Transvascular accumulation of Sialyl Lewis X conjugated liposome in inflamed joints of collagen antibody-induced arthritic (CAIA) mice*

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Summary. The aim of the current study was to investigate the specific accumulation of the Sialyl Lewis X (SLX) liposome in inflammation in the collagen-antibody induced arthritic (CAIA) model mice. The SLX-liposome encapsulating fluorescent substance (Cy5.5 or Cy3) was prepared for this study. The SLX-liposome was administered intravenously via the mouse caudal vein. After 1 to 24 h, the accumulation of SLX-liposome was observed using in vivo fluorescent imaging equipment (eXplore Optix), or the knee joints were removed for histological analysis. The in vivo fluorescent imaging showed that the signal was confined to the inflammatory site in the CAIA mice in an inflammatory dependent manner. The signal intensity was stronger at 24 h than at 1 h after injection. In the histological sections, the fluorescent signals were detected in the periarticular soft-tissue, especially in the hyperplastic synovium, including a pannus invasion with inflammatory cells in the CAIA. Intense signals were observed in vessel-like structures 1 h after injection; these were co-labeled with the vascular endothelial cell marker (CD31) and E-selectin, a ligand of the SLX-liposome expressed on activated endothelial cells. The diffused signals from the vessels increased time-dependently at 6 to 24 h after injection. This is the first report to examine the exact localization of the SLX-liposome by encapsulated fluorescence in hyperplastic synovial tissue of CAIA mice. These results suggest the feasibility and potential use of SLX-liposome as a vehicle for the active targeting of drug delivery to inflammatory tissue.

Introduction

In inflammation, the vascular endothelium plays an important role in leukocyte recruitment and infiltration into the affected tissue (Steeber and Tedder, 2000; Szekanecz and Koch, 2008). The cytokine-activated endothelial cell is marked-in part-by increased cell surface expression of various adhesion molecules, including E- and P-selectins, vascular cell adhesion molecule 1 (VCAM-1), and intracellular adhesion molecule 1 (ICAM-1) (Steeber et al., 2005; Simone et al., 2008). Of these markers, E-selectin exhibits the most distinct activation-dependent and endothelial-selective pattern of expression (Koch et al., 1991; Kriegsmann et al., 1995, Everts et al., 2003a). For the anti-inflammatory therapeutics, an approach which allows a selective targeting of anti-inflammatory drugs and a simultaneous diminishing
of systemic side effects is ideal (Ding et al., 2006). Therefore, E-selectin ligands represent attractive affinity moieties for delivery diagnostics and therapeutic agents to the activated endothelium (Barthel et al., 2007).

Liposome is a biocompatible nanoscale vehicle that can be filled with drugs. Liposomes have been extensively tested in experimental animals as carriers for drug delivery. The major advances in liposome research have allowed liposomes to avoid detection by the body's immune system, specifically, the cells of the reticuloendothelial system (Klibanov et al., 1990; Allen et al., 1991; Maruyama et al., 1992). These liposomes, known as "stealth liposomes", are constructed with polyethylene glycol (PEG) studding the outside of the membrane (Maruyama 2002). Such PEG modified liposomes have prolonged the circulation time in blood and consequently allow the development of a targeted drug delivery system.

To date, two major potential ligands have been reported for E-selectin targeting liposomes, categorized as immunoliposomes and glycoliposomes. The cellular uptake of immunoliposomes conjugated with the anti-E-selectin antibody was investigated in activated human endothelial cells (Kessner et al., 2001; Everts et al., 2003a; Tan et al., 2003). Furthermore, E-selectin-directed targeted drug delivery was demonstrated in a mouse glomerulonephritis model and in a local skin inflammation model of the mouse using liposomes conjugated with anti-E-selectin antibodies (Everts et al., 2003b; Asquesildottir et al., 2008). Sialyl Lewis X (SLX) carbohydrate is a well clarified E-selectin ligand expressed on the surface of leukocytes. The feasibility of a glycoliposome conjugated with SLX for drug transfer into activated endothelial cells has been demonstrated in vitro (Stahn et al., 2001). A novel type of SLX-liposome was developed, on which the SLX tetrasaccharides were bound via human serum albumins, and tris (hydroxymethyl) aminomethane (Tris) with Cy3 encapsulated in the SLX-liposome was examined in undecalcified cryosections with immunohistochemical identification of the activated vascular endothelium.

Materials and Methods

Animals and arthritis induction

All animal research was conducted in accordance with the requirements of the Okayama University Animal Research Committee. DBA/1J mice (Shimizu Laboratory Supplies, Kyoto) were used to evaluate the in vivo distribution of SLX-liposome in the synovial tissue of CAIA mice. All of the mice in the current study were 6- to 7-weeks-old. The mice were housed in wire cages with a sawdust bottom in an air-conditioned room at constant temperature under standard diurnal conditions, fed a standard commercial diet, and given tap water ad libitum. Arthritis was induced by an arthritogenic cocktail of five monoclonal antibodies (mAbs) to type II collagen (Chondrex, Redmond, WA, USA) combined with LPS stimulation according to the method by Terato et al. (Terato et al., 1992; Nishida et al., 2004) with a slight modification to increase the efficacy of the arthritogenic cocktail. The DBA/1J mice were injected intravenously with 2 mg of mAb on day 0 followed by an intraperitoneal injection of 50 μg of LPS on day 3. These mice were used for the experiments 10 days after mAb injection. They were monitored for the clinical evaluation of arthritis every day after the mAb injection. Arthritis was scored using a range of 0-4 points, according to the criteria of Terato et al., (Terato et al., 1992). Each limb was graded individually on a scale of 0-4 (maximum cumulative clinical arthritis score 16 per mouse), where 0 = normal, 1 = mild but definite redness and swelling of the ankle or wrist or redness and swelling of any degree in any single digit, 2 = moderate to severe redness and swelling of the ankle and wrist, 3 = redness and swelling of the entire foot including the digits, and 4 = maximally inflamed limb, with the involvement of multiple joints.

Histological analysis of hind limbs

The mice (n = 3) were euthanized by systemic perfusion with 4% paraformaldehyde under general anesthesia. Both hind limbs of CAIA and normal control mice were dissected and fixed in the same solution for 24 h. The samples were decalcified by incubation in 5% EDTA (pH7.4) for 10 days, dehydrated in a graded series of ethanol, and embedded in paraffin. Hematoxylin and eosin (HE) staining and toluidine blue staining were performed on standard sagittal sections of 4 μm.
In vivo fluorescent imaging by eXplore Optix

The SLX-liposome encapsulating the near-infrared (NIR) fluorescent substance (Cy5.5) for in vivo fluorescent imaging was purchased from GE Healthcare Bioscience (Tokyo). In vivo fluorescent imaging was performed as described previously (Hirai et al., 2007). Fifty μl of the SLX-liposome were administered intravenously from the mouse caudal vein at day 9 after mAb injection (n = 2, Fig. 1A). After 1 or 24 h, the accumulation of the SLX-liposome was observed using in vivo fluorescent imaging equipment (eXplore Optix; GE Healthcare, Buckinghamshire, UK) (excitation, 680 nm; emission, 700 nm). The fluorescent signal intensity was compared with that obtained before liposome injection in the same mouse (n = 2).

Immunohistochemical analysis of hind limbs by the undecalcified cryosection

An immunohistochemical analysis was performed on the knee or the ankle joints from the mice injected with the SLX-liposome (K1) or non targeted-liposome (K0) encapsulating fluorescent substance (GE Healthcare Bioscience, Tokyo). The samples were collected at 0, 1, 6, 12, and 24 h after liposome injection (n = 3-5 for each time point). The sagittal sections were cut using the undecalcified cryosection method previously described by Kawamoto (Kawamoto and Shimizu, 2000; Kawamoto, 2003). Briefly, the sample was placed in a stainless steel container filled with a 4% carboxymethylcellulose (CMC) compound (Finetec Co. Ltd., Tokyo). The CMC compound was completely frozen in hexane, which was cooled with liquid nitrogen. Serial articular cartilage sections (5 μm) were cut on a cryomicrotome (Leica CM1900 rotatory microtome, Leica; Wetzlar, Germany) equipped with a knife made of tungsten carbide (TC-65, Leica). The adhesive film (Cryofilm, Finetec Co. Ltd.) was used according to the manufacturer’s instructions. Sections were dehydrated with 100% ethanol for 1 min and fixed with 4% PFA in phosphate buffered saline (PBS) for 10 min. The sections were rinsed with PBS, blocked with 2% BSA in PBS (blocking buffer) for 1 h and incubated with primary antibodies against CD31 or CD62E (BD Pharmingen; 1:50 dilution in the blocking buffer). The sections were then washed and incubated with FITC-labeled anti-rat IgG (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. The sections were counterstained with 0.0025% calcein (Funakoshi Co. Ltd., Tokyo). Images were captured using an Olympus BX50 light microscope (Olympus, Tokyo) equipped with an AxioCam digital camera (Carl Zeiss, Oberkochen, Germany), followed by image manipulation with AxioVision software (Carl Zeiss). The serial sections were stained with HE to assess the general morphology and then were examined under a light microscope.

Results

Arthritis induction by a new arthritogenic cocktail of five monoclonal antibodies (mAbs) to type II collagen

The CAIA mice were created using a new arthritogenic cocktail of five mAbs to type II collagen (Chondrex) as described in the Materials and Methods. This is the first report characterizing this model. By day 4, clinically apparent arthritis was observed, with marked swelling or redness of the limb joints in all mAb-treated mice. The clinical symptoms of active arthritis reached a peak on day 7 (Fig. 1A). On day 10, the knee joints were dissected and assessed histologically. A histological analysis revealed marked synovial proliferation, infiltration by inflammatory cells, and bone erosion and bone and cartilage destruction in the knee joints of CAIA mice (Fig. 1B, C).

In vivo fluorescent imaging analysis

To ascertain the reproducibility of the SLX-liposome (GE Healthcare Bioscience) to the inflamed region in the present CAIA mice, in vivo fluorescent imaging analysis was conducted. After administration of the SLX-liposome encapsulating a near-infrared (NIR) fluorescent substance (K1-Cy5.5), a fluorescent signal of Cy5.5 was monitored in the inflamed region (the hind paws) of the same mouse and compared with that before injection. There was a slight increase in the accumulated signals after 1 h (Fig. 2B) and a marked increase in signals after 24 h (Fig. 2C) in the CAIA mice, while there was no obvious increase detected in the control normal mice administered with the SLX-liposome (K1-Cy5.5; Fig. 2D–F).

Assessment of fluorescent signals of the SLX-liposome in the undecalcified cryosection

To obtain detailed information on the SLX-liposome pharmacodynamics at the inflamed joints of the CAIA mice, the fluorescent signal of Cy3 encapsulated in the SLX-liposome was examined in undecalcified cryosections. A cryosection using cryofilm and a tungsten carbide blade enabled a confirmation of the accumulation and transition of the Cy3 signals in the inflamed joint. Linear vascular-like signals were evident at 1 h after the
Fig. 1. Legend on the opposite page.
Synovial accumulation of SLX-liposome in CAIA mice

liposome-administration (Fig. 3A, arrowheads), and those were subsequently diffused in the synovial tissues by 24 h after the administration of the SLX-liposome (K1-Cy3; Fig. 3B), while no pronounced signal accumulation was observed in the control mice administered with untargeted liposomes (K0-Cy3; Fig. 3C, D). To elucidate the relative localization of the fluorescent signal derived from the SLX-liposome and the vascular structure, immunostaining was performed using CD31 as a vascular endothelium marker and E-selectin (CD62E) as an activated endothelial cell marker. At 1 h after the SLX-liposome administration, the Cy3 fluorescence was confined to the vessels in the synovium double-labeled with CD31 (Fig. 4B) and, notably, was co-stained with the anti-E-selectin antibody (Fig. 4C). In addition, the time-dependent Cy3 signal transition was tracked for 24 h. At 6 h after the SLX-liposome administration, the Cy3 signals co-labeled with CD31 (Fig. 5B, arrowheads) were fewer than those at 1 h (Fig. 5A). Cy3 fluorescent leakage to the surrounding tissue became more evident time-dependently at 24 h than at 6 and 12 h (Fig. 5C, D).

Discussion

The current study investigated the specific accumulation of the Sialyl Lewis X (SLX) liposome encapsulated with a fluorescent substance to inflamed sites in CAIA mice.

The CAIA mice used for this study were created with a new arthritogenic cocktail of five mAbs to type II collagen (Chondrex). There are no previous reports of CAIA mice created with this new cocktail. Eight CAIA mice exhibited similar clinical signs according to the clinical evaluation described by Terato et al. (1992). A histological analysis could further demonstrate the severe pathological manifestations characterized by bone erosion, bone and cartilage destruction, and the

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**Fig. 1.** Induction of anti-collagen antibody-induced arthritis and histological appearances of the mouse knee joints. A: Protocol of the arthritis induction and changes in clinical scores during the experimental period. The DBA/1J mice were injected intravenously (i.v.) with 2 mg of the new cocktail of five mAbs to type II collagen on day 0 followed by an intraperitoneal (i.p.) injection of 50 µg of LPS on day 3. The mice (n = 8) were monitored for the clinical evaluation of arthritis in each limb every day after the mAb injection. B–E: Light micrographs of sagittal sections of the knee joints on day 10 after mAbs injection (B, C) and control joints without arthritis (D, E), stained with H.E. (B, D) and toluidine blue (C, E). Bone erosion (arrow), bone and cartilage destruction by pannus (arrowheads), and synovial proliferation and inflammatory cell infiltration are seen in the knee joints of CAIA mice (B, C). F: femur, M: meniscus, S: synovium, T: tibia. Bar = 50 µm

**Fig. 2.** Assessment of SLX-liposome accumulation in inflamed regions by an in vivo fluorescent imaging system. SLX-liposome (K1-Cy5.5) was administered from the tail vein (50 µl/mouse) in CAIA (A–C) or control (D–F) mice. The inflamed regions (the back side of the right hind limb) of the same mouse were observed using eXplore Optix (GE Healthcare) before administration (A, D), and at 1 h (B, E) and 24 h (C, F) after injection. The data were normalized using the eXplore Optix software program (GE Healthcare).
infiltration of inflammatory cells in the synovial space and pannus formation in these mice. This protocol is therefore considered to be a useful arthritic model.

The undecalcified cryosection method using cryofilm and tungsten carbide blade as described in Materials and Methods was used to preserve the fluorescent signal. Although there is a subtle limitation in the fixation conditions due to the cryofilm material, this method is useful for fluorescent-labeled immunohistochemistry and for tracking fluorescent substances. Hence, the tracking of Cy3 encapsulated in the SLX-liposome could be compared with the labeling of vascular endothelial cell markers (Fig. 4). The initial targeting of the SLX-liposome to the activated endothelial cells marked by CD62E was observed at 1 h after the injection, and then a gradual leakage of the Cy3 signal to the surrounding synovial tissues was seen between 6 to 24 h after the injection (Fig. 5).

Previous reports showed a shift of the fluorescence of the liposome from blood vessels stained with acridine
Fig. 4. Immunohistochemical identification of the SLX-liposome localization on the activated vascular endothelial cells. Cy3 signals encapsulated in the SLX-liposome (K1-Cy3) was co-labeled with the vascular endothelial cell marker (CD31 in B, green) and E-selectin (CD62E in C green), a ligand of the SLX-liposome, which is expressed in the activated-endothelial cell. A: HE staining of the hyperplastic synovium (S) and bone tissue (Bo) in the CAIA mouse knee joint. The inset indicates the region of interest. B, C: Notice a number of double-labeled signals, indicating the presence of the SLX-liposome targeted to the activated endothelial cells at 1 h after K1-Cy3 injection. Bars = 100 μm (A), 20 μm (B, C)

Fig. 5. Temporal changes in SLX-liposome distribution in the hyperplastic synovium of CAIA mice. Cy3 signals (magenta) were located in the blood vessels (CD31: green) at 1 h after K1-Cy3 injection and colocalized with CD31 at 1 h and 6 h (A, arrowheads in B). Subsequently leaked signals around the blood vessels increased time-dependently at 6–24 h (B–D). Bar = 20 μm
orange to the surrounding tissue using an in vivo laser scanning microscope (IV100, Olympus; Hirai et al., 2007). The IV100 is a new intravital laser scanning microscope for small animal imaging. It is beneficial for in vivo fluorescent imaging with minimum invasion in combination with appropriate counterstaining such as acridine orange. However, acridine orange is not a specific staining marker for blood vessels and cannot distinguish the activated status of endothelial cells. The current results certified the time-dependent transition of fluorescence to the surrounding tissue with a vascular marker as a reference.

However, there are still technical limitations associated with this study. The status of the SLX-liposome and the encapsulated materials is unclear. After the liposome is extravasated, is it still intact or broken? Or is it incorporated by any cells? Further analysis by electron microscopy will provide additional answers to these questions. A new tool, e.g. the SLX-liposome containing gold colloid particles, is still required in order to understand the mechanisms of this drug delivery system.

In conclusion, CAIA mice using a new cocktail of five mAbs to type II collagen were generated and the exact localization of the SLX-liposome encapsulated with fluorescent substances was histologically examined in hyperplastic synovial tissues of these mice. These results suggest that SLX-liposome targeting to the activated endothelial surface marker, E-selectin, may be a potentially useful vehicle for the active targeting of drug delivery to inflamed tissues, including arthritis.

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