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Prednisolone phosphate-containing TRX-20 liposomes inhibit cytokine and chemokine production in human fibroblast-like synovial cells: a novel approach to rheumatoid arthritis therapy

Takashi Harigai, Hitomi Hagiwara, Yumi Ogawa, Takanobu Ishizuka, Shinichi Kaneda and Junji Kimura

Abstract

To evaluate the potential of using prednisolone phosphate (PSLP)-containing 3,5-dipentadecyloxybenzamidine hydrochloride (TRX-20) liposomes to treat rheumatoid arthritis (RA), we examined their ability to bind human fibroblast-like synovial (HFLS) cells and their effects in these cells. To test for binding, Lissamine rhodamine B-1, 2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (rhodamine)-labelled PSLP-containing TRX-20 liposomes were added to HFLS cells, and the fluorescence intensity of the rhodamine bound to the cells was evaluated. Rhodamine-labelled PSLP-containing liposomes without TRX-20 were used as a negative control. To evaluate the uptake of liposomes by the HFLS cells, we used TRX-20 liposomes containing 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) and *p*-xylene-bis-pyridinium bromide (DPX), and observed the cells by fluorescence microscopy. The effects of the PSLP in TRX-20 liposomes on HFLS cells were assessed by the inhibition of the production of two inflammatory cytokines (interleukin 6 and granulocyte macrophage colony-stimulating factor) and one inflammatory chemokine (interleukin 8). The interaction of the PSLP-containing TRX-20 liposomes with HFLS cells was approximately 40 times greater than that of PSLP-containing liposomes without TRX-20. PSLP-containing TRX-20 liposomes bound to HFLS cells primarily via chondroitin sulfate. TRX-20 liposomes taken up by the cell were localized to acidic compartments. Furthermore, the PSLP-containing TRX-20 liposomes inhibited the production of the inflammatory cytokines and the chemokine more effectively than did the PSLP-containing liposomes without TRX-20. These results indicate that PSLP-containing TRX-20 liposomes show promise as a novel drug delivery system that could enhance the clinical use of glucocorticoids for treating RA.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by inflammation, cell proliferation and the destruction of cartilage and bone. Factors that play important roles in its pathogenesis include inflammatory cytokines and chemokines, especially tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), interleukin-8 (IL-8) and granulocyte macrophage colony-stimulating factor (GM-CSF) (Feldmann et al 1996; Kavanaugh 2002). In the inflamed joints of RA, synovial membranes become stratified and thickened because of an abnormal proliferation of synovial cells, which are considered to be the major providers of inflammatory cytokines and chemokines.

Glucocorticoids, such as prednisolone phosphate (PSLP), have long been used as the most potent anti-inflammatory drugs for treating inflammatory diseases, including RA (Almawi et al 2002; Moreland & O'Dell 2002). However, they can have both beneficial and adverse effects. Glucocorticoids control inflammation by inhibiting the production of inflammatory cytokines and chemokines, thereby suppressing the progression of rheumatic conditions. However, high doses of glucocorticoids cause some adverse systemic reactions (e.g., glucocorticoid-dependent osteoporosis, infections), therefore their dosage needs to be limited when they are used in actual treatment (Moreland & O'Dell 2002).

Studies have shown that the direct intra-articular administration of glucocorticoids elicits a local anti-inflammatory effect without causing systemic adverse reactions (Green &

Research and Development Center, Terumo Corporation, 1500 Inokuchi, Nakai-machi, Ashigarakamigun, Kanagawa 259-0151, Japan

Takashi Harigai, Hitomi Hagiwara, Yumi Ogawa, Takanobu Ishizuka, Shinichi Kaneda, Junji Kimura

Correspondence: S. Kaneda, Research and Development Center, Terumo Corporation, 1500 Inokuchi, Nakai-machi, Ashigarakamigun, Kanagawa 259-0151, Japan. E-mail: Shinichi_Kaneda@terumo.co.jp

Foong 1993; Caldwell 1996). Intra-articular administration is, however, effective only at the sites of treated joints and cannot be used frequently. Moreover, drug clearance from the joint cavities is rapid. If the action of glucocorticoids could be limited to inflammatory sites instead of the whole body, and the duration of their action at the affected parts prolonged, it is likely that their effectiveness would be enhanced with fewer adverse reactions.

Liposomes have been studied as a tool to deliver a drug to its target sites (Lasic & Martin 1995). The intra-articular administration of methotrexate-containing liposomes effectively suppressed the progression of RA compared with the administration of free methotrexate (Williams et al 2001). In addition, some reports demonstrated that a single intravenous administration of liposomes containing methotrexate or clodronate suppressed swelling in an animal model of RA (Richards et al 1999; Williams et al 1999).

We previously reported 3,5-dipentadecyloxybenzamidine hydrochloride (TRX-20) liposomes as a novel drug delivery system via the intravenous route (Harigai et al 2001). It has been demonstrated that TRX-20 liposomes selectively bind to some subendothelial cells, such as smooth muscle cells or mesangial cells, instead of the endothelial cells that compose blood vessel walls, and this binding is typically established by way of chondroitin sulfate. At inflammatory lesions, synovial cells (Kraan et al 2002), like smooth muscle cells and mesangial cells, markedly proliferate and play an important role in the inflammatory process. Here, we investigated the interaction between PSLP-containing TRX-20 liposomes and synovial cells using human fibroblast-like synovial (HFLS) cells derived from an RA patient's cells. We studied the effect of PSLP-containing TRX-20 liposomes on the function of HFLS cells by assessing their ability to inhibit the production of inflammatory cytokines and an inflammatory chemokine, compared with liposomes that did not contain TRX-20.

Materials and Methods

Materials

TRX-20 was synthesized as described previously (Harigai et al 2001). Other materials were obtained from suppliers: prednisolone phosphate (PSLP) (Diosynth bv, Netherlands); hydrogenated soy phosphatidylcholine (HSPC) (Lipoid, Germany); cholesterol (Merck, Germany); distearoylphosphatidylethanolamine-polyethylene glycol (Mr = 5000) (PEG-DSPE) (NOF Co.); Lissamine rhodamine B-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (rhodamine), 8-hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (HPTS) and *p*-xylene-bis-pyridinium bromide (DPX) (Molecular Probes, Inc., Eugene, OR); Sepharose 4 Fast Flow (Amersham Biosciences Co., Piscataway, NJ); tissue culture plastics (Falcon labware, Becton, Dickinson & Co., Franklin Lakes, NJ); fetal bovine serum (Trace Scientific Pty Ltd, Australia); antibiotic–antimycotic liquid (final concentration: 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 250 ng mL^{-1} amphotericin B) (Invitrogen Co. Netherlands). Glycosaminoglycan (GAG) lyases: chondroitinase ABC protease free (chondroitinase ABC) and heparitinase I were from Seikagaku Co. Bafilomycin A_1 was from Sigma (St Louis, MO). TNF-α was from Roche Diagnostics GmbH (Switzerland). The enzyme-linked immunosorbent assay (ELISA) kits for IL-1β and GM-CSF were purchased from Amersham Biosciences Co.; IL-6 and IL-8 were from Immunotech (France). Other reagents used in the study were of reagent grade.

Preparation of liposomes

Rhodamine-labelled PSLP-containing TRX-20 liposomes were prepared by the method described previously (Harigai et al 2001), with the following exceptions: the PSLP was dissolved in physiologic saline; and the unencapsulated PSLP was removed by chromatography with a Sepharose 4 Fast Flow column. Rhodamine-labelled PSLP-containing liposomes (without TRX-20) were composed of HSPC and cholesterol (molar ratio 56:46). HPTS/DPX-containing TRX-20 liposomes were prepared with PBS containing 2 mM HPTS and 30 mM DPX as quencher reagent of HPTS fluorescence. Unencapsulated HPTS and DPX were removed by Sepharose 4 Fast Flow column chromatography. The outer surface of all liposomes used in this study was modified with 0.75 mol% PEG-DSPE

HFLS cells and media

HFLS cells, derived from patients with RA, were purchased from Cell Applications, Inc. (donors: 38- and 50-year-old females). The cells were grown in Synoviocyte Growth Medium (Synoviocyte Basal Medium (TNF-α not included) and Synoviocyte Growth Supplement (information on composition not published, but contained 10% fetal bovine serum (FBS)) at 37°C in a humidified atmosphere (95% air, 5% CO_2), and passaged with a Subculture reagent kit (Cell Applications, Inc.). In all experiments, cells from the 3rd to 5th passage were used. Phosphate-buffered saline (PBS) tablets were from Dainippon Pharmaceutical Co. Experiments using human cells were carried out under the approval of The Terumo Corporation Research and Development Center Biohazard Committee, including ethics judgments.

Binding assay of PSLP-containing TRX-20 liposomes

Cells were seeded (1×10^4 cells/cm^2) into 6-well plates. After 2 days, the medium was changed to fresh Synoviocyte Growth Medium (2 mL/well). The cells were then incubated with rhodamine-labelled PSLP-containing liposomes with or without TRX-20 (250 nmol total lipid/well) at 37°C for 24 h. After the incubation, the cells were washed twice with PBS and lysed by adding RIPA buffer. Each lysate was transferred into an Opaque White 96-well Tissue Culture Plate, and the fluorescence was measured using a Fluoroskan II (Labsystems Oy, Finland).

Treatment of cells with GAG lyases

Cells grown to subconfluence in 6-well plates were rinsed twice with PBS, and then incubated in Hank's Balanced Salt Solution (HBSS) containing 50 mM CH_3COONa, 3% bovine

serum albumin, and 20 mU mL^{-1} chondroitinase ABC or heparitinase I at 37°C for 60 min. After enzymatic digestion of the surface GAG, the cells were incubated with rhodamine-labelled PSLP-containing TRX-20 liposomes in Synoviocyte Growth Medium, shaken gently 10 times, and the fluorescence intensity of the rhodamine was measured as described above. The total lipid of the liposomes was calculated from the fluorescence intensity and was indicated as a ratio to the total cell protein.

Time course of uptake of HPTS/DPX-containing TRX-20 liposomes

Cells were incubated with HPTS/DPX-containing TRX-20 liposomes (250 nmol total lipid/dish) in Synoviocyte Growth Medium (2 mL/dish) at 37°C for 4 h and washed twice with PBS, and then fresh Synoviocyte Growth Medium was added. A fluorescence image was obtained using a Carl Zeiss Axiovert 200M microscope and an AxioCam CCD camera operated under the control of AxioVision version 3.0 software (Carl Zeiss Jena GmbH, Germany). Two fluorescence microscopy filter sets were used to observe the fluorescence of distinct HPTS states at two different excitation wavelengths. The filter set (No. 6) was a band-pass excitation filter (436/10 nm), a beam splitter (460 nm) and a long-pass emission filter (470 nm). The other filter set was a band-pass excitation filter (365/12 nm) from filter set No. 1, a beam splitter and the long-pass emission filter from filter set No. 6. The final image was processed for colour tone correction using the same conditions as the image for comparison, using Photoshop version 7.0 (Adobe systems Inc., San Jose, CA).

Bafilomycin A_1 treatment

Cells were incubated with HPTS/DPX-containing TRX-20 liposomes as described above. After 48–50 h, cells were incubated with 100 nM bafilomycin A_1 in dimethyl sulfoxide (DMSO) at 37°C for 60 min. Fluorescence images were then obtained as described above.

Effect of PSLP-containing TRX-20 liposomes

Cells were cultured in 12-well plates with incubation medium (Synoviocyte Basal Medium (TNF-α not included) containing 10% inactivated FBS and antibiotic–antimycotic liquid) at 37°C for 48 h. The culture medium was then changed to incubation medium containing TNF-α (1 ng mL^{-1}) and the cells were incubated with PSLP-containing liposomes with or without TRX-20 (1 μM PSLP concentration). After 48 h, the incubation medium was collected and subjected to ELISAs using IL-1β, IL-6, IL-8 and GM-CSF determination kits.

Statistical analysis

Log conversion of the measured values was carried out, because the cytokine and chemokine concentrations changed exponentially after inhibition. To check the experimental system, Student's *t*-test was performed between PSLP-non-added (Non-PSLP) and the TNF-α non-stimulated control and between Non-PSLP and PSLP, and the Tukey test was carried out among Non-PSLP, PSLP-containing TRX-20 liposomes and PSLP-containing liposomes without TRX-20.

The statistical analysis software was SAS System release 6.12 (SAS Institute Inc., Cary, NC), used via EXSAS version 5.10 (Arm Co., Osaka).

Results

Binding properties of PSLP-containing TRX-20 liposomes

The interaction of PSLP-containing liposomes, with or without TRX-20, with cultured HFLS cells was determined as the total binding efficiency (sum of cell binding and intracellular uptake). TRX-20 clearly enhanced the interaction of liposomes with HFLS cells. The total efficiency of binding of PSLP-containing liposomes to HFLS cells was represented as total lipid (nmol) from the liposomes, calculated from the fluorescence intensity of rhodamine, per total mg protein. The total binding efficiency of PSLP-containing TRX-20 liposomes was 1235 nmol lipid/mg protein, while that of PSLP-containing liposomes without TRX-20 was 31 nmol lipid/mg protein (n = 4). The effect of pretreatment with GAG lyases on the binding of PSLP-containing TRX-20 liposomes to HFLS cells was also investigated. The total binding efficiency of PSLP-containing TRX-20 liposomes was decreased to 35% compared with control, in which the enzyme was not processed by digestion with chondroitinase ABC (n = 3). On the other hand, 98% total binding efficiency remained with digestion of heparitinase I.

Time course of uptake

Uptake of HPTS/DPX-containing TRX-20 liposomes by HFLS cells was observed by fluorescence microscopy. In this study, we detected HPTS fluorescence by excitation with a 436/10 or a 365/12 filter. The localization of liposomes was evaluated using the pH-dependent spectral properties of HPTS, a fluorescence indicator for pH measurements, as the probe.

HFLS cells were incubated with HPTS/DPX-containing TRX-20 liposomes for 4 h. The cells were washed and fresh medium was added. Figure 1 shows the binding and uptake properties of the HPTS/DPX-containing TRX-20 liposomes. When the HPTS/DPX-containing TRX-20 liposomes were in Synoviocyte Growth Medium, the signals of a neutral pH were detected when filter 436/10 was used for excitation, and when the liposomes were in a low-pH environment, the signals were detected using the 365/12 filter for excitation.

After 4 h of incubation, the signals of a neutral pH were detected on the cell surface, but the signals of a low pH were weak, indicating that the liposomes were at a neutral pH. After 24 h of incubation, almost no signals of a neutral pH were detected. In contrast, the strength of the signals of a low pH had increased, indicating that liposomes were in a low-pH environment. After 48 h of incubation, strong signals of a low pH were detected.

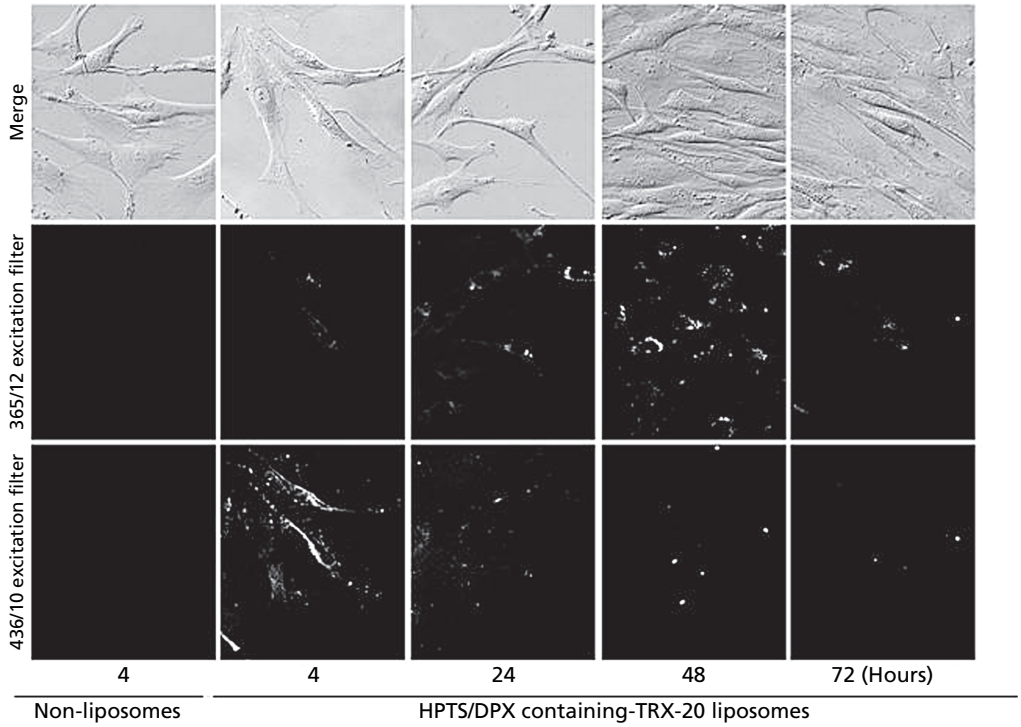

Figure 1 Time course of uptake of HPTS/DPX-containing TRX-20 liposomes by HFSL cells. Fluorescence micrographs of living HFLS cells (passage 3) incubated with HPTX/DPX-containing TRX-20 liposomes (total lipids 250 nmol). Cells were incubated with the liposomes for 4 h, washed twice with PBS, then cultured for the incubation period indicated.

Intracellular fate of HPTS/DPX-containing TRX-20 liposomes

Observation of the pH-dependent spectral properties of HPTS suggested that the TRX-20 liposomes were probably present in intracellular acidic organelles. To demonstrate cytoplasmic localization after the uptake of TRX-20 liposomes, HFLS cells were treated with bafilomycin A_1, a known inhibitor of endosomal and lysosomal acidification. We reasoned that if HPTS/DPX-containing TRX-20 liposomes were localized to acidic compartments, the appropriate excitation wavelength of HPTS should change after treatment with bafilomycin A_1. HFLS cells were treated with 100 nM bafilomycin A_1 at 37°C for 1 h after incubation with HPTS/DPX-containing TRX-20 liposomes for 48–50 h. The fluorescence signals were changed by treatment with bafilomycin A_1. The signals of a low pH elicited with the 365/12 filter decreased, whereas the signals of a neutral pH elicited with the 436/10 filter was increased (data not shown). These changes suggested that HPTS/DPX-containing TRX-20 liposomes were in acidic compartments.

Effect of PSLP-containing TRX-20 liposomes

In this experiment, the cells were stimulated by TNF-α to induce the production of two cytokines (IL-6 and GM-CSF) and a chemokine (IL-8). Free PSLP inhibited the production of IL-6, GM-CSF and IL-8. In this experimental system, PSLP-containing TRX-20 liposomes inhibited the production of IL-6, GM-CSF and IL-8 more markedly than did PSLP-containing liposomes without TRX-20 (Figure 2). IL-1β was not detected under the conditions of this experiment (data not shown).

Discussion

In our previous study (Harigai et al 2001), we reported that TRX-20 liposomes might serve as a novel drug delivery system by establishing that they specifically bound to certain cell types. Here, we report on the characteristics and effectiveness of PSLP-containing TRX-20 liposomes as a step towards assessing their usefulness in the treatment of RA with glucocorticoids.

One of the important clinical issues with glucocorticoid treatment is that the dose is limited by their adverse effects, including glucocorticoid-dependent osteoporosis (Moreland & O'Dell 2002). This is because glucocorticoids have a broad action spectrum and can extend their actions to cells that are not their primary targets (Newton 2000; Almawi et al 2002). In this respect, molecular targeting therapy using anti-TNF-α or anti-IL-6 receptor antibodies is a very sophisticated modality because it restricts the target molecules (Choy et al 2002; Maini & Feldmann 2002). However, such therapy alone cannot control the progression of RA completely, because the disease involves very complicated processes that progress as various cells and biomolecules become involved and affect

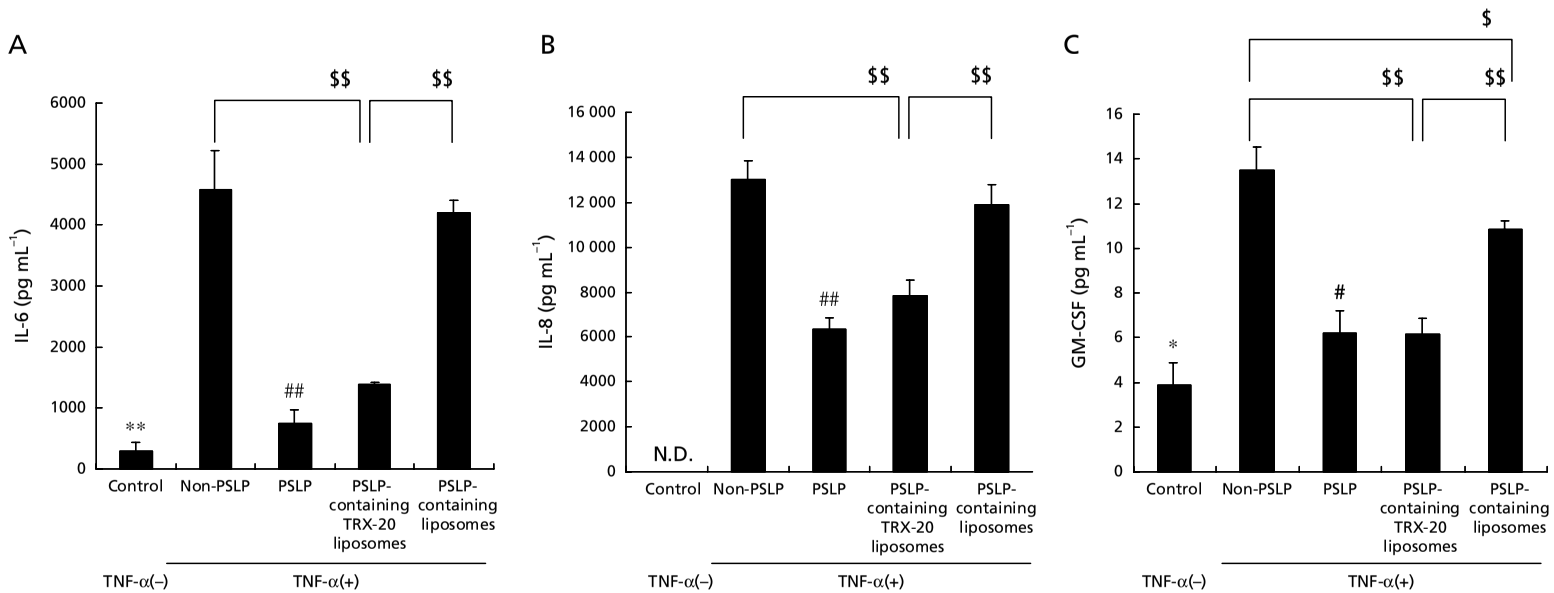

Figure 2 Effect of rhodamine-labelled PSLP-containing TRX-20 liposomes on the production of two cytokines and a chemokine. The incubation medium of TNF-α-stimulated HFLS cells was collected after a 48-h treatment with PSLP or PSLP-containing liposomes with or without TRX-20 (1000 nM PSLP concentration). HFLS cells were of the third passage. A. IL-6 protein concentration. B. IL-8 protein concentration. C. GM-CSF protein concentration as assayed by ELISA. *$P < 0.01$, **$P < 0.001$, Non-PSLP vs TNF-α non-stimulated control; #$P < 0.01$, ##$P < 0.001$, PSLP vs control (Student's *t*-test). \$$P < 0.05$, \$\$$P < 0.001$, Non-PSLP, PSLP-containing TRX-20 liposomes and PSLP-containing liposomes without TRX-20 (Tukey test). N.D., not detected.

one another (Kavanaugh 2002). On the other hand, if glucocorticoids with strong and extensive actions could be delivered to target cells specifically, it might be possible to obtain better therapeutic effects than with free glucocorticoid, while suppressing adverse reactions (Green & Foong 1993). Therefore, we studied the effect of PSLP-containing TRX-20 liposomes on HFLS cells, which have a major role in the pathogenesis of RA.

The interaction of HFLS cells with PSLP-containing TRX-20 liposomes was approximately 40 times higher than that with PSLP-containing liposomes without TRX-20. The interaction of PSLP-containing TRX-20 liposomes with HFLS cells occurred mainly via the chondroitin sulfate that is present on the cell surface and in the extracellular matrix. In addition, the HFLS cells took up HPTS/DPX-containing TRX-20 liposomes, and increasing uptake was shown with time for 48 h, (Figure 1) (Daleke et al 1990; Straubinger et al 1990; Van Bambeke et al 2000). Moreover, after cellular uptake, the HPTS/DPX-containing TRX-20 liposomes were shown to be present in a low-pH cellular compartment, which was confirmed to be an acidic compartment by bafilomycin A_1 treatment (Yoshimori et al 1991; Brisson et al 1999; Forgac 1999). These findings indicated that PSLP-containing TRX-20 liposomes interact more effectively with the chondroitin sulfate on their target cells than do the PSLP-containing liposomes that do not contain TRX-20 and that the liposomes were taken up by cells where they were translocated to acidic compartments.

The progression of joint inflammation and joint destruction in RA is dependent on the activation of a network consisting of inflammatory cytokines and chemokines (Feldmann et al 1996). In this study, PSLP-containing TRX-20 liposomes suppressed the production of two cytokines and one chemokine by HFLS cells when they were stimulated with TNF-α; this is thought to represent the uppermost stream of the cytokine network of RA (Feldmann et al 1996). PSLP-containing TRX-20 liposomes suppressed the production of inflammatory cytokines and a chemokine of synovial cells to a level comparable with that of free PSLP, and worked more effectively than PSLP-containing liposomes without TRX-20 (Figure 2). In this experiment, PSLP dissociated to prednisolone and phosphoric acid in culture medium. In this condition, prednisolone can pass easily through the cell membrane and exert its effect at low doses, because of its hydrophobicity. However, when prednisolone is administered to the whole body, it is difficult to maintain effective concentrations of it in inflammatory regions without adverse effects. To improve this situation, we designed the lipid composition of the liposomes to avoid leakage of PSLP and deliver it to the target region. The most important feature of TRX-20 liposomes is their increase in binding affinity to target cells. We expect the liposomes to accumulate in the target tissue with intravenous administration without rapid leakage of PSLP and to maintain effective concentrations of PSL in target tissues for a long period of time. On the other hand, in in-vitro studies, delay of release of PSLP from liposomes resulted in weaker effects than observed with free PSLP.

Synovial cells produce and secrete a variety of inflammatory cytokines and chemokines and thus contribute to the chemotaxis, proliferation and maintenance of leucocytes (Miyazawa et al 1998). They also affect the secretion of proteinases that are related to bone destruction, and the differentiation and proliferation of osteoclasts (Takayanagi et al 1997; Miyazawa et al 1998). In this study, we demonstrated that PSLP-containing TRX-20 liposomes could suppress the inflammation-activating effects of synovial cells by inhibiting the production of representative cytokines and a chemokine. We therefore suggest that, because of its ability to target synovial cells, this may be a highly useful method for treating RA, given the cascade of progression of this disease. We have studied the effects of PSLP-containing TRX-20 liposomes in animal models of nephritis (Liao et al 2001). We are also studying the accumulation of liposomes in synovial tissue and effects on experimental arthritis in rats and will clarify the potential for treatment of RA in the near future.

Conclusions

PSLP-containing TRX-20 liposomes bound to HFLS cells primarily via chondroitin sulfate. By this mechanism, they could suppress the production of inflammatory cytokines and chemokines more markedly (with an effect comparable with that of free PSLP) than could PSLP-containing liposomes without TRX-20.

In actual clinical practice, it is necessary to consider carefully the balance between efficacy and the adverse reactions of PSLP, to maintain its effective concentrations at the lesion, and it is often difficult to administer the needed dose. However, PSLP-containing TRX-20 liposomes, like the drug delivery system using PEG-modified liposomes, are likely to have the Enhanced Permeation and Retention (EPR) effect, which should improve the dosage issue (Lasic & Martin 1995; Harigai et al 2001). The liposomes keep the drug concentration in the blood and tissue within its effective range by accumulating at the lesion, without causing the adverse reactions associated with oral prednisolone preparations. In addition, it is conceivable that, with the action of TRX-20, the liposomes can effectively deliver the PSLP into the synovial cells, which play an important role in the pathogenesis of RA, thereby securing effective concentrations within synovial cells more easily than do PSLP-containing liposomes without TRX-20. Although our data were obtained from in-vitro experiments, they strongly support the idea that PSLP-containing TRX-20 liposomes will be a novel treatment modality to enhance the usefulness of glucocorticoids in the treatment of RA.

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