LIPOSOMAL TARGETING OF GLUCOCORTICOIDS TO SYNOVIAL LINING CELLS STRONGLY INCREASES THERAPEUTIC BENEFIT IN COLLAGEN TYPE II ARTHRITIS

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ABSTRACT

Objective. To investigate the effect of a single intravenous treatment with glucocorticoids encapsulated in long-circulating PEG-liposomes on both joint inflammation as well as cartilage destruction and to investigate the phenomenon of selective homing of these liposomes in the inflamed synovium.

Methods. Mice with collagen type II – induced arthritis (CIA) were i.v. treated with liposomal and free prednisolone phosphate (PLP) a few days after the first signs of the disease. Paw inflammation was scored during one week post-treatment, after which sections of the knee joints were prepared for assessment of cartilage damage. In addition, arthritic mice were treated with colloidal gold-containing liposomes. At 24 hours post-injection knee joint sections were prepared in which the location of liposomes was visualized using silver enhancement.

Results. Treatment of CIA with 10 mg/kg liposomal PLP resulted in a complete resolution of paw inflammation. The effect was visible until 1 week post-treatment. 10 mg/kg free PLP could only become slightly effective after repeated daily injections. Although the paw-inflammation recurred at 1 week after treatment with liposomal PLP, knee joint sections prepared at this time point indicated that the cartilage damage was still reduced. Localization of gold-labeled liposomes in the inflamed joints was seen in the proximity of blood vessels, in the cellular infiltrate, but mainly in the synovial lining, which is crucial in the arthritic process. Unaffected joints did not take up liposomes.

Conclusions. This study shows that by using the property of LCL to target the synovial lining selectively in inflamed joints, the anti-inflammatory activity glucocorticoids can be greatly increased, showing also a beneficial effect at the level of cartilage destruction.
INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disorder, characterized by joint inflammation and cartilage destruction (1,2). An important role in the pathogenesis of joint inflammation is ascribed to the synovial lining layer that surrounds the connective tissue in the joints. Normally, the synovial lining consists of a few cell layers of mainly fibroblast-like and macrophage-like synoviocytes. In RA however, the synovial lining layer expands as a result of newly arrived macrophages from the periphery (3,4). Macrophages in the synovial lining of arthritic joints have been shown to produce many pro-inflammatory cytokines, attract new inflammatory cells and produce enzymes that can damage the cartilage (3,5,6).

Liposomes were shown to be valuable for targeting macrophages as these cells efficiently phagocytose them upon exposure (7-9). Intra-articular administration of a liposomal form of pro-apoptotic clodronate resulted in selective depletion of the phagocytic lining cells, which resulted in a marked decrease of joint inflammation (10,11). To realize macrophage depletion at inflamed synovia via the systemic route, Camilleri et al. and Richards et al. successfully employed small-sized liposomes that localized in inflamed joints after i.v. injection (12,13,14). However, as the majority of i.v. injected liposomes are generally taken up by the mononuclear phagocyte system (MPS) in liver and spleen, systemic treatment with liposomal clodronate may result in unintended elimination of hepatosplenic phagocytes, which play an important role in the immune defense of the body (15,14).

Effective suppression without elimination of synovial macrophages can be accomplished with glucocorticoids (GC). GC can strongly reduce the generation and release of pro-inflammatory cytokines and cartilage-degrading enzymes by macrophages in arthritic joints (16,17,18). Besides activated macrophages, also the pro-inflammatory activity of fibroblasts, lymphocytes and endothelial cells is suppressed, and this may explain the striking anti-arthritic activity of GC. However, serious adverse effects limit systemic use of glucocorticoids in arthritis patients (19,20). Moreover, high and frequent dosing is necessary to achieve sufficient activity in the joints, since target localization is usually poor as a result of efficient clearance (21).

For efficient delivery of GC into inflamed joints via systemic treatment, the use of small-sized liposomes coated with poly(ethylene glycol) (PEG) may be advantageous. PEG has been shown to be very effective in reducing recognition and rapid removal of liposomes from the circulation by the MPS, enabling liposomes to stay in the circulation for a prolonged period of time (22-24). The long-circulation property provides the liposomes the opportunity to substantially extravasate and accumulate in inflamed tissue (25). A study in arthritis patients treated with $^{99m}$Tc-labeled PEG-liposomes indicated that PEG-liposomes can remain in the circulation with a half-life as long as 50 hours and that they selectively extravasate in inflamed joints (26).

The objective of this study was to evaluate the capacity of long-circulating PEG-liposomes to target the inflamed synovium and to expose phagocytic cells in the lining to
encapsulated GC. In the murine model of type II collagen-induced arthritis (CIA) the effect of liposomal GC on paw inflammation was investigated and compared with the effect of unencapsulated (‘free’) GC. Knee sections were prepared to evaluate the effect of liposomal GC on cartilage damage. Gold-labeled liposomes were used to determine the exact location of uptake in the inflamed tissue and to evaluate whether liposome localization is indeed inflammation-driven.

Our results indicate that selective targeting of the inflamed synovial lining can be realized with long-circulating PEG-liposomes. Encapsulation of GC in these liposomes results in a dramatic increase of the anti-inflammatory activity of GC in the inflamed synovium. Besides a complete resolution of paw inflammation, a significant reduction of cartilage damage still visible at 1 week post-treatment was achieved. The approach of targeted delivery to the inflamed synovium mediated by PEG-liposomes may strongly improve the value of GC in the treatment of arthritis.
METHODS

GC-containing PEG-liposomes
Liposomes were prepared by the film-extrusion method (27). Briefly a lipid solution was prepared in ethanol, containing dipalmitoyl phosphatidylcholine (DPPC) (Lipoid GmbH, Ludwigshafen, Germany), PEG 2000–distearoyl phosphatidylethanolamine (DSPE) and cholesterol (Sigma Chemical Co., Poole, UK) in a molar ratio of 1.85:0.15:1.0. The lipid solution was transferred to a round-bottom flask and a lipid film was created by rotary evaporation. The film was hydrated with a solution of 100 mg/ml prednisolone phosphate (PLP) in sterile water. The resulting lipid dispersion was sized by multiple extrusion through polycarbonate filter membranes. Unencapsulated prednisolone was removed by dialysis against 0.9% phosphate buffered saline using Slide-A-Lyzer dialysis cassettes with a molecular weight cut-off of 10,000 (Pierce, Rockford, IL, USA). Mean particle size was determined by dynamic light scattering with a Malvern 4700 system (Malvern Ltd., Malvern, UK). The liposomes were sized to a diameter between 90 and 100 nm. Phospholipid content was determined with a phosphate assay (28) in the organic phase after extraction liposomal preparations with chloroform. The aqueous phase after extraction was used for determining the PLP content. With high performance liquid chromatography using a mobile phase of acetonitril-water with pH of 2, connected to an UV-detector, which was set at 254 nm, both prednisolone and its phosphate ester could be measured in one single run. Each ml liposomal preparation contained around 4.5 mg PLP and an average of 60 µmol phospholipid.

Gold-labeled PEG-liposomes
Colloidal gold containing PEG-liposomes were prepared as described by Huang et al. with some modifications (29). The method of preparation is similar to the method described above for the preparation of PLP-PEG-liposomes except for the hydration step, which was performed with a freshly prepared tetrachloroaurate solution in citrate buffer. Sizing was performed at 4 °C as described above. Immediately after extrusion, colloidal gold was formed by incubation of the dispersion at 37 °C. To remove non-encapsulated gold, the preparation was eluted on a Sephacryl S1000-SF column (Pharmacia, Uppsala, Sweden).

Collagen-induced arthritis
The Dutch Committee of Animal Experiments approved all animal studies. Male DBA/1lacJ mice between 10 and 12 weeks of age (20-25 g) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). To induce arthritis, 100 mg of bovine type II collagen dispersed in complete Freund’s adjuvant containing 2 mg/ml of heat-inactivated Mycobacterium Tuberculosis (both purchased from DIFCO laboratories, Detroit, MI, USA) was injected subcutaneously at the base of the tail. At day 21 a booster injection of 100 mg of bovine type II collagen was given intraperitoneally. At day 24 post-induction, the first signs of joint
inflammation became visible. From that day on, the mice were regularly examined for the
visual signs of inflammation. The severity of the joint inflammation was blindly graded by
assigning a score to each paw from 0 to 2, based on erythema, swelling and deformation of
the joints. The sum of the grades for each animal is the clinical score and varies from 0-8 (30).

Therapeutic efficacy
All mice were treated on day 28 post-induction, when the average score of all mice in the
experiment is about half the maximal scores. At day of treatment, groups of 6-7 mice were
formed with equal average clinical scores. 10 mg/kg PLP both encapsulated in PEG-
ilosomes and in unencapsulated form was given as a standard dose. This dose was based
on clinical experience with so-called ‘pulse’ treatment of arthritis patients involving single or
repeated injections of 1 gram methylprednisolone (31,32). All preparations were given
intravenously in the tail vein. Regarding multiple injections of free glucocorticoid, each
following day treatment was repeated at the same time during 5 days. The effect of
treatment on clinical scores and body weight was monitored 1 week post-treatment.

Histology
At 1 week post-treatment knee joints were dissected and fixed in 4% formaldehyde in
phosphate-buffered saline (PBS). In addition the joints were decalcified with 5% formic acid
in PBS during 7 days. After dehydration and embedment in paraffin, sections of the knee
joint were cut that included patella, femur, menisci and tibia. These sections were mounted
on gelatin-coated microscopic slides, stained with hematoxylin and eosin and examined
using a light microscope (Leica, DMR, Germany). As a measure of inflammation, infiltrate
and exudates were scored by two blind observers. Infiltrate is defined as the influx of
leukocytes in the synovium. Exudate is defined as the influx of leukocytes in the joint cavity.
The scores varied between 0 and 3, 0 being no cellular influx or exudate whereas 3 indicates
maximal cellular influx or exudate. Besides inflammation also cartilage matrix erosion was
scored using the same scale, 0 being no cartilage erosion whereas 3 indicates maximal
cartilage erosion found at that time-point. Both the tibia and the femur were evaluated.

Visualization of gold-liposomes
A separate group of mice was treated with colloidal gold-containing PEG-liposomes to
visualize the exact location of the liposomes in the synovium. 24 hours after treatment knee
joints were dissected and decalcified in EDTA/PVP (polyvinylpyrrolidone) in TRIS buffer
during 2 weeks. After freezing in liquid nitrogen, sections were cut in a cryostat (Microm,
HM500M, Walldorf, Germany). These sections were mounted on Superfrost microscopic
slides (Menzel Gläser, Germany). Silver enhancement of colloidal gold was performed with
Sigma silver enhancer kit (Sigma, St. Louis, MO, USA) and terminated by incubating with a
0.5% sodium thiosulphate solution in distilled water. The sections were then stained with
hematoxylin and eosin and examined using light microscopy.
Statistical analysis
For statistically assessing and comparing therapeutic efficacy in different groups the nonparametric Wilcoxon/Kruskal-Wallis test (rank sums) was used. For evaluating differences between groups regarding infiltrate and exudate, one-way analysis of variance was used. For statistical evaluation of the effect on cartilage damage Fisher’s test was used. P values of less than 0.05 were considered significant.
RESULTS

Therapeutic activity

Figure 1 A shows the anti-inflammatory activity of free and liposomal PLP at a dose of 10 mg/kg in the murine CIA model. A single dose of free PLP did not result in a significant effect on paw inflammation scores. However, the same dose encapsulated in PEG-liposomes resulted in a complete disappearance of paw inflammation at 5 days post-treatment. In Figure 1 B the effect of repeated daily injections of 10 mg/kg free PLP is shown in comparison to 10 mg/kg and 1 mg/kg liposomal PLP. Surprisingly, also repeated administration with 10 mg/kg PLP had only limited effect. The effect is significant compared to the control mice but not significantly different from the effect of a single injection of a ten-fold lower dose of liposomal PLP.

Figure 1. Paw inflammation scores after single treatment with 10 mg/kg PLP-PEG-liposomes (♦), 1 mg/kg PLP-PEG-liposomes (◊), 10 mg/kg unencapsulated PLP (∆), compared to both empty PEG-liposomes (■) and saline (●) as controls. In contrast to liposomal PLP, a single treatment with 10 mg/kg unencapsulated PLP had no significant effect (A). Multiple treatment with 5 daily injections of 10 mg/kg unencapsulated PLP (▲) had significant effect at day 32 and 35 but the effect was not better than 1 mg/kg PLP-PEG-liposomes (B). Each point represents the mean of 7 mice ± SEM. Arrows indicate treatment.

Histological evaluation of inflammation and cartilage damage

Figure 1 shows that 1 week post-treatment paw inflammation scores are recurring. However, the knee sections prepared at this time point still show a profound therapeutic effect of liposomal PLP on the integrity of the cartilage layers (Figure 2). In most of the tissue sections taken from control mice the cartilage appeared highly damaged whereas in the majority of the treated mice loss of cartilage was hardly visible. Figure 3 reveals only little difference regarding infiltrate and exudate as parameters for inflammation. These results correspond with the recurrence of the inflammation score as shown in Figure 1. Scoring of
the cartilage loss, however, revealed a significant beneficial effect in the treated group (Fishers’s test, p < 0.05). In 4 of the 7 mice treated with liposomal PLP no cartilage degradation was detected whereas in the control group all mice showed profound loss of cartilage.

Figure 2. Effect of liposomal PLP on cartilage loss 1 week post-treatment. (A) Knee joint section after treatment with saline and (B) The same knee joint after treatment with 10 mg/kg PLP-PEG-liposomes. Original magnification x 200. T, tibia; F, femur, JS, joint space; C, cartilage layer.

Figure 3. Histological evaluation of the effect on exudate and infiltrate as measures for inflammation and on cartilage erosion of 10 mg/kg liposomal PLP at 1 week post-treatment. Data indicate the mean of 7 mice ± SEM.

Localization of PEG-liposomes in the inflamed synovium
The localization of colloidal gold containing PEG-liposomes in the knee sections of CIA-mice was visualized by silver enhancement and shown in Figure 4. Microscopic evaluation revealed that most of the liposomes accumulated in the region of the synovial lining. The majority of visualized gold appeared to be cell-associated. In areas around certain blood vessels the density of liposomes appeared to be relatively high. Despite the severity of the arthritis model, some mice had knee joints without inflammation. Interestingly, hardly any liposomes were visible in joints that did not show signs of inflammation (Figure 5). Clearly, the presence of an inflammatory process is a key factor for the liposomes in order to be able to localize in the synovium.
Figure 4. Visualization of gold-labeled PEG-liposomes in the inflamed knee joint. (A) Original magnification X 50. (B) A magnification of X 100 of an area surrounding the synovial lining. Insert: magnification of X 200 of an area surrounding blood vessels. Gold particles are visible as black dots. Note that the liposomal gold is mainly localized in the synovial lining and some around blood vessels. Relatively few gold is visible in the cellular infiltrate. P, patella; F, femur; JS, joint space; CI, cellular infiltrate; SL, synovial lining; V, vessels.

Figure 5. Visualization of gold-labeled PEG-liposomes in an unaffected knee joint. Original magnification X 50. Note the absence of cellular infiltrate and gold-liposomes the synovial lining. P, patella; F, femur; JS, joint space; SL, synovial lining.

Figure 6. Spleen localization of gold-labeled PEG liposomes. (A) Original magnification X 100. (B) A section of the white pulp surrounded by gold-labeled macrophages that reside in the red pulp. WP, white pulp; RP, red pulp.
Localization of PEG-liposomes in the spleen

Silver enhancement of tissue sections from the spleen shows that almost all liposomes that were taken up by the spleen localized in the marginal zone and the red pulp. Visualized gold appeared to be largely cell-associated. Hardly any gold was detected in the white pulp (Figure 6).
DISCUSSION

In the previous chapter we reported that encapsulation of PLP in long-circulating PEG-liposomes resulted in a strong increase of the anti-inflammatory activity of PLP as compared to the free drug. A single i.v. injection of liposomal PLP could induce a complete resolution of rat adjuvant arthritis. Mechanistic studies with PLP encapsulated in different types of liposomes revealed that the observed increased therapeutic efficacy was a result of the specific property of small long-circulating PEG-liposomes to selectively home to inflamed paws. The present study focuses on the anti-arthritic effect of PLP-PEG-liposomes in murine CIA, which is an experimental arthritis model with a different etiology. This model also allowed a more in-depth investigation of the anti-arthritic effect by histological evaluation of inflamed joints. Furthermore, it became possible to visualize the exact location of the PEG-liposomes inside the inflamed synovium after extravasation from the vasculature.

The data in this study confirm the profound anti-inflammatory activity of PLP-PEG-liposomes. The reduction of paw inflammation scores after a single injection of 10 mg/kg was rapid and complete resolution of arthritis was achieved at day 5 post-treatment. The therapeutic effect lasted for more than a week. 10 mg/kg unencapsulated PLP was only effective after repeated injection but this strategy, which was based on the clinical ‘pulse’ treatment regimen employed in RA patients, was not more beneficial than a single injection of 1 mg/kg liposomal PLP. Although after 1 week post-treatment joint inflammation clearly recurred, cartilage erosion was still reduced in mice treated with PLP-PEG-liposomes. Apparently, treatment with liposomal PLP results in a profound delay of the cartilage erosion process. This finding may be of importance as treatment of RA with i.v. ‘pulse’ GC is often criticized for the lack of ability to induce a delay in the progression of joint erosion (33,34).

Knee joint histology performed after injection of gold-labeled PEG-liposomes revealed that the liposomes mainly localized in the synovial lining. Higher magnifications showed that almost all gold particles were cell-associated. The fact that a clear distinction was observed between cells that are positive for gold staining and cells without intracellular gold, strongly suggests that PEG-liposomes selectively localized in cells with phagocytic capacity after extravasation in the inflamed synovium. As it was shown before that gold-labeled PEG-liposomes are stable in vivo, it is assumed that all intracellular gold originated from phagocytosed liposomes (29). The affinity of liposomes for phagocytes was also clearly visible in the spleen, in which the phagocyte-rich red pulp showed strong liposome uptake whereas the T cell-rich white pulp showed no sign of liposome uptake at all. As the PEG-coating was used for its capacity to shield liposomes from recognition by MPS phagocytes, the extensive uptake by tissue phagocytes was somewhat unexpected. However, other researchers also report on similar observations with PEG-liposomes targeted to sites of bacterial infection (35,36).

Besides the synovial lining also some gold staining was visible in the infiltrate and around the blood vessels, which brings up two possible explanations for the observed localization of liposomes in the inflamed synovium. First, the gold found in the inflamed
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synovium is associated with infiltrating monocytes/macrophages that phagocytosed liposomes in the periphery or second, gold around some blood vessels are liposomes extravasating from the circulation without the help of leukocytes. Schifferlers et al. addressed this issue and found support for the second explanation (35). Also, the fact that Camilleri et al. and Richards et al. report their small clodronate containing liposomes to eliminate synoviocytes despite the depletion of peripheral phagocytes, points in the direction of a phagocyte-independent localization mechanism (12,13,14).

As the onset of CIA varies, some knees of the CIA mice did not yet show signs of inflammation at the day of treatment. Tissue sections from these knee joints did not reveal any sign of liposome localization. Clearly, PEG-liposomes show selectivity for inflamed synovia, which further supports the suggested role of inflammation-enhanced vascular permeability as an essential phenomenon for liposome localization.

The affinity of PEG-liposomes for macrophages in inflamed synovia makes them highly attractive carriers for GC. Macrophages play an important role in the onset and progression of arthritis, as they produce the pro-inflammatory cytokines TNF-α, IL-1, IL-6, generate chemokines, tissue-degrading enzymes and play a role in the presentation of auto-antigens to T cells (5,6). Selective depletion of activated macrophages from the inflamed joint via intra-articular injection of liposomal clodronate was indeed shown to be a promising treatment approach (10,11). Depletion of macrophages via the systemic route appeared to be less attractive, since elimination of macrophages from liver and spleen was shown to strongly affect MPS-functioning (15). GC may be more interesting to encapsulate in liposomes for a systemic treatment approach as GC can effectively downregulate the pro-inflammatory effector production without eliminating the macrophages.

The small PEG-liposomes used in this study offer the advantage of reduced uptake by the MPS, along with a prolonged circulation property resulting in enhanced accumulation at inflamed sites (22-26). The extensive phagocytosis of PEG-liposomes we observed within the inflamed tissue will inevitably result in exposure of the macrophages to high intracellular concentrations of GC, which may be one plausible explanation for the strong anti-inflammatory effect. However, as GC can easily pass cellular membranes, the drug may escape from the intracellular compartment, suppressing other inflammatory cells in the synovium as well. Besides their role as target cell in arthritis, macrophages may therefore also play a crucial role in the release of GC from liposomes and the generation of relatively high and prolonged concentrations of active drug in the synovium. Previously we showed that the liposomal formulation used in our studies was highly stable in vivo and that leakage of GC from the liposomes without the involvement of an external trigger is not likely. The suggested need for activated macrophages to release GC from liposomes may add an additional form of target selectivity to the liposomes besides selective accumulation at inflames sites. Further studies are however necessary to address the uptake and intracellular processing of liposomal GC by macrophages.
Our study indicates that the observed property of PEG-liposomes to selectively target the inflamed synovial lining can strongly enhance the beneficial effects of GC in arthritis. The observed affinity of PEG-liposomes for macrophages may lead to high concentrations in inflammatory cells at arthritic joints combined with less exposure of healthy non-target tissues to GC.
REFERENCES