

行政院國家科學委員會專題研究計畫 成果報告

肥胖對痛風性關節炎疾病嚴重度之影響 - leptin, 痛風結晶 物與金屬蛋白酵素角色之探討

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骨關節炎是常見的關節病變，尤其是超過 65 歲年長者得病機率極高。肥胖一向被視為骨關節炎致病的重要危險因子之一，其原因可能是來自於關節承受過度重量所致，但是除了單純物理原因外是否有其他機制參與其中卻仍然是個待解的謎。脂肪激素(adiponectin)是一種專由脂肪細胞所分泌的蛋白質，其在血液中含量極高，最近更發現脂肪激素具有抑制糖尿病與心血管疾病之功能。在我們初步的結果發現骨關節炎患者血液中脂肪激素表現量比正常人低，而此初步結果是全新的發現。本計畫已確認脂肪激素確實在骨關節炎病人中有異常表現，並利用細胞實驗研究其脂肪激素對軟骨細胞之影響。

本計劃已完成下列實驗目標：

- (1) 分析既有之檢體資料庫中關節炎病人之血液與滑膜液中脂肪激素蛋白表現量為何。
- (2) 分析骨關節炎病人之血液與滑膜液中脂肪激素蛋白多元體結構分佈為何。
- (3) 探討脂肪激素受體在關節組織(包含軟骨、滑膜、硬骨等)中表現量為何。
- (4) 利用細胞實驗探討脂肪激素對軟骨細胞之新血管生成與發炎反應影響。

目前很多科學家皆認為脂肪組織在關節病變中佔有極重要之角色，但卻苦無太多證據支持。本計畫內容發現脂肪激素在關節病變中確實有異常表現，相信本計畫之結果對此領域應有一定之貢獻。

Obesity has been associated with an increased risk of osteoarthritis (OA). However, the mechanism by which obesity contributes to OA remains uncertain. Adiponectin, an adipocyte-derived hormone, has shown anti-diabetic and anti-atherogenic properties. In the present study, we aimed to investigate the role of adiponectin in OA development. We demonstrated that OA patients expressed significantly lower levels of plasma adiponectin than control subjects, and that adiponectin was present in OA synovial fluid (SF) whose expression levels negatively correlated with the clinical severity of OA, though not statistically significant. FPLC and ELISA studies revealed the distribution and abundance of the adiponectin complexes in plasma and SF from patients with OA. The percentage of high molecular weight (HMW) per total adiponectin in OA SF was lower than in OA plasma, while that of the hexamer form was similar and the trimer form was higher. The expression levels of adiponectin receptors AdipoR1 and AdipoR2 were examined in human OA tissues by RT-PCR. AdipoR1 was abundantly expressed in cartilage, bone and synovial tissues, whereas AdipoR2 was rarely detected. Finally, the effects of adiponectin on primary chondrocyte functions were studied using antibody-based protein array and RT-PCR. The patterns of mRNA expression and protein production strongly indicate that adiponectin is involved in the modulation of anti-angiogenesis in chondrocytes by up-regulating TIMP-2 and down-regulating IL-1 β -induced MMP-13. Together these findings clearly demonstrate the significance of adiponectin in the pathophysiology of OA, which may indicate a potentially important link between obesity and OA.

Introduction

Adiponectin is a newly discovered cytokine that is secreted exclusively by adipose tissue [1-2]. It is abundantly present in the circulation in three molecular forms: trimer, hexamer, and a high molecular weight (HMW) species [3]. Early studies have suggested that adiponectin plays a key role in the control of energy homeostasis because its plasma levels are inversely correlated with body mass index (BMI), intra-abdominal fat, and indices of insulin resistance [4-5]. In addition, the reciprocal association of adiponectin and C-reactive protein levels in both human plasma and adipose tissues has been described, suggesting that adiponectin may participate in the development of atherosclerosis [6]. Two adiponectin receptors, AdipoR1 and AdipoR2, have recently been cloned from both human and murine tissues. AdipoR1 is abundantly expressed in skeletal muscle, whereas AdipoR2 is predominantly expressed in the liver [7]. AdipoR1 and AdipoR2 are predicted to contain seven transmembrane domains, but are structurally and functionally distinct from G-protein-coupled receptors. Studies have also indicated that AdipoR1 and AdipoR2 may be able to form homo- and hetero-multimers [8-9]. Another receptor, the more recently reported T-cadherin, has been shown to bind the hexamer and HMW forms of adiponectin [10].

Osteoarthritis (OA) is the most common form of chronic arthritis yet little is known about its aetiopathogenesis. The mechanisms underlying the clinical and pathological progression of structural changes in OA are multiple and complex. It is believed that OA is a consequence of mechanical and biological events that destabilize the normal coupling of degradation and synthesis within articular joint tissues. The disease process affects not only the articular cartilage but also the entire joint structure including the subchondral bone, ligament, cartilage, capsule, synovial membrane, and periarticular muscles [11-12]. Obesity has been strongly implicated as a possible risk factor for the development of knee, hip, and perhaps hand OA [13-15]. It has been suggested that increased joint loading resulting from obesity would increase the risk of developing knee and hip OA. At this stage, it is difficult to arrive at an explanation for why obesity would be associated with hand OA. More recently, it has been reported that obesity could

have a systemic metabolic effect that influences the development of OA [16]. One recent study has also suggested that another adipocytokine leptin may act as a regulator of chondrocyte metabolism [17]. While these results are groundbreaking, more data will be important to further substantiate the role of obesity in the modulation of metabolic processes in OA. The present study is an extension to the above work and reports the significance of adiponectin in the development of OA.

Materials and Methods

Patient Samples. The characteristics of the study subjects are summarized in Table 1. Blood samples were obtained from 26 patients with knee OA (age range, 38 - 84 years). Synovial fluid (SF) and synovial tissue samples were prepared as previously described [18-19]. SF was isolated from 29 patients with knee OA. Patients with OA were selected according to the criteria developed by American College of Rheumatology [20-21]. The knee radiographs taken from hospital records were evaluated by two independent orthopedists. Kellgren-Lawrence criteria [22] were used for classification and grading of OA, and reliability was tested against Ahlback criteria [23]. The clinical severity of OA ranged from I to V, with grade I being the least severe and grade V the most severe. Synovial tissues, cartilage, and bone were obtained during surgery from patients with OA (n = 4) and from subjects with bone fractures (n = 2) who served as controls. Tissue samples were obtained from the diseased knee and hip joints. All patients were from Taipei Medical University Hospital. The Research Ethics Board at the Taipei Medical University approved the use of all specimens.

Measurement of adiponectin. Adiponectin concentrations in plasma and SF from patients with OA were measured by using a commercial enzyme-linked immunosorbent assay (Human Adiponectin ELISA Kit; B-Bridge International Inc., San Jose, CA). The absorbance was read on an automated ELISA plate reader at 450 nm. Values were calculated from a standard curve. Patient samples were pre-treated according to the instructions of the manufacturer. The results are expressed as means $\pm$ SD.

Analysis of oligomeric state of adiponectin in plasma and SF. After the preparation of patient samples, the distribution of the oligomeric state of adiponectin in plasma and SF was determined by gel filtration chromatography, as described in detail previously [24]. Briefly, human plasma and SF samples were fractionated in a HiLoad 16/60 Superdex 200 column (Amersham Pharmacia Biotech) equilibrated with PBS, followed by elution with PBS at a flow rate of 0.5 ml/min with 1.0-ml fractions. Fractions were analyzed for the concentration of individual isoforms of adiponectin using ELISA as described above.

Generation of chondrocytes from human cartilage and cell culture. Cartilage explants were minced and digested overnight with rotation at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 1 µg per ml of bacterial collagenase (Sigma, St. Louis, MO). Cells released by enzymatic digestion were filtered, washed, and pelleted by centrifugation. Primary human chondrocytes were cultivated in DMEM/F12 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO₂. All culture media and reagents were purchased from GIBCO BRL (Grand Island, NY) unless otherwise stated. For all experiments, primary chondrocytes were used at passages 3 to 7.

Measurement of cytokine release using antibody arrays. Primary chondrocytes were starved overnight in serum-free DMEM/F12, and pretreated with recombinant full-length human adiponectin (R&D Systems, Minneapolis, MN; 30 µg/ml) for 18 h and subsequently with recombinant human IL-1 β (R&D Systems, Minneapolis, MN; 5 ng/ml) for 6 h. Following treatments, the supernatants were collected and assayed for angiogenesis-related cytokines using the Human Angiogenesis Antibody Array I (RayBiotech, Aurora, CO) consisting of 20 different cytokine antibodies spotted in duplicate onto a membrane. Array experiments were performed according to the manufacturer's instructions.

RNA preparation and RT-PCR analysis. Total RNA was extracted from freshly isolated

synovial tissue, cartilage, and bone of OA patients and normal subjects as well as from primary chondrocytes using TRIzol Reagent (Gibco/BRL, Grand Island, NY). The sequences of the sense and anti-sense primers are listed in Table 2. RT-PCR was performed with BD Advantage 2 PCR Enzyme System (BD Biosciences Clontech). The RT-PCR reaction was carried out in a 25- μ l volume containing 25-50 ng of cDNA template, 10 pmol of each sense and anti-sense primers for a specific cDNA, and 2.5 μ l 10X Advantage 2 PCR Buffer, 0.25-0.5 μ l 50X Advantage 2 Polymerase Mix, 1 μ l 10 mM dNTP mix, and sterile water. For MMP3, the PCR program consisted of 35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 2 min. For MMP13, the amplification cycles were 95°C for 1.5 min, 55°C for 2 min, and 72°C for 3 min for 35 cycles. For GAPDH, the amplification cycles consisted of 30 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min. For AdipoR1 and AdipoR2, the conditions were 95°C for 5 min, followed by 30 cycles of 15 s at 95°C, 1 min at 60°C, and 1 min at 72°C. For T-cadherin, the conditions were 94°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. For TIMP-1, the conditions were 94°C for 5 min, followed by 45 s at 94°C, 45 s at 56°C, and 45 s at 72°C. All PCR amplifications were preceded by an initial denaturation for 5 min at the given temperature, followed by a final extension cycle at 72°C for 10 min. The PCR products were analyzed by agarose (1.5%) gel electrophoresis and visualized by ethidium bromide staining.

Statistical analysis. Data were analyzed by SAS (Statistical Analysis System, version 8.0, SAS Institute Inc., North Carolina, USA). Differences (between experimental groups) were analyzed by Student's unpaired *t* test, and linear regression model was used to examine the relationship between selected variables. *P* < 0.05 was accepted for statistical significance.

Results

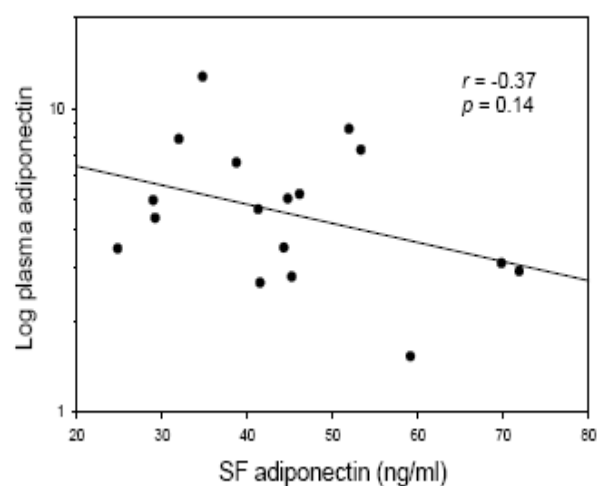
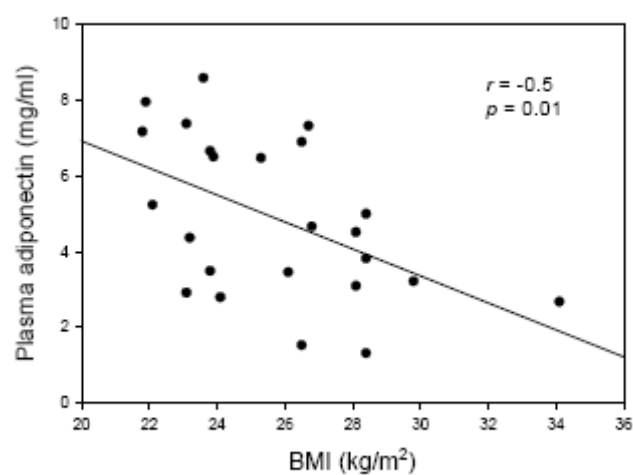
Table 1. Physical and biochemical characteristics of OA patients

Note. Values are expressed as means $\pm$ SD.

	Plasma	SF
<i>n</i>	26	24
Age (years)	65.4 ± 10.8	60.9 ± 14
BMI (kg/m ²)	25.8 ± 3.1	25.2 ± 3.4
Adiponectin	5.3 ± 1.3 (μg/ml)	0.044 ± 0.017 (ng/ml)
	(1.3 ~ 12.7)	(19.4 ~ 97.7)

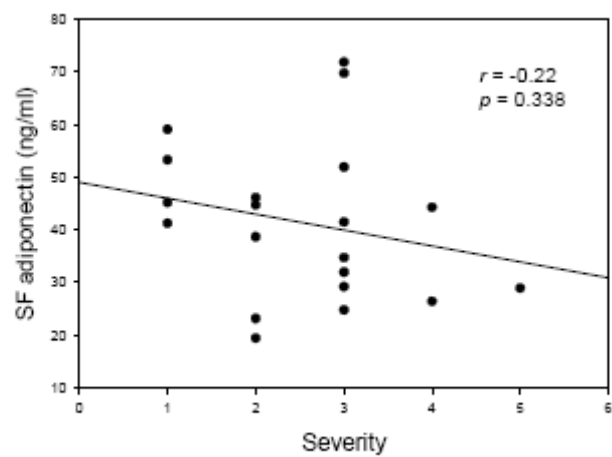
Abbreviations: BMI, body mass index; SF, synovial fluid.

Figure 1



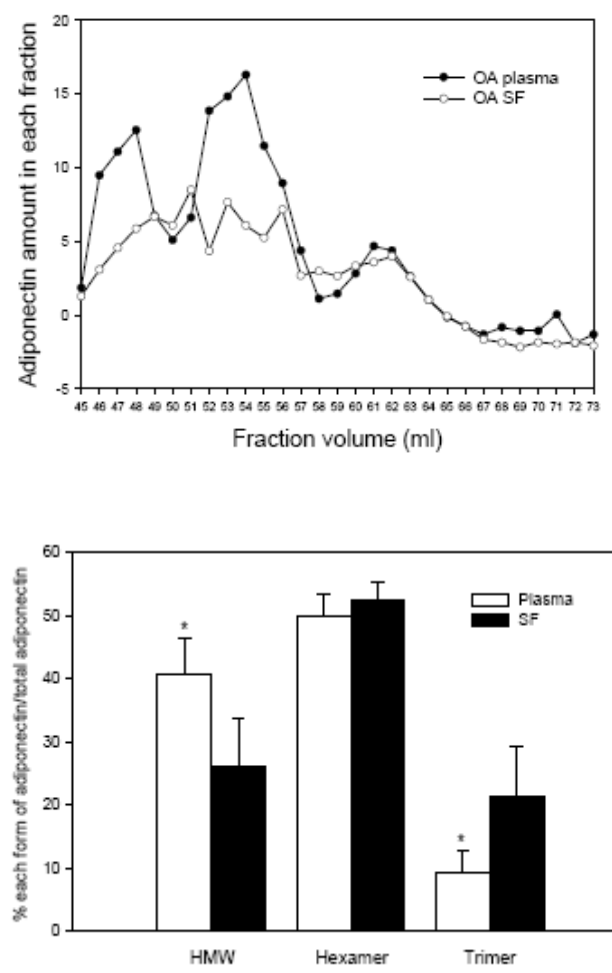
Adiponectin expression in OA patients. (A) Plasma adiponectin levels expressed in relation to subject BMI. (B) Relation between SF adiponectin levels and log plasma adiponectin in OA patients.

Figure 2



Relation between SF adiponectin levels and the severity of OA.

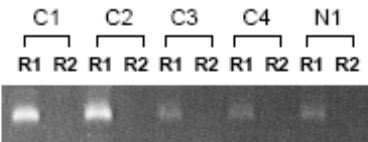
Figure 3



Analysis of oligomeric states of adiponectin in OA plasma and synovial fluid. (A) Elution profiles of adiponectin fractionated on a gel filtration column. Patient samples for analysis were prepared as described under “Materials and Methods”, loaded into a HiLoad 16/60 Superdex 200 column, and eluted in PBS. The gel filtration fraction numbers are indicated. An aliquot of each fraction was subjected to ELISA system (B). The content of adiponectin HMW, hexamer, and trimer is expressed relative to the total amount of adiponectin. Statistically significant differences are indicated.

Figure 4

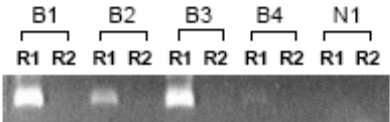
OA cartilage



OA synovial tissue

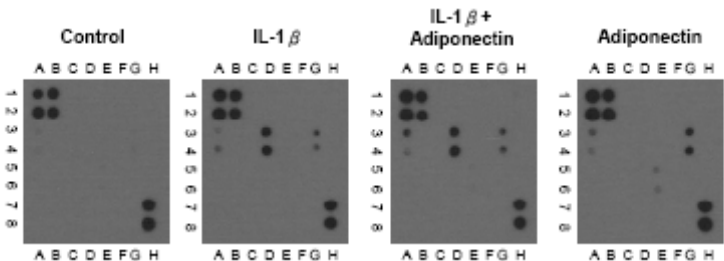


OA bone



Expression of AdipoR 1 and AdipoR2 mRNA in OA bone, cartilage, and synovial tissues. Adiponectin receptors 1 and 2 mRNA levels were analyzed by RT-PCR using cDNA prepared from OA tissue RNA. The number for each patient is listed above the bracket. *R1* and *R2*, adipoR1 and adipoR2, respectively. Representative results from 3 experiments are shown.

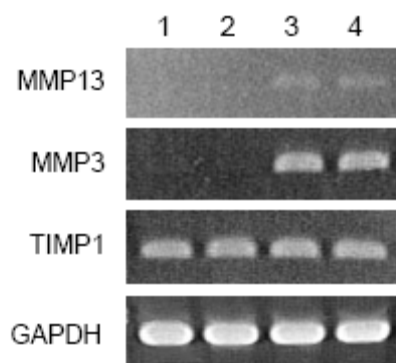
Figure 5



A	B	C	D	E	F	G	H
POS	POS	NEG	NEG	Angiogenin	EGF	EAN-78	bFGF
POS	POS	NEG	NEG	Angiogenin	EGF	EAN-78	bFGF
GRO	IFN- γ	IGF-1	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
GRO	IFN- γ	IGF-1	IL-6	IL-8	LEPTIN	MCP-1	PDGF-BB
PIGF	RANTES	TGF- β 1	TIMP-1	TIMP-2	Thrombospondin	VEGF	VEGF-D
PIGF	RANTES	TGF- β 1	TIMP-1	TIMP-2	Thrombospondin	VEGF	VEGF-D
BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	POS
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Protein microarrays of cytokines secreted by human primary chondrocytes after exposure to adiponectin. (A) Chondrocytes were treated in serum-free medium with or without 30 μ g/ml adiponectin in the presence or absence of 5 ng/ml IL1 β . The respective media were used to detect cytokine release. The comprehensive map of this array is shown in panel (B).

Figure 6



Effects of adiponectin on MMP13, MMP3, and TIMP-1 mRNA expression in human primary chondrocytes. Confluent chondrocytes were treated for 24 h with 30 μ g/ml adiponectin and subsequently with 5 ng/ml IL1 β for 24 h. Total RNA was collected for analysis as described in “Materials and Methods”. Lanes: 1, adiponectin only; 2, untreated control; 3, IL1 β only; 4, IL1 β and adiponectin. Representative results from 3 experiments are shown.

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