Development of heparin nanoparticles: synthesis, physicochemical/biochemical characterization and application to arthritis therapy

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Preface

Rheumatoid arthritis is the long-lasting chronic inflammatory disease that affects about 1% of global population. It leads to irreversible joint damage and systemic disorders, and decrease of quality of life of patients worldwide. Although recent treatment targeting and inhibition of pro-inflammatory cytokines with biologic drugs is effective as a short-term treatment but has limitation including the suppression of the whole immune system and increased infection risk. Thus, novel specific anti-inflammatory therapy is necessary to increase therapeutic efficiency and minimize the side effects.

It was proposed to develop delivery systems to selectively target Toll-like receptor 4 (TLR4) as a source of inflammation in arthritic synovium. Augmented number of immune cells overexpressing TLR4 in the joints of arthritic patients suggest that antagonism of the receptor of activated immune cells may result in inhibition of inflammation through the suppression of persistent cytokine production. Heparin has attracted much attention as a biomaterial because of low toxicity and high biocompatibility, to develop amphiphilic nanoparticles as promising drug carriers for various drugs, genes and imaging agents. In addition to this, its anti-inflammatory properties can be enhanced towards the particulate carrier systems. There is limited knowledge about the mechanisms of activity and application of heparin-based nanoparticles as anti-inflammatory agents. In this thesis, therefore, novel heparin-lipid nanoparticles for selective TLR4 targeting were first developed. Then, mechanism and structure-activity relationship of anti-inflammatory effect was investigated. Finally, therapeutic effect of these nanoparticles was evaluated in murine model of rheumatoid arthritis.

This dissertation consists of three parts. First, synthesis and physicochemical characteristics of heparin/D-erythro-sphingosine nanoparticles are shown in Chapter I. In Chapter II, the anti-inflammatory activity of these nanoparticles is investigated *in vitro* using cells such as macrophages and dendritic cells. Antagonistic effect of these nanoparticles against TLR4 was elucidated. Furthermore, structure-activity relationship of synthesized

nanoparticles was studied and functional groups responsible for the effect were revealed. In Chapter III, therapeutic effect of the nanoparticles is evaluated *in vivo* using collagen type II induced arthritis mice model. Studies in this dissertation demonstrate the development of novel heparin-lipid nanoparticles, their physicochemical/biochemical characterization and therapeutic application in arthritis therapy. The data supports a potential role for suppression of TLR4 signaling as a novel therapeutic approach in patients with rheumatoid arthritis. This work is to the best of my knowledge original, except where acknowledgements and references were made to previous works. Neither this, nor any other considerably similar work has been or is being considered to any other degree or diploma at any other institution.

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Chapter I

Synthesis and physicochemical characterization of heparin/D-erythro-sphingosine nanoparticles

I.1.Introduction

Polymeric or macromolecular micelles have extensively been studied as vehicles for targeted delivery of various drugs, genes and imaging agents. The micelles are self-assembled colloidal particles comprising amphiphilic molecules such as two-block copolymers and lipid-grafted macromolecules. Among many carrier systems that have been developed, heparin-based nanoparticles are one of the attracting ones [1-12]. In addition to low toxicity and high biocompatibility like other materials, heparin has a variety of biological activities beyond anti-coagulation [13-15]. The intrinsic properties of heparin can provide additional functionality towards the particulate carrier systems. One typical example is anti-angiogenic therapy to suppress tumor growth [16, 17]. Lipid-conjugated heparin derivatives retain an ability to bind to angiogenic factors such as fibroblast growth factors and vascular endothelial growth factors, so that it can significantly decrease endothelial cell proliferation [18, 19]. Another interesting property of heparin is its anti-inflammatory activity [13], although related reports dealing with heparin-based nanoparticles are limited [20]. Due to its high negative charge, heparin can non-specifically bind and inhibit proteins such as cytokines, growth factors, cytotoxic peptides, and tissue-destructive enzymes which involved in inflammation thereby limiting the activation of inflammatory cells, and their accumulation in tissues [14]. Effect of heparin in a range of inflammatory diseases was supported by a number of pre-clinical and clinical trials [15]. Underlying mechanisms responsible for anti-inflammatory effect of heparin are yet to be clarified [21], but notably heparin inhibits recruitment of leukocytes to inflammatory sites via blockade of P- and L- selectins which critically require 6-O sulfation of glucosamine residues [22]. Furthermore, heparin inhibits adhesion and migration of leukocytes in the endothelium by binding to cell surface proteins such as β2-integrin adhesion molecule CD11b/CD18 and platelet/endothelial cell-adhesion molecule 1 [13]. However, the clinical studies showing the effectiveness of heparin in these conditions are very limited either due to its anticoagulant effect or non-specificity of action.

Here, self-assembling nanoparticles composed of glycol-split non-anticoagulant heparin – D-erythro-sphingosine conjugates were prepared and their physicochemical characteristics were investigated.

I.2. Discussion

Self-assembling nanoparticles composed of glycol-split heparin/D-erythro-sphingosine conjugates (NAHNP) were synthesized by carbodiimide cross-linking chemistry. Chemical structure of the conjugates was confirmed by ^{1}H NMR. The conjugates provided spherical self-assemblies in water with mean diameters in a range of 110-160 nm. Size of particles tends to decrease with increasing degree of substitution of D-erythro-sphingosine. Nanoparticles had a highly negative zeta-potential presumably due to sulfo groups of heparins. The critical micellization concentration was relatively lower depending on the degree of substitution. Partitioning coefficient of pyrene between the micellar and aqueous phases indicated that increasing degree of substitution of D-erythro-sphingosine makes an inner core of self-assemblies more hydrophobic. Nanoparticles comprising higher substituted lipid conjugates could form more stable self-assemblies, and thus might be more promising candidates for *in vivo* drug delivery and therapeutics.

Chapter II

Characterization and implications for anti-inflammatory effect of heparin nanoparticles *in vitro*

II.1. Introduction

There has been considerable interest in the potential anti-inflammatory properties of heparin as it can bind and inhibit proteins critically involved in inflammation, limiting the activation of inflammatory cells, as well as their accumulation in tissues [20, 21]. However, disadvantages of clinical use of heparin for inflammation include a lack of selectivity of action and anticoagulant activity inducing hemorrhagic complications.

Here, it was demonstrated that NAHNP acts asa selective TLR4 antagonists and have much greater anti-inflammatory activity than native heparin. This means that the heparin/D-erythro-sphingosine nanoparticles can block an initial step of pro-inflammatory reactions in primitive immune cells which is a different target from that of the above-mentioned action. TLR family members are critical for the development of innate and adaptive immunity in response to pathogen and endogenous ligands generated in damaged tissues [28]. TLRs, particularly signaling through TLR4 have also been implicated in both the establishment of diseases such as arthritis [29-33], Alzheimer's disease [34], chronic myositis [35], systemic lupus erythematosus [36] and their maintenance. Under the circumstances when the immune system is disbalanced, inhibition of TLR4 signaling appears to be important in limiting the redundant response during the inflammation. Further it was demonstrated that, NAHNP blocks the production of pro-inflammatory cytokines from *E. coli* lipopolysaccharide (LPS)-mediated stimulation of macrophages and dendritic cells*in vitro*. Macrophages and dendritic cells as essential cells of the innate immune system are the major source of pro-inflammatory cytokines after stimulation with LPS, a selective TLR4 agonist [37, 38]. *In vitro* experiments of the underlying mechanism suggested the inhibitory effect of nanoparticles was due to downregulation of myeloid differentiation factor 88 (MyD88)-dependent nuclear factor-κB (NF-κB) signaling via TLR4 but not other TLRs. In addition, the structure-activity relationship for the anti-inflammatory effects was investigated and functional groups necessary for the activitywere elucidated.

These results shed light on synergistic effects of anti-inflammatory drugs with the heparin-based nanoparticulated carriers.

II.2. Discussion

Unlike native heparin, nanoparticles significantly inhibited *E. coli* lipopolysaccharide (LPS)-induced production of pro-inflammatory cytokines in both primary mouse macrophages and DC2.4 dendritic cell line. *In vitro* experiments of the underlying mechanism using mouse macrophages suggested the inhibitory effect of nanoparticles was due to downregulation of MyD88-dependent NF-κB signaling via TLR4 but not other TLRs. Effect of NAHNP was higher than native heparin nanoparticles (HPNP) indicating that glycol-splitting of non-sulfated uronic acids increases anti-inflammatory activities of particles. Experiments using nanoparticles of desulfated heparins suggested that 6-O-sulfate groups of D-glucosamine residue was essential for effective inhibition, while removal of 2-O-sulfo and 3-O-sulfo groups as well as replacement of N-sulfo groups with N-acetyl unaltered anti-inflammatory activity. In addition, comparisons among different aliphatic amine-heparin conjugates suggested that decrease in alkyl chain length of NAHNP resulted in loss of inhibitory activity.

Chapter III

Anti-inflammatory effect of heparin/D-erythro-sphingosine nanoparticles on type II collagen-induced arthritis in mice

III.1. Introduction

The involvement of Toll-like receptors in the pathogenesis of rheumatoid arthritis is supported by an increasing number of studies [51-57]. Notably, expression of TLR4 is highly increased in the synovium of rheumatoid arthritis patients [58, 59] and TLR4mutant mice are protected from experimental arthritis [60-62]. It is thought that extracellular endogenous ligands present in the arthritic joints activate TLR4 and contribute to maintaining inflammation [52, 53, 56, 63-65]. Recently, it was demonstrated that during arthritis, immune complexes containing citrullinated proteins greatly increase inflammation through MyD88-dependent pathway via TLR4 and activated Fcγ receptors [66]. Signaling activated by TLR4 ligands induces proinflammatory cytokine expression from TLR4-overexpressing cells such as macrophages, dendritic cells and fibroblasts in arthritic synovium [67]. Furthermore, TLR4 expressed on $CD4^+$T cells promotes autoimmune inflammation [68]. The generation of cytokines such as TNF-α, IL-6 and IL-1β regulated by transcription factor NF-κB is important in the pathogenesis of rheumatoid arthritis. Systemic inhibition of these cytokines with biologic drugs is effective as a short-term treatment but might also suppress the whole immune system and increase infection risk [69]. Evidence supports a role for TLR4 in the pathogenesis of rheumatoid arthritis [70-78], thus targeting the receptor of cell populations secreting distinct cytokines might be an effective approach to suppressing inflammation.

Heparins conjugated with D-erythro-sphingosine which was shown to blockade pro-inflammatory cytokine production from *E. coli* lipopolysaccharide (LPS)-induced macrophages and dendritic cells, canform stable self-assemblies, and thus might be promising candidates for *in vivo* drug delivery and therapeutics.Anti-inflammatory effect of heparin has been widely described in the literature although the mechanisms responsible for the effects are complex and incompletely understood [21]. The primary role of heparin as anti-inflammatory agent was closely linked to its ability of binding and inhibiting proteins such as selectins and growth factors involved in inflammation and angiogenesis [13]. *In vitro* studies showed that NAHNP suppressed the

production of TNF-α, IL-6 and IL-1β from LPS-stimulated macrophages and dendritic cells by inhibiting TLR4-mediated NF-κB signaling pathway. This suggests that the heparin nanoparticles can block the activation of TLR4-overexpressing primitive immune cells such as macrophages and dendritic cells in arthritic synovium which is a different target of heparin from that of the above-mentioned activity.

In this context, here the potential anti-inflammatory effect and therapeutic activity of NAHNPs in the collagen-induced arthritis model (CIA) was investigated.Thesefindings and potential benefits of these nanoparticles as a novel specific treatment for rheumatoid arthritisare discussed.

III.2. Discussion

Rheumatoid arthritis is a chronic, systemic inflammatory disease, which damages synovium and leads to destruction of cartilage and bone loss. In arthritis synovium the generation of cytokines such as TNF-α, IL-6 and IL-1β regulated by NF-κB is pivotal in the pathogenesis of the disease. Type II collagen (CII)- induced arthritis was developed in DBA/1J mice by subcutaneous immunization with CII emulsified in complete Freund's adjuvant and boosted intraperitoneally with CII emulsified in incomplete Freund's adjuvant. Heparin nanoparticles were administered by intraarticular injections once per day starting from onset of the disease. Intraarticular administration of NAHNP to type II collagen-induced arthritis mice significantly suppressed progression of the disease from the onset of arthritis symptoms. Pharmacological activity of the nanoparticles was associated with suppression of TNF-α, IL-1β and IL-6 in joints and sera, as well as decreased levels of circulating auto-antibodies. Nuclear localization of RelA *in vivo* was significantly inhibited in NAHNP treatment. These results suggest that selective inhibition of TLR4–NF-κB signaling with hydrophobically modified heparin derivatives composited to nanostructures provides an effective therapeutic approach to inhibit chronic inflammation in an animal model of rheumatoid arthritis.

Conclusion

In conclusion, novel heparin derivatives with self-assembling properties were developed, demonstrating their anti-inflammatory effects mediated through the inhibition of the TLR4–NF-κB pathway. This is the first research showing the hydrophobically modified heparin derivatives function as TLR4 antagonists, in addition to uncovering their structure-activity relationship. Moreover, heparin nanoparticles were found to suppress inflammation of CII-induced murine arthritis model. These results provide a new option of drug delivery and therapeutics against rheumatoid arthritis.

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