Malta). The right carotid artery was exposed by a midline neck skin incision, and a guide wire (Transend 0.010 inch; Boston Scientific, Natick, MA) was inserted from the external carotid artery and advanced retrogradely into the common carotid artery. Thereafter, a single-lumen compliant balloon catheter (Sentry 3.5 x 10 mm; Boston Scientific) was navigated along the guide wire. The balloon was visible through the vascular wall. After adjusting the proximal end of the balloon to the bifurcation, it was inflated with 0.2 ml of saline and maintained for 10 seconds. Balloon inflation was performed six times, and the external carotid artery was ligated after angioplasty. Balloon angioplasty was completed 10 mm proximal to the carotid bifurcation.

E-selectin expression in the carotid artery after balloon angioplasty

E-selectin expression was examined on days 1, 2, 3, and 7 after balloon angioplasty. Immunohistochemical analysis was performed on frozen sections using mouse anti-human CD62E monoclonal antibody (10 μg/ml, clone# 68-5H11, BD Pharmingen) as the primary antibody. A Vectastain Elite ABC kit (Vector Laboratories, Peterborough, UK) was used to visualize the primary antibodies.

Active Targeting system with doxorubicin liposomes

Doxorubicin accumulation was investigated in frozen sections of the injured

carotid arteries to confirm the efficiency of Dox-Lipo-SLX. Dox-Lipo-SLX, Dox-Lipo, free doxorubicin, or saline was administered intravenously 24 hr after balloon angioplasty. The doxorubicin content was adjusted to 0.08 mg/kg in all three groups by addition of saline. Rats were sacrificed, and the carotid arteries were harvested 24 hr after administration. Doxorubicin accumulation was examined in the frozen sections by phase contrast and fluorescence microscopy (540 nm excitation, 605 nm emission, Keyence Biozero, Keyence Co., Osaka, Japan). In some samples, the carotid arteries were opened longitudinally and extended on the slides. The fluorescence from carotid arterial walls was observed by phase contrast and fluorescence microscopy. After washing with PBS, some samples were homogenized in acid alcohol, and doxorubicin was extracted for 24 hr at $4^{o}C$. After removing debris following centrifugation, the dose of extracted doxorubicin was quantified using a spectrofluorophotometer (470 nm excitation, 550 nm emission).

Efficacy of doxorubicin liposomes in the prevention of restenosis

To evaluate whether Dox-Lipo-SLX inhibits stenosis within two weeks of balloon angioplasty, rats were assigned to four groups (n=7): Dox-Lipo-SLX, Dox-Lipo, free doxorubicin, and no treatment (negative control). Dox-Lipo-SLX, Dox-Lipo, free doxorubicin, or saline was administered three times intravenously in each respective group on days 1, 2, and 5 after balloon angioplasty. Dox-Lipo-SLX, Dox-Lipo, and free doxorubicin were adjusted to achieve a corresponding doxorubicin content of 0.08 mg/kg/injection. Rats were sacrificed, and the carotid arteries were harvested on day 14

after balloon angioplasty. A histological examination was then performed by hematoxylin-eosin, elastica van Gieson, PCNA, and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) staining using a rabbit PCNA polyclonal antibody (PCNA(FL-261); sc-7907, Santa Cruz Biotechnology, CA), LSAB+system-HRP (Dako, Denmark), and the Apop tag peroxidase In situ apoptosis detection kit (Intergen, NY). The intimal hyperplasia area (IHA) inside of the internal elastic lamina, the residual lumen area (RLA), and the circumferential length of the external elastic lamina (CLEE) present in the injured artery were measured at three points (3, 4, and 5 mm from the bifurcation) using the volumetric soft in Keyence Biozero. An average of three data points was used for evaluation. The area inside the internal elastic lamina (IAIE) and CLEE were measured proximally to the angioplastic region (about 15 mm from the bifurcation). The efficacy of Dox-Lipo-SLX in preventing stenosis was evaluated using the residual lumen ratio [(RLA in the injured artery)/(IAIE at the proximal point in the carotid artery)]. Remodeling was evaluated using the remodeling ratio [(CLEE in the injured artery)/(CLEE at the proximal point in the carotid artery)]. Intimal hyperplasia was evaluated by IHA. To analyze cell proliferation and cell apoptosis, the numbers of PCNA-positive and TUNEL-positive cells present in the intima, media, and adventitia were counted for each section.

Rat body weight was measured prior to both angioplasty and sacrifice to evaluate possible side effects of each treatment. Weight changes were evaluated as increasing folds [(weight before sacrifice)/(weight before angioplasty)].

Statistical Analyses

Statistical analyses were performed using the Student's t-test. A value of $P<0.05$ was considered as statistically significant.

RESULTS

Expression of E-selectin and doxorubicin taken up by HSMCs

Cells expressing E-selectin were observed (**Fig. 1A**), and doxorubicin fluorescence exhibited higher intensity in cells that received Dox-Lipo-SLX in comparison to those that did not (**Fig. 1B-D**). Intracellular doxorubicin was distributed predominantly in the cytoplasm, and doxorubicin fluorescence decreased after the inhibition assay, which utilized an anti-E-selectin antibody (**Fig. 1F**). By quantifying extracted doxorubicin, the cells treated with Dox-Lipo-SLX were found to contain 3.5-fold or over ten-fold more doxorubicin than those treated with Dox-Lipo or free doxorubicin (**Fig. 1G**). These findings indicate that SLX on the surface of liposomes can recognize E-selectin on HSMCs and that Dox-Lipo-SLX can accumulate *in vitro*. Figure 1E shows HSMC autofluorescence under the same conditions used for the other fluorescence micrographs.

E-selectin expression in the carotid artery after balloon angioplasty *in vivo*

E-selectin expression was observed in the intima and media, predominantly on days 2 and 3 (**Fig. 2B-C**) and weakly on day 1 after angioplasty (**Fig. 2A**). E-selectin expression was oriented laterally on the vascular walls, which might indicate the degree of vascular wall injury induced by balloon angioplasty, and disappeared on day 7 (**Fig. 2D**). These findings indicate that vascular injury induced E-selectin expression in the intima and media.

Active Targeting system with Dox-Lipo-SLX *in vivo*

Doxorubicin fluorescence in rat models treated with Dox-Lipo-SLX was observed in the intima and media of injured carotid arteries, potentially indicating the accumulation of liposomes containing doxorubicin (**Fig. 3A**). The Dox-Lipo-SLX accumulation was focal, and there was significantly more doxorubicin fluorescence observed in rats treated with these liposomes compared to those treated with either Dox-Lipo (**Fig. 3B**) or free doxorubicin (**Fig. 3C**). In some sections treated with Dox-Lipo-SLX, doxorubicin accumulated in the vascular walls (**Fig. 3E**), corresponding to E-selectin expression (**Fig. 3F**). Figure 3D shows the autofluorescence of vascular walls. When the injured carotid arteries treated with Dox-Lipo-SLX were opened, some accumulated doxorubicin fluorescence was observed (**Fig. 4A**). Moreover, no doxorubicin liposome fluorescence was observed in open, uninjured carotid arteries from the side opposite to the injured site of the same rats, without arterial wall autofluorescence (**Fig. 4B**). By quantifying the amount of doxorubicin *in vivo*, 12-13 ng of doxorubicin was detected in the injured arteries treated with Dox-Lipo-SLX 24 hr after administration, whereas only 3 ng was found in the injured arteries of rats receiving other treatments or the contralateral arteries (normal vessels) of rats administered Dox-Lipo-SLX (**Fig. 5**). These findings support that, *in vivo*, Dox-Lipo-SLX might be able to recognize E-selectin and accumulate in its presence. Dox-Lipo-SLX may be able to function as an active targeting DDS *in vivo*.

Efficacy of Dox-Lipo-SLX to prevent stenosis

The residual lumen ratio of the group treated with Dox-Lipo-SLX was significantly larger than all other groups (**Fig. 6A-B**), and concomitantly the remodeling ratio was significantly lower (**Fig. 6C**). The intimal hyperplasia area was lowest in the group treated with Dox-Lipo-SLX (**Fig. 6D**). With regard to cell proliferation and apoptosis, the numbers of PCNA-positive cells were lowest in the Dox-Lipo-SLX group compared to all other groups (**Fig. 6E**), and the numbers of TUNEL-positive cells in Dox-Lipo-SLX were significantly higher (**Fig. 6F**). There was a significant difference in the residual lumen ratio, the remodeling ratio, and the numbers of apoptotic cells between the Dox-Lipo-SLX group and all other groups. However, the observed difference in the intimal hyperplasia area or the numbers of PCNA-positive cells was not significant. As doxorubicin might predominantly inhibit vascular remodeling, there could be a significant difference in the residual lumen ratio, including the vascular remodeling evaluation and the remodeling ratio. The observed differences between the Dox-Lipo-SLX and Dox-Lipo groups are hypothesized to be due to active targeting of DDS with Dox-Lipo-SLX in the *in vivo* treatment experiments.

Each treatment was well tolerated, and no side effects due to Dox-Lipo-SLX or Dox-Lipo were noted during body weight monitoring in body. The fold increases weight were 1.109 ± 0.03324 (mean$\pm$SEM) for the group treated with Dox-Lipo-SLX, 1.089 ± 0.06457 for Dox-Lipo, 1.143 ± 0.0664 for free doxorubicin, and 1.137 ± 0.07417 for the control, revealing no significant differences.

DISCUSSION

Active targeting DDS with Dox-Lipo-SLX

The mutual recognition of E-selectin and SLX has been well clarified in literature reports [15,16]. Doxorubicin fluorescence was most intense in cells treated with Dox-Lipo-SLX. Furthermore, it was diminished *in vitro* by the addition of an anti-E-selectin antibody. Doxorubicin accumulation was observed in the intima and media corresponding to E-selectin expression, and the Dox-Lipo-SLX group demonstrated many apoptotic cells. These findings suggest that the active targeting DDS utilizing Dox-Lipo-SLX may be useful in the angioplasty model. In contrast to Dox-Lipo-SLX, the distribution of doxorubicin within the vascular tissue upon treatment with Dox-Lipo or free doxorubicin was diffuse. These findings indicate that Dox-Lipo-SLX has an affinity for E-selectin and effectively delivers doxorubicin to cells expressing E-selectin *in vitro* and *in vivo*. It may be reasonable and beneficial to control the delivery of particles by exploiting the affinity between SLX and E-selectin, as E-selectin assists in the rolling of lymphocytes on activated endothelial cells and is expressed on the endothelium of atherosclerotic lesions [17,18]. Furthermore, E-selectin has been reported to contribute to intimal hyperplasia [11]. Although active targeting DDSs mediated by antibodies, so-called immunoliposomes [19] or peptides [20,21], has been reported, there are few successful *in vivo* reports demonstrating targeted delivery *via* blood circulation. Further evaluation of conditions addressing an active targeting DDS is necessary to achieve greater accumulation in lesions.

The main causes of restenosis after angioplasty include intimal hyperplasia and

vascular remodeling [22,23], with vascular remodeling having a greater effect on restenosis in the balloon angioplasty model than intimal hyperplasia [24,25]. Therefore, the residual lumen area ratio, which includes both intimal hyperplasia as well as vascular remodeling, and the remodeling ratio were evaluated. The residual lumen ratio in the Dox-Lipo-SLX group was significantly higher than all other groups, and the remodeling ratio was significantly lower. These results suggest that Dox-Lipo-SLX may be effective for the prevention of restenosis, considering that vascular remodeling has a greater effect on this condition. However, to improve efficacy, this therapeutic approach may not only be needed to prevent remodeling, but also to inhibit intimal hyperplasia. This therapeutic approach loaded on liposomes should be evaluated in the future.

The phenotypic change in VSMCs usually occurs 2-3 days after balloon angioplasty, with the accumulation of inflammatory cells in the rat PTA model. E-selectin protein facilitates the adherence of leukocytes to the vascular endothelium at the site of inflammation in acute leukemia and in rheumatism [26,27]. In the present study, we investigated E-selectin protein expression after balloon angioplasty and found that most expression occurred 2-3 days after balloon angioplasty, simultaneously with the phenotypic change in VSMCs. The correlation between phenotypic change and doxorubicin accumulation with Dox-Lipo-SLX could result in greater treatment efficacy. An active targeting DDS that recognizes E-selectin might be useful for directing the specific effects on cells, changing their phenotype, as well as modulating the migration and proliferation.

Comparison of Dox-Lipo-SLX and a passive targeting system

Passive targeting systems function via enhanced permeability and retention effects. Uwatoku *et al*. reported that NK911 nanomicelles, including doxorubicin, with a passive targeting DDS function prevented restenosis [28]. They showed that the presence of 1 to 10 mg/kg of doxorubicin was needed in the NK911 to prevent restenosis. This is approximately 10 to 100 times more than that required in the present study. One to 10 mg/kg of doxorubicin can induce cardiomyopathy, which is a cumulative condition and increases the risk of congestive heart failure when the total dose of doxorubicin administered to patients reaches values of 500 mg/mm^2 [29]. The doxorubicin dose indicated in Uwatoku's report might not be feasible for patients with vascular stenosis, as unpleasant side effects could depend on the amount administered. Therefore, decreasing the amount of doxorubicin administered is important; in the present study, the dose used was 1/10 to 1/100 of that used in the passive targeting system. In either case, the active targeting DDS has great potential for enhancing efficacy or decreasing unpleasant side effects in other normal organs. The different outcomes obtained with Dox-Lipo-SLX and Dox-Lipo may be a consequence of active versus passive targeting. Dox-Lipo may represent a passive targeting system, although its *in vivo* circulation time is somewhat short.

Potential of active targeting DDSs for preventing stenosis after angioplasty

Restenosis of coronary artery stenosis has been effectively addressed using

drug-eluting stents (DESs). However, DESs may pose problems concerning late stent thrombosis [30,31]. Stent technology, including bare metal stents, cannot solve some feasibility problems associated with lesions in small vessels, as well as long, bifurcating, and multiple lesions [32]. On the other hand, DDS could prevent restenosis after angioplasty, even in cases for which DES cannot be used. In addition, DDS could be indicated for thin vessels that preclude the use of stents, such as intracranial or peripheral arteries.

Limitations

One limitation of this study is that human arteriosclerotic arteries differ from normal rat arteries. Though intimal hyperplasia and vascular remodeling of the rat carotid artery is caused only by mechanical injury, restenosis in human arteriosclerotic arteries may involve inflammation of compressed vulnerable plaques and the hyperplastic network of the vasavasorum [33]. Therefore, intimal hyperplasia proliferation would occur continuously. Further determination of E-selectin expression in humans is necessary to better understand the long-term proliferative activity of intimal hyperplasia to decide when DDS should be used for clinical cases.

CONCLUSION

This study demonstrates that Dox-Lipo-SLX, which represents nanoliposomes containing doxorubicin coated with SLX, may be effective in preventing stenosis after rat carotid artery injury. In addition, prevention of restenosis after angioplasty may be a good application of active targeting DDSs, utilizing the affinity that exists between SLX and E-selectin.

ACKNOWLEDGEMENTS

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Figure Legends

Figure 1.   (A) E-selectin expression (green) in human aortic smooth muscle cells (HSMCs) following lipopolysaccharide (LPS) treatment. Doxorubicin accumulation (red) in HSMCs expressing E-selectin with (B) Dox-Lipo-SLX, (C) Dox-Lipo, (D) free doxorubicin, and (E) no treatment. (F) Observed doxorubicin accumulation in HSMCs after LPS treatment and blocking with anti-E-selectin antibodies. Magnitude = 400x. (G) Measurement of doxorubicin accumulation in HSMCs following treatment with Dox-Lipo-SLX, Dox-Lipo, free doxorubicin, or no treatment. *P<0.001*

Figure 2.   E-selectin expression in the carotid artery walls of rats with respect to time after angioplasty. (A) Day 1, (B) day 2, (C) day 3, and (D) day 7 after the procedure. Bars = 50 μm.

Figure 3.   (A) Doxorubicin accumulation (red) in carotid artery walls after angioplasty that was treated with Dox-Lipo-SLX. Doxorubicin accumulation after treatment with (B) Dox-Lipo, (C) free doxorubicin, and (D) saline as a control. Comparison of (E) doxorubicin accumulation with Dox-Lipo-SLX and (F) E-selectin expression. The arrows indicate E-selectin expression on endothelial cells (F). Bars = 50 μm.

Figure 4.   Observation of fluorescence in opened rat carotid arteries treated with Dox-Lipo-SLX. (A) Scattered accumulation of doxorubicin in the opened, injured arteries. (B) Little accumulation in opened, uninjured arteries obtained from the

opposite side of the same rat. Bars = 50 µm.

Figure 5. Doxorubicin dose delivered to injured carotid arteries or uninjured carotid arteries from the contralateral side of treated rats. Samples were collected 24 hr after administration.　Injured A: injured arteries. Contralateral A: uninjured arteries from the contralateral side of the treated rats. The results are expressed as the mean±SEM (n=3 each). *P<0.01, **P<0.001*

Figure 6.　Effects of active targeting DDSs in preventing stenosis after angioplasty. (A) Photomicrographs (H&E staining) of the balloon-injured rat carotid arteries. (B) Residual lumen ratio, (C) remodeling ratio, (D) initial hyperplasia area, (E) numbers of PCNA-positive cells, and (F) numbers of TUNEL-positive cells for all four treatment groups. The results are expressed as the mean±SEM (n=7 each). *P < 0.05, ** P < 0.01、***P < 0.001.*