發現退化性關節炎的致病細胞素

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計畫主持人：張明熙
共同主持人：林啟禎

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Abstract:
To explore if any novel cytokine involve in the pathogenesis of osteoarthritis, we employ proteomics approach to analyze the protein in the synovial fluid from patients of OA, RA, GOUT and AS. We have not identified any novel proteins present in the synovial fluid from one patient of each group. We will continue to improve the technique of proteomics and analyze more samples. In addition, we discovered that RA patient contained more IL-19 and IL-20 in the synovial fluid than the other patients. We will further explore the molecular mechanism of IL-19 and IL-20 in pathogenesis of RA.

Keywords: osteoarthritis, rheumatoid arthritis, cytokines, IL-19, IL-20, synovial fluid

Introduction
細胞激素(cytokine)與關節炎有著密切之關係。特別是在類風濕性關節炎(Rheumatoid Arthritis；RA)患者之組織及關節液中已有前發炎反應之細胞激素，例如 TNF-α，IL-1，IL-6，IL-10，GM-CSF 及趨化激素(chemokine)IL-8 已知有明顯之過量的表現。但是，關節炎之分類中屬於非發炎性關節炎之退化性關節炎(Osteoarthritis；OA)在知其原因為與關節內軟骨退化，脫水，磨損有關之外，細胞激素是否也與 OA 有相關，目前並不十分清楚。有研究顯示，在以 IL-1, TNF, IL-17, IL-18 及 LPS 刺激軟骨細胞(Chondrocyte)之後，會產生 nitric oxide (NO) (Lotz M, (1999), Rheumatic Dis Clin N Am. 25: 269-281)。另外也有取得 OA 患者之軟骨細胞後發現了 IL-1 之前驅體 IL-1α及 IL-1 之拮抗體(antagonist) intracellular IL-1 receptor antagonist (IL-1 IRA) (Weissbach et al., (1998) Biochem Biophys Research Commun, 244, 91-95)。這些研究顯示，不僅 OA 的確與細胞激素有所關聯，也說明了探索細胞激素在 OA 的角色，對瞭解 OA 的致病原因是有其必要性的。
Purpose:
要偵測 OA 相關的細胞激素，除了以免疫學的偵測方式，或是分子生物學的手法來進行之外，蛋白質體學研究的進步，也提供了一種快速鑑定的方式。在本計劃中，以 OA 患者之關節液為實驗組，並收取 RA, GOUT and AS 患者之關節液作為對照組，在經過樣品化處理後，進行二維電氣泳動分析，在萃取出患者之特有蛋白後，以質譜儀進行成分分析，之後，再以分子生物學之方法找出其全長核甘酸序列後，將其表現並進行其功能研究。

Material and Method:
Collection of synovial fluid
We collected synovial fluid from 16 OA patients, 6 RA patients, 6 GOUT patients and one AS patients.

Preparation of synovial fluid:
Hyaluronidase was added to synovial fluid (25 U / 1 ml synovial fluid). The fluid was shaked at 37 ℃ for at least 30 min and passed through mesh. The flow through was collected and equal volume of histopaque was added to the fluid. The sample was centrifuged for 30 minutes and the supernatant was collected for proten analysis by 2-D gel.

Generation of monoclonal and polyclonal antibodies against hIL-19 and hIL-20
We used recombinant IL-19 and IL-20 as antigens to immunize mice and rabbit to generate monoclonal and polyclonal antibody.

Generation of IL-19 monoclonal antibody.
BALB/cJ mice were immunized subcutaneously every week for 4 weeks with recombinant human IL-19 protein (100 µg/mouse) emulsified with an equal volume of Freund's complete/incomplete adjuvant. Three days before fusion, three mice were boosted by intravenous injection of the antigen without adjuvant. Spleen cells (1.2 × 10^8) from immunized mice were fused with X63-Ag8-6.5.3 myeloma cells (1.5 × 10^7) in the presence of PEG 4000 (Merck & Co., Inc., Whitehouse Station, NJ). After fusion, the cells were distributed into 24-well plates and cultured in HAT medium for 14 days. Using ELISA, culture supernatant was tested for the presence of antibody reacting with human IL-19 (hIL-19). For the cloning of the selected hybridoma cell, the limiting dilution was carried out for twice. The hybridoma cells were cultured in Dulbecco's Modified Eagle's medium (GIBCO BRL, Invitrogen) containing 15% fetal calf serum, 1% penicillin/streptomycin, 2% L-glutamine, and 1% adjusted NaHCO3.
solution. The isotype of the selected antibody was IgG as determined by isotyping ELISA. The antibody was purified from ascites using Protein A chromatography.

Generation of anti-hIL-19 polyclonal antibody.

Human IL-19 polyclonal antibody was generated by injection of human IL-19 recombinant protein into a rabbit following the standard procedure. Serum samples were collected and the antibody was purified using protein A chromatography. Selection and characterization of monoclonal antibodies (MoAbs) against human IL-20 followed the standard protocols. The epitope specificity of a panel of MoAbs was determined by testing the ability of pairs of MoAbs to bind simultaneously to the antigen in ELISA. In brief, one antibody was attached to a solid substrate (capture antibody) followed by attachment of the antigen (IL-20), and the ability of the second antibody (detection antibody) labeled with biotin to bind with the antigen was monitored.

Result:

Proteomics Analysis
We collected synovial fluid from four groups of OA, RA, GOUT, and AS patients. The samples were analyzed by proteomics. As shown in Fig 1, synovial fluid from patients of OA, RA, GOUT, and AS did not contain any major difference in the protein. It may be due to the sensitivity of the detection method by proteomics. Therefore, we use ELISA approach to analyze if the synovial fluid contained any novel cytokine of IL-10 family.

ELISA Analysis
Figure 2 showed that RA patients contained highest level of IL-19 in their synovial fluid. Figure 3 showed that RA patient contained highest level of IL-20 in their synovial fluid. The level of IL-20 in two RA patients were 10,000 fold more than other patients including OA and GOUT. Clinical symptoms demonstrated that these two patients have very serious joint deterioration and need artificial joint replacement soon. Therefore, the result demonstrated that IL-19 and IL-20 play important role in inflammation of joint in pathogenesis of RA.

Future Work:
It is known that cytokines are involved in the inflammation of arthritis, especially in rheumatoid arthritis (RA) which is an multisystem autoimmune disease. Some studies
indicate that several kinds of pro-inflammatory cytokines eg, TNF-α, IL-1, IL-6, IL-10, and GM-CSF, besides chemokine, eg IL-8 are identified from the biopsies analysis of synovial tissue and synovial fluid from RA patients. On the other hand, osteoarthritis (OA) is characterized as a non-inflammation disease loss of joint cartilage and the imbalance in the anabolic and catabolic activity of chondrocytes. A study indicates that nitric oxide can be induced by the treatment with cytokines eg IL-1, TNF, IL-17, IL-18 from chondrocytes (Lotz M, (1999), Rheumatic Dis Clin N Am. 25; 269-281). Another research indicates that IL-1 precursor; IL-1α and intracellular IL-1 receptor antagonist; IL-1 IRA exist in the chondrocytes (Weissbach et al., (1998) Biochem Biophys Research Commun, 244, 91-95).

The results of our studies suggest that IL-19 and IL-20 may not directly involve in the pathogenesis of the OA disease but other undiscovered novel cytokines may involve in the OA disease. Identification of these cytokine remains to be explored. We will further improve our technique of protiomics analysis including modification of purification protocol of protein from synovial fluid. In addition, we analyzed only one patient from OA, RA, GOUT and AS patient. We need to analyze more samples to get more clear picture of protein in synovial fluid from these four groups. Our novel discovery of high IL-19 and IL-20 in synovial fluid from RA patients is also very interesting to us. We will explore the molecular mechanism by analyzing transcripts of synovial membrane and culture fibroblast from the membrane.

Figure 1
Please see the attachment “Figure 1 of proteomics”

(A): RA   (B): GOUT   (C): AS   (D): OA
Figure 1

Proteomics

A

Transferrin

Albumin

α-HS-glycoprotein

Microglobulin/
Haptoglobin

Ig light chain

ApoA1

ApoJ

B

Transferrin

Albumin

Microglobulin/
Haptoglobin

Ig light chain

ApoA1

ApoJ

C

Transferrin

Albumin

Microglobulin/
Haptoglobin

Ig light chain

ApoA1

D

Transferrin

Albumin

Microglobulin/
Haptoglobin

Ig light chain

ApoA1
Figure 2

Comparison of hIL-19 Levels in Synovial Fluid
Figure 3

Comparison of hIL-20 Levels in Synovial Fluid