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Effect of Low Molecular Weight Heparin and Different Heparin Molecular Weight Fractions on the Activity of the Matrix-Degrading Enzyme Aggrecanase: Structure–Function Relationship

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Abstract The matrix-degrading enzyme aggrecanase has been identified in cartilage and is largely responsible for cartilage breakdown. The present study determined the efficacy of different heparin molecular weight fractions (HMWFs) and low molecular weight heparins (LMWHs) on aggrecanase activity. Aggrecanase activity was determined using biotinylated peptide substrate, which was immobilized onto streptavidin-coated 96-well plates; aggrecanase enzyme was then added. Proteolysis of the substrate at the specific amide bond was detected using specific antibody for the neoepitope generated. HMWFs ranging from 1,700 to 12,000 Da demonstrated a concentration-dependent inhibitory efficacy of aggrecanase activity, with a K_i ranging from 5,000 nM down to 1 nM as a function of the molecular weight. The higher the molecular weight distribution, the greater the inhibitory efficacy of the heparin fragments toward aggrecanase activity. The absence or presence of antithrombin did not alter the affinity of heparin in inhibiting aggrecanase. Additionally, tissue factor pathway inhibitor at various levels did not alter the activity of aggrecanase. LMWHs demonstrated different levels of potency in inhibiting aggrecanase activity as a function of their average molecular weight distribution. Tinzaparin (average molecular weight = 6,500 Da) and enoxaparin (average molecular weight = 4,500 Da) demonstrated a K_i of 20 and 80 nM, respectively. The aggrecanase inhibitory effect of LMWH might contribute to blocking inflammation and tumor invasion by inhibiting aggrecanase activity and maintaining an intact extracellular matrix barrier. J. Cell. Biochem. 95: 95–98, 2005.

Key words: heparin; low molecular weight heparin; aggrecanase; inflammation; invasion; anti-inflammatory; anticancer

Heparin and low molecular weight heparins (LMWHs) indirectly modulate various coagulation factors, such as factors Xa, IIa, IXa, and XIa, through antithrombin (AT); they also have multiple activities beyond their anti-Xa and anti-IIa effects, including stimulation of the release of vascular tissue factor pathway inhibitor (TFPI) through an AT-independent mechanism [Mousa, 2002; Mousa et al., 2003].

Aggrecanase has been identified in cartilage and is largely responsible for cartilage breakdown [Westling et al., 2002]. This enzyme is involved in inflammatory disorders. Inhibition of aggrecanase activity may protect the extracellular barrier, which may reduce inflammatory disorders or potential for invasion of cancer. This study outlines the effects of the carbohydrate-based heparin and LMWH on the activity of the carbohydrate-dependent, matrix-degrading enzyme aggrecanase.

MATERIALS AND METHODS

Bovine cartilage culture medium was used as a source of aggrecanases. A biotinylated 41-amino-acid peptide (QTVTWPDMELPLPRNITEGE-ARGSVILTVKPIFEVSPSPLK[Biotin]), a specific substrate for aggrecanase I, was synthesized by Quality Controlled Biochemical, Inc. (Hopkins, MA) and esterified at the four carboxylates [Miller et al., 2003].

Peptide substrate for aggrecanase I was immobilized on streptavidin-coated 96-well plate by adding 100 μl of 70 nM peptide stock solution.

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After overnight incubation, plates were washed three times with 50 mM Tris buffer, pH 7.4. The dried plates were sealed and stored at 4°C until used. Assays were performed by adding 100 μl of aggrecanases (cartilage cultured medium) with the different heparin fractions, different LMWHs, r-TFPI (Leo Pharmaceutical, Inc.; Ballerup, Denmark), or polyanionic proteins from soybean obtained from DuPont (Wilmington, DE) at various concentrations for K_i determination (0.1–5,000 nM). Incubation was carried out at 37°C for 4 h. Reaction was stopped by removing the enzyme solution and washing the wells with 5 mM Tris buffer. A primary antibody [Hughes et al., 1995] and HRP-conjugated goat anti-mouse secondary antibody (Pierce; Chicago, IL) were used. Developed absorbance was then read at 450 nm using a Molecular Device (Sunnyvale, CA) Spectromax 250 plate reader.

The potential effect of the carbohydrate-based heparin moieties on the activity of the carbohydrate-dependent matrix-degrading enzyme aggrecanase was examined. The matrix-degrading enzyme aggrecanase has been identified in cartilage and is largely responsible for cartilage-aggressing breakdown. The present study determined the efficacy of different heparin molecular weight fractions (HMWFs) and LMWHs (Leo Pharmaceutical) on aggrecanase activity. Aggrecanase activity was determined using biotinylated peptide substrate [Miller et al., 2003], which was immobilized onto streptavidin-coated 96-well plates; aggrecanase enzyme type I (ADAM TS4) was then added. Proteolysis of the substrate at the specific amide bond was detected using specific antibody for the neoepitope generated [Miller et al., 2003].

RESULTS

Matrix-Degrading Enzyme (Aggrecanase)

HMWFs ranging from 1,700 to 12,000 Da demonstrated a concentration-dependent inhibitory efficacy of aggrecanase activity, with a K_i ranging from 5,000 nM down to 1 nM as a function of the molecular weight (Table I). The higher the molecular weight distribution, the greater the inhibitory efficacy of the heparin fragments toward aggrecanase activity, with very low affinity with the pentasaccharide moiety (1,700 Da). Additionally, TFPI at various levels (1–5,000 nM) did not alter the activity of aggrecanase. LMWHs demonstrated different levels of potency in inhibiting aggrecanase activity as a function of their average molecular weight distribution. Tinzaparin (average molecular weight = 6,500 Da) and enoxaparin (average molecular weight = 4,500 Da) demonstrated a K_i of 20 and 80 nM, respectively. Additionally, polyanionic proteins from soy with various molecular weights did not show any inhibitory effect on aggrecanase I (data not shown). These data suggest that the large carbohydrate in aggrecan is important for substrate recognition by aggrecanase. The aggrecanase inhibitory effect of LMWH could contribute to blocking inflammation by inhibiting aggrecanase activity and maintaining an intact extracellular matrix barrier. This is in addition to the potent endothelial cell TFPI release by LMWH, which would contribute to the inhibition of vascular inflammation and thrombosis.

The higher the molecular weight distribution of the heparin fragments, the greater the inhibition of aggrecanase I activity, with very low affinity for the pentasaccharide (Table I). LMWHs demonstrated different levels of potency in inhibiting aggrecanase I activity as a function of their average molecular weight distribution (Table II) and perhaps due to other structural features.

TABLE I. Effects of Heparin Molecular Weight Fraction (HMWF) on Aggrecanase I Activity

Molecular weight of HMWF (Da)	Mean K_i (nM) ± SD[a]
12,623	0.75 ± 0.25
8,672	0.90 ± 0.20
7,023	10.50 ± 0.55
5,074	125.5 ± 15.0
3,524	2,100 ± 250
1,700	>5,000

Data represent mean ± SD, n = 3.
[a]Inhibition observed in the absence of antithrombin.

TABLE II. Effects of Low Molecular Weight Heparin (LMWH) on Aggrecanase I Activity

LMWH (molecular weight, Da)	Mean K_i (nM) ± SD[a]
Tinzaparin (average 7,500)	20.25 ± 2.5
Fraxiparin (average 5,500)	75.55 ± 6.5
Enoxaparin (average 4,500)	79.25 ± 9.5

Data represent mean ± SD, n = 7.
[a]Inhibition observed in the absence of antithrombin.

TABLE III. Effect of LMWH Versus TFPI on Aggrecanase Activity

Compound	Mean K_i (nM) ± SD
Tinzaparin (average 7,500 Da)	20.50 ± 2.5
rTFPI	>5,000

Data represent mean ± SD, n = 3.
TFPI, tissue factor pathway inhibitor.

The absence or presence of AT (using human plasma with AT III plasma 150 μg/ml) did not alter the affinity of heparin in inhibiting aggrecanase I; likewise, TFPI at various levels did not alter the activity of aggrecanase I (Table III).

DISCUSSION

Cartilage degradation is a critical feature of osteoarthritis and inflammatory joint. Aggrecan, a key protein in cartilage, impacts much of the compressibility and elasticity associated with normal joint function. Proteolytic degradation of aggrecan is associated with clinical symptoms of arthritis and related joint disorders [Sandy et al., 1992]. Peptide fragments resulting from aggrecan proteolysis are found at elevated levels in the synovial fluids and cartilage matrix of arthritis patients. These peptide fragments reflect the cleavage of aggrecan at two specific sites [Sandy et al., 1992]. Two structurally related enzymes were identified in bovine cartilage culture that are highly specific at cleaving the aggrecan at the Glu 373/Ala 374 site [Tortorella et al., 2000].

Erosion of cartilage is a major feature of joint diseases (e.g., osteoarthritis and rheumatoid arthritis), which leads with time to a loss of joint function. Proteolytic cleavage of the aggrecan core protein is a key event in the progress of these joint diseases. Aggrecan degradation has been believed to be mediated by a putative proteinase, aggrecanase. Recent studies have shown the loss of glycosaminoglycan (GAG) from injured cartilage [DiMicco et al., 2004]. Heparin fragments and LMWH demonstrated potent inhibition of aggrecanase activity that might contribute to the anti-inflammatory benefits of heparin and LMWH. The inhibition of aggrecanase activity by LMWH might blunt tumor invasion by maintaining an intact extracellular matrix barrier [Okada, 2000]. In agreement with our data, studies with sulfated GAG demonstrated direct inhibition of aggrecan activity [Munteanu et al., 2002]. The degradation of aggrecan and reduction in tissue levels of aggrecan by articular cartilage explant cultures stimulated with retinoic acid or rHuIl-1alpha was inhibited by heparin and heparan sulfate in a dose-dependent manner and by calcium pentosan polysulfate but not by chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, and keratin sulfate [Munteanu et al., 2002]. Studies from our laboratory showed that other polyanions (such as polyanionic proteins in soy) did not exhibit any effect on aggrecanase activity. These results suggest that GAGs have the potential to influence aggrecan catabolism in articular cartilage and this effect occurs in part through direct inhibition of aggrecanase activity.

CONCLUSIONS

LMWH inhibits aggrecanase I, which depends on the molecular weight distributions of LMWHs. Heparin fragments of higher molecular weight (up to 8,000 Da) resulted in the highest potency in inhibiting aggrecanase I. The effects of heparin and LMWH appear to be AT and TFPI independent. These data suggest that the large carbohydrate in aggrecan is important for substrate recognition by aggrecanase I.

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