Modulation of Perlecan Protein towards Chondrocyte Secretion Factors at the Articular Cartilage in Hyperglycemic Animal Model

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ABSTRACT

High carbohydrate influences the cartilage microcellular environment and chondrocytes. Perlecan (HSPG2) conducts an essential role as chondrocytes mechano-transducer and chondrocytes secretion factors. This research aims to fulfill the research gap about hyperglycemia which influences to perlecan of articular cartilage. About twenty male rats were divided into four groups: group I (sugar-treated rats 1.00 mg/kg of BW), group II (sugar-treated rats 1.5 mg/kg of BW), group III (2.00 mg/kg of BW), and control. The Anterior Cruciate Ligament Transection (ACLT) used to generate an unstable joint to be osteoarthritis condition at the right knee. Then, sugar was administrated for two months. Level expression of the protein was analyzed using Western Blotting. The result showed that perlecan expression was decreased within all sugar-treated rats group as compared to control. The IGF-1 expression was decreased, whereas TGF-β was slightly increased within all treatment groups. Moreover, the expression of FGF-2 was higher in all treatment groups. Of note, IL-1β expression was only elevated in group II and III. The interaction of perlecan to the chondrocyte secretion factors was determined by the cartilage condition within hyperglycemia.

Keyword: anabolic, catabolic, chondrocyte, hyperglycemia, perlecan

INTRODUCTION

The articular cartilage condition was commonly determined by chondrocytes and microenvironment within the cartilage matrix. Various components were based inside the cartilage matrix, including micro-molecules and macromolecules, such as perlecan. This perlecan or Heparan Sulphate ProteoGlycan type 2 (HSPG2) had a function as chondrocytes mechano-transducer [1] and chondrocytes differentiation factor [2-4] to maintain the cartilage matrix [5]. Chondrocytes in the healthy articular cartilage belong to pre-hypertrophic phase [6] which produces higher anabolic factors than catabolic factors [7] as a homeostatic system. Moreover, the previous study showed obviously that hyperglycemia influence the modification of perlecan molecules in aorta endothelial [8], and the reduced number of...
glucosaminoglycans (GAG) chains on the perlecan core protein. It is similar to the hyperglycemia condition which could reduce the Heparan Sulphate (HS) chain of perlecan core protein [8]. This loss of perlecan molecules might cause unstable homeostatic as a final impact received by chondrocytes. Importantly, hyperglycemia caused the accumulation of Advanced Glycation End Products (AGEs) and activation of AGES receptor (RAGEs) which affect to the production of proteoglycans, and synthesis of matrix metalloproteinase 1 (MMP-1) and type II collagen mRNA by chondrocytes [9]. Those AGEs mediate the inflammation that affects the unstable homeostatic of chondrocytes. It is indicated by decreasing production of anabolic factors and increasing production of catabolic factors [10].

However, the influence of hyperglycemia condition to the chondrocytes and perlecan at the articular cartilage remains indistinct. Therefore, this research aims to investigate the influence of hyperglycemia condition to the chondrocytes and perlecan at the articular cartilage which related to anabolic and catabolic factors.

EXPERIMENT

Animal treatments

Twenty male rats (*Rattus norvegicus*) of Wistar strain aged 2-3 months with weight 160-200 grams were used in this research. All animal experiment was characterized in a healthy condition which determined by shiny feathers, clear eyes, and active movement. This in vivo research was conducted in the Biosains Institute of Brawijaya University, Malang. All animal experiments were approved by Ethics Animal Research Committee of Brawijaya University No. 604-KEP-UB.

Rats were acclimated for a week, then Anterior Cruciate Ligament Transaction (ACLT) at right knee was performed according to the previous research [11]. All of the treatment groups were treated for wound healing within two weeks. This condition was maintained for sixth weeks to develop an unstable joint as osteoarthritis condition. Furthermore, rats were divided into four groups: group I (sugar-treated rats 1.00 mg/kg of BW), group II (sugar-treated rats 1.5 mg/kg of BW), group III (sugar-treated rats 2.00 mg/kg of BW), and control. In brief, sugar was administrated by oesophageal oral about three times a day for two months. Both the control and treatment group were given water and 30 gr of food (Comfeed®) per animal model. Food was given once a day in the evening. After the treatment, rats were anesthetized with diethyl ether before sacrificed. Furthermore, the right knee was removed from the body and the articular cartilage was collected to be sectioned horizontally linear to the epiphytic plate.

Western Blotting

A sample for western blot analysis was prepared by washed 0.25 gr of cartilage bone with PBS 1X for three times. Homogenization of cartilage bone was performed by cold mortar. The homogenate was added with extract buffer and centrifugated at 10,000 rpm in 4°C for 20 minutes. The supernatant was collected and the concentration was measured by nanodrop spectrophotometer. Moreover, the cartilage protein was separated by 12.5% SDS polyacrylamide gels electrophoresis and blotted by using PVDF membrane. Nonspecific protein binding was blocked by 5% skim milk in PBS. Next, the membrane was incubated with anti-rabbit perlecan, anti-mouse IGF1, anti-mouse TGF-β, anti-mouse FGF-2 and anti-mouse IL-1β (Santa Cruz Biotechnology) in 4 °C overnight. After that, membranes were washed with PBS 1X and incubated with polyclonal anti-rabbit IgG and anti-mouse IgG (KPL, Gaithersburg, Maryland, USA) labeled AP. The antigen-antibody complexes were visualized with Western Blue Stabilized Substrate (KPL, Gaithersburg, Maryland, USA) and
the reaction was stopped by aquadest. The membranes were washed with PBS1X and incubated with polyclonal anti-rabbit IgG and anti-mouse IgG (KPL, Gaithersburg, Maryland, USA) labeled AP. The antigen-antibody complexes were visualized with Western Blue Stabilized Substrate (KPL, Gaithersburg, Maryland, USA) and stopped by the addition of sterile distilled water. The Labeled membrane was visualized by ChemiDoc Imaging (Bio-Rad Laboratories Inc., Hercules, USA) and quantified by Quantity One software. (Bio-Rad Laboratories Inc., Hercules, USA).

**Statistical analysis**

Statistical analysis of western blot data obtained from perlecan protein expression, IGF-1 and TGF-β expression (anabolic factors), and FGF-2, and IL-1β expression (catabolic factors) was presented as mean ± standard deviation (SD) from duplicate experiment to evaluate between the group of treatment. However, analysis of all data within the group experiment was conducted by one-way ANOVA with post-hoc Turkey HSD. The \( p \)-value <0.05 was considered as statistically significant.

**RESULT AND DISCUSSION**

The average protein expression by perlecan, IGF-1, TGF-β, FGF-2, and IL-1β are presented in Figures 1A and 1B. Result demonstrated that protein expression of perlecan was decreased within all treatment group. The highest expression of IGF-1 shown in the group III (2.00 mg/kg of BW sugar-treated rats group), whilst the lowest expression was exhibited by group I. There were no significant differences between control and other groups. The TGF-β expression was decreased at the group I (1.00 mg/kg of BW sugar-treated rats group), and it was slightly increased within group II and III. In addition, another protein expression such as IL-1β was also diminished within the group I (1.00 mg/kg of BW sugar-treated rats group), but it was increased within group II and III. Furthermore, group II and III which treated with sugar 1.5 mg/kg of BW and 2.00 mg/kg of BW had slightly lower expression of FGF-2 than control. However, FGF-2 expression has the highest expression overall as compared to another protein expression.

After the treatment, the level of perlecan was decreased, it was followed by increased carbohydrate intake within all four groups. Control group (6.39± 0.57) had higher perlecan levels than group I (5.59 ± 0.57), while group II (4.71 ± 1.52) was lower than group I. However, the lowest expression of perlecan protein was demonstrated by group 3 (4.59 ± 0.57). The decreased level of perlecan was followed by the diminished level expression of IGF-1. This decreased expression was relatively irregularly as compared to control. For the expression level of TGF-β, the lowest level was found within the group I (1.20 ± 1.73). As exhibited in Fig. 1, under hyperglycemia condition, the secretion of anabolic factors by chondrocytes was declined which followed by the decreased level expression of perlecan protein. It suggested that hyperglycemia affects the secretion of anabolic factors that changing the level expression of perlecan protein.
Figure 1. Level expression of perlecan, IGF1, TGF-β, FGF-2, IL-1β of articular cartilage of animal models. C= Control, 1 = sugar-treated rats 1.00 mg/kg of BW group, 2 = sugar-treated rats 1.5 mg/kg of BW) group, 3 = (2.00 mg/kg of BW) group.

According to Figure 1C, it is shown that group I had slightly decreased expression of FGF-2 (1.254 ± 0.57) as compared to the control (1.256 ± 1.52). Furthermore, group II (1.217
± 1.0) and group III (1.196 ± 1.52) were also shown low expression of FGF-2. However, those decreased level of FGF-2 was not significant. Analysis of IL-1β expression was showed that group I (7.433 ± 1.52) has lower expression compared to the control group (8.073 ± 1.00). Furthermore, expression of IL-1β was increased within group II (8.560E2 ± 1.00000) and group III (9.500E2 ± 1.00000), respectively, compared to control. It appears that decreased level of perlecan is accompanied by an increased level expression of IL-1β as a catabolic factor. It is suggested that the hyperglycemia had an effect on the secretion of catabolic factors by decreasing the expression of the level of perlecan.

Previously, the effect of hyperglycemia on the protein expression of perlecan at articular cartilage has remained unclear. However, the present study is attempting to investigate the influence of hyperglycemia on the protein expression of perlecan at articular cartilage. It also investigates the expression of anabolic factors and catabolic factors. From these studies, there is an exists complex interplay of hyperglycemia as mechanotransduction and secretion factors of chondrocytes. Hence, it can either support or inhibit the homeostasis conditions of cartilage matrix. However, understanding that interaction was essential to step forward to the preventive issue and therapeutic interventions for cartilage pathologies, as known as osteoarthritis.

The pathological condition of osteoarthritis was indicated by chondrocytes which failed to maintain homeostasis in an articular matrix. It is started with microenvironment changing that influenced by the internal and external manner of a particular matrix. The microenvironment of cartilage is a dynamical condition which influences the chondrocytes as a permanent resident of articular cartilage [12]. Furthermore, perlecan as a chondrocytes mechanotransducer plays a crucial role to support the regeneration of articular cartilage matrix [13]. Another function of perlecan to chondrocyte is controlled by various cytokines and growth factors in the cartilage matrix which needed to be explored more. Moreover, the effect of hyperglycemia on the perlecan at articular cartilage is still unclear.

A previous study demonstrated that hyperglycemia influence the perlecan modification at aortic endothelial. The modification was showed by reducing one of four GAG chain within perlecan. However, this modification resulted in the elimination of one of the HS chain [14]. HS chain has been shown as an important regulator of FGF activity by acting as co-receptor at the surface of the cell [15,16]. It works to prevent the attachment of FGF to the surface of aortic endothelial by sequestration in the extracellular matrix [17,18]. This condition may lead to the contribution of atherosclerosis progression.

The investigation study about the effect of hyperglycemia on the joints is needed to be elevated since the cause of morphological change in perlecan articular cartilage affects the chondrocytes changing. Present studies showed that protein expression of perlecan is affected by hyperglycemia. It is exhibited by the lowest level expression of perlecan in group III (4.5933E2 ± .57735) compared to the control (6.3967E2 ± .57735). Group II (4.7167E2 ± 1.52753) known has lower perlecan expression than group I (5.5967E2 ± .57735). However, both group I and II had a level expression below the control. This diminished level expression of perlecan was followed by an increasing level of sugar intake within hyperglycemia condition. It could be explained by the glycation process of perlecan which caused by hyperglycemia. The higher dose of sugar intake influence would be decreased the protein expression of perlecan.

Decreased level of perlecan at the pericellular matrix influence the chondrocyte secretion function, for instance, imbalance secretion of anabolic and catabolic factors [19]. A recent study showed that perlecan was needed to maintain cartilage health. The anabolic factors such as IGF-1 and TGF-β had a similar result. Both of them were slightly decreased under
hyperglycemia condition. IGF-1 is a growth factor which needed to stimulate the growth of cartilage whilst preventing the cartilage for further degradation [20]. Based on the result, the group I exhibited the lowest expression of IGF-1, then slightly increasing at group II and group III, but it still lower than control.

According to the respond of young rat models to the hyperglycemia, the TGF-ß is known to act as growth factors, which involved at chondrocyte differentiation phase [21]. It also had a function to inhibit the chondrocyte hypertrophy. Generally, the level of TGF-ß was decreased by aging. However, the present result demonstrated that expression of TGF-ß was slightly decreasing due to an animal model in this research is relatively young. Moreover, catabolic factors such as FGF-2 known as an inhibitor of cartilage growth [22]. Within hyperglycemia condition, the level of perlecan was decreased and it was followed by the expression of FGF-2 which also diminished. Moreover, decreased level of perlecan affected to the elevated level of FGF-2 which had contact with the chondrocytes cell membrane. Therefore, it induces the carbohydrate diets which impacted to higher secretion of catabolic factors at osteoarthritis. For IL-1ß expression, the result showed that the expression of this pro-inflammatory cytokine was slightly increased. This result was suspected as a response of ACLT and decreased level expression of perlecan.

CONCLUSION
This study concludes that in hyperglycemia condition, perlecan articular cartilage was decreased and appears to be hyperglycemia-modification protein target. Results of these present studies need to be forwarded to the research of chondrocytes anatomical cellular changing due to the influence of hyperglycemia to perlecan, as complementary of osteoarthritis pathogenesis.

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