대한침구학회지 제19권 제5호(2002년 10월)
The Journal of Korean Acupuncture & Moxibustion Society

원 저

The Effect of Bee Venom Acupuncture on Protease Activity and Free Radical Damage in Synovial Fluid from Collagen-induced Arthritis in Rats

Kim Min-jeong, Park Sang-dong, Lee A-ram, Jang Jun-hyouk, Kim Kyung-ho and Kim Kap-sung

Department of Acupunture & Moxibustion, College of Oriental Medicine Dong-Guk University

국문초록

쥐의 Collagen 유발 관절염의 활액에서 단백분해효소의 활성 및 유리기 손상에 미치는 봉독약침의 억제효과

김민정 · 박상동 · 이아람 · 장준혁 · 김경호 · 김갑성

동국대학교 한의과대학 침구과 교실

봉독은 祛風濕, 止痛, 解痙平喘, 消腫降圧의 효능으로 오랫동안 통증과 염증성 질환을 치료하는데 이용되어져 왔는데 최근에는 면역관련질환치료에 응용하여 좋은 결과가 보고되고 있다. 본 연구는 Rheumatoid arthritis와 유사한 형태의 대표적 실험모델로 알려진 실험용 쥐의 Type II collagen 유발 관절염(Type II collagen induced arthritis; CIA)의 활액에서 봉독약침이 단백분해효소와 유리기 손상에 미치는 면역억제효과를 알아보기 위해 실행되었다. 본 실험에서는 CIA가 유발된 실험용 쥐에 봉독약침(5㎕/kg)을 처리한 실험군과 대조군으로서 CIA 유발 쥐에 생리식염수를 처리한 군(CIA군), 정상적인 쥐에 생리식염수로 처리한 군(정상군)으로 구분하여 각 군들의 일련의 표본에서 세포질, 리소좀, 간질성 단백분해효소의 활성과 유리기로 인한 단백질 손상정도를(carbonyl 유도체를 측정하여)서로 비교하였다. 그 결과 각 군의 활액표본에서는 많은 종 류의 단백분해효소가 정상군보다 CIA군에서 유의하게 활성이 높았으며, 봉독약침(5㎕/kg)을 처리한 군에서 이 효소들의 활성이 유의하게 감소하였다. 그러나 각 군들의 혈장표본에서는 이 효소들의 활성은 서로 유

· 접수 : 2002년 8월 24일 · 수정 : 2002년 9월 8일 · 채택 : 2002년 9월 14일
· 교신저자 : 김갑성, 경북 경주시 용강동 357, 동국대 경주한방병원 침구과
Tel : 054-770-1558 E-mail : kapsung@unitel.co.kr

의한 차이가 없었다. 이는 혈장속의 면역반응과 연관되리라고 추측되는 단백분해효소들의 활성변화는 병인적 측면에서 RA와 같은 염증성관절 질환과는 큰 상관성이 없다는 것을 의미한다. Carbonyl 유도체 측정으로 평가한 유리기 손상은 활액과 혈장표본에서 모두 봉독약침(5㎕/kg)을 처리한 군에서 유의성 있게 감소하였다. 이상의 결과로 볼 때 단백분해효소와 유리기의 활성은 RA의 병인학적 측면에서 모두 잠재적인 중요성을 가지고 있으므로 향후 새로운 RA치료법은 이들 단백분해효소의 활성저해와 유리기의 소거능을 포함해야 한다고 사료되며 봉독약침은 이러한 2가지 효능을 포함한 효과적인 치료라고 평가된다.

Key words : Free radicals; Proteases; Type II collagen-induced arthritis; Bee venom acupuncture

I. Introduction

Bee venom therapy has been used for many years in traditional oriental medicine. In modern oriental medicine, bee venom therapy is being widely used for acupuncture to cure some immune-related diseases, especially RA and satisfactory results are obtained[1]. However, little is known about the mode of action of bee venom therapy on RA.

Normal joint function depends upon the structural integrity of the constituent cartilage and bone components, which in turn is dependent upon an equilibrium between the processes of tissue synthesis and degradation during cartilage and bone remodelling. It is generally accepted that proteolytic enzymes are involved in the catabolic aspect of normal tissue remodelling[2,3], and that altered activity of these enzymes is responsible for cartilage destruction and bone erosion associated with destructive disorders such as RA[2]. RA is chronic inflammatory disease with progressive articular damage and is often associated with systemic manifestations. It is characterized by inflammatory cell infiltration of proliferated synovial linings, and subsequent tissue erosion. As a result, pathological destructions of bone and cartilage in joint are followed. Although increased protease activity has been implicated in the pathogenesis of RA, differences in mechanism associated with these disorders remains to be elucidated. The potential role of many other protease types (e.g. macropain, alanyl-, arginyl-, leucyl-, pyroglutamyl aminopeptidases) present in synovial fluid in the pathogenesis of RA remains to be determined[4]. Reactive oxygen free radical species (ROS) have been implicated in the pathogenesis of degenerative joint disease[5]. ROS are highly reactive transient chemical species with the potential to initiate cellular damage (to proteins, lipids, etc.) in joint tissues[5]. ROS are formed during normal aerobic metabolism in all cells (e.g. via leakage from the mitochondrial electron transport chain), and following phagocyte activation during infection/inflammation ; cells are normally protected from ROS induced damage by a variety of endoge-

nous ROS scavenging proteins, enzymes and chemical compounds[5].

The relative contribution of proteolytic enzymes (cytoplasmic/lysosomal/matrix) and ROS to tissue damage in RA is currently unknown; this in turn is of importance for the development on a rational basis of novel pharmacological therapeutic strategies for patients with RA based on administration of protease inhibitors and/or ROS scavenging agents.

Since CIA is clinically similar to human RA, CIA in rats and mice has been frequently used as experimental model for this disorder. In the present investigation, we studied the inhibitory effects of BVA on protease activity and free radical damage from CIA rats. The objectives were as follows : (i) to compare relative activity levels of a comprehensive range of individual proteolytic enzyme types in synovial fluid samples ; and (ii) to compare changes in protease activities and determined corresponding protein carbonyl levels(a marker of free radical induced tissue damage) in synovial fluid, and hence to evaluate the effect of BVA on proteolytic enzymes and ROS to the pathogenesis of each disorder.

Ⅱ. Materials and methods

1. Materials

Lewis rats were purchased from Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology(Taejon, Korea). They were allowed at least 1 week to adapt to the environment(25±3℃, 55±5% humidity and 12hrs light/dark cycle) and were used at 7 weeks of age.

Bee venom was obtained from Monmouth Pain Institute, Inc.(New Jersey, USA) as an i.p injection grade for human. Each vial contained 10㎖ of bee venom. For i.p. injection into rats, randomly selected vials were suspended in normal saline at a concentration of 5㎕/100㎕.

All reagents (including enzyme assay substrates) were obtained from Sigma Co. or Bachem(Bubendorf, Switzerland) and were of analytical grade where available.

2. Methods

1) Arthritis induction

Type II collagen(Sigma, Co., St. Louis, MO, USA) extracted from bovine articular cartilage was dissolved overnight at 4℃ in 0.1㏖/ℓ acetic acid at 2.0㎎/㎖, after which the solution was emulsified in an equal volume of complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, MI, USA) in an ice-cold water bath. Arthritis was induced by an intradermal injection of 0.1㎖ of the cold emulsion into the base of the tail.

2) Experimental groups and bee venom treatments

Experimental rats were divided into three groups. (1) In BVA group, bee venom was subcutaneously acupunctured on Shinsu(B23) and both knee joints at dosages of 5㎕/㎏,

daily for 14 days(7 days before the initiation of the arthritis and 7 days after the initiation of the arthritis). (2) CIA group was given the injection of saline and phosphate-buffered saline on the same regions at dosages of 5㎕/kg for the 14 days. (3) In normal group, saline and phosphate-buffered saline was also injected on same regions at dosages of 5㎕/kg for the 14 days without induction arthritis.

3) Sample collection

Synovial fluid samples were obtained from rats. The diagnosis of CIA was based on clinical criteria described previously[6]. Samples were obtained from knee joints by arthrocentesis in rats presenting with symptomatic joint effusion (and hence by definition in the active phase of disease), centrifuged (5000×*g*, 10 min) and stored at −40℃ prior to laboratory analysis.

4) Proteolytic enzyme assays

Enzyme (0.05㎖ synovial fluid/plasma) was incubated with the appropriate assay medium (total volume 0.3㎖) at 37℃(10-120min), and the reaction terminated by addition of 0.6㎖ of ethanol. The fluorescence of the liberated aminoacyl 7-amino-4-methylcoumarin (AMC) was measured by reference to a tetraphenylbutadiene fluorescence standard block(λ_{ex} 380㎚, λ_{em}440㎚). Assay blanks were run in which the enzyme was added to the medium immediately before ethanol addition. Assay conditions were modified for samples with high enzyme activity such that the extent of substrate utilization never exceeded 15%. Stock substrate solutions (2.5 ㎜ol/ℓ) were prepared in 10% ethanol. Using the assay procedures described above, it is possible to accurately quantitate the activity levels of specific individual cytoplasmic, lysosomal or matrix protease types in synovial fluid/plasma samples, without significant cross-assay interference. Assay of cathepsin D activity was based on the spectrophotometric procedure of Pennington[7]. The reaction was terminated by addition of 0.5ml 10% PCA, the samples centrifuged at 2000×*g* for 10min, and the absorbency of acid soluble peptides determined at 280nm. Assay blanks were run as above.

5) Protein carbonyl assay and levels of free radical-induced protein damage (determined as protein carbonyl derivative) in synovial fluid from CIA and BVA rats.

The protein carbonyl assay used was based on the method of Levine et al.[8]. Streptomycin sulphate (10%, w/v, in 50㎜ol/ℓ Hepes buffer, pH 7.5) was added to synovial fluid samples equivalent to a final concentration of 1% (w/v) to precipitate any nucleic acids present. The samples were allowed to stand at room temperature for 15 min and then centrifuged at 11000×*g* for 15 min. The pellet was discarded and the supernatant retained. For each sample, the supernatant was divided into two aliquots, an equal volume of 20% (w/v) trichioroacetic acid (TCA) added to each, fol-

lowed by centrifugation at 3000×*g* for 15 min. The supernatants were discarded, and the pellet from one sample aliquot reconstituted in 0.5㎖ of 10㎜ol/ℓ 2,4-dinitrophenylhydrazine in 2㏖/ℓ hydrochloric acid, and the pellet from the second aliquot in 0.5ml of 2㏖/ℓ HCl (for the assay reagent blank). The samples were stood at room temperature for 1 h, 0.5ml of 20% TCA added, followed by centrifugation at 3000×*g* for 5 min (the supernatants are discarded). The pellets were then washed three times with 1 ml of ethanol/ethyl acetate (1:1, v/v) to remove unbound reagent, standing the samples for 10 min before centrifugation and discarding the supernatant each time. The protein pellet was redissolved in 1㎖ of 6㏖/ℓ guanidine solution in 2㎜ol/ℓ potassium phosphate adjusted to pH 2.3 with trifluoroacetatic acid and incubated at 37℃ for 15 min. For each sample the spectrum was read between 360 and 400nm using a Pye-Unicam SP8-100 spectrophotometer (1cm pathlength cell), and the carbonyl concentration calculated from the maximum absorption (relative to the reagent blank) using a value for the extinction coefficient of 22000 M^{-1} cm^{-1}. The carbonyl content is expressed as nmoles of carbonyl per mg of synovial fluid protein.

The ability of the assay procedure described above to determine levels of free radical-induced protein carbonyl in synovial samples from OA/RA cases was validated as follows. Samples of synovial fluid obtained from a control subject without degenerative joint disease were gassed to saturation with either N_2O for subsequent generation of hydroxyl (OH^-) radicals, or with O_2 (following addition of 20mmol/l sodium formate as a scavenger of OH^- radicals) for subsequent generation of superoxide (O_2^{--}) radicals. Generation of OH^- or O_2^{--} radicals in vitro via ^{60}Co gamma radiolysis of synovial fluid samples was based on the method of Davies[9]. Quantification of free radical dosage rate (equivalent to 99krad/h) was determined by standard dosimetric techniques[10]. Samples were irradiated for time periods between 2 and 20h, with subsequent analysis of free radical-induced protein carbonyl group formation determined as described above(relative to corresponding non-irradiated samples).

6) Protein determination

Synovial fluid protein levels, for calculation of specific protelytic enzyme activity measurements and specific protein carbonyl measurements above, were determined by the method of Lowry et al.[11] using bovine serum albumin as standard.

7) Analytical methods

Protein contents were determined by a protein assay kit of Bio-Rad Laboratories(Richmond, CA, USA).

8) Statistical analysis

Results were expressed as means±SD. Statistical analysis was performed by Student's T-test with $P<0.05$ for significance.

Ⅲ. Results

1. Effect of BVA on cytoplasmic protease activity in synovial fluid

In terms of absolute activity level comparison, the activity of arginyl aminopeptidase, leucyl aminopeptidase, pyroglutamyl aminopeptidase, dipeptidyl aminopeptidase III was significantly increased in CIA samples compared with normal samples. However, BVA(5㎕/kg) significantly reduced these enzyme activities to about 1/10 each <Table Ⅰ>.

2. Effect of BVA on lysosomal protease activity in synovial fluid

For the lysosomal proteases, synovial fluid samples from CIA rats, the relative levels of activity were cathepsin H, cathepsin L, dipeptidyl aminopeptidase II, dipeptidyl aminopeptidase I and cathepsin B. It is apparent from this data that the relative activity levels for corresponding enzyme types in RA samples is broadly similar. In terms of comparison of absolute levels of enzyme activity, all of the lysosomal proteases, were significantly increased in CIA samples, compared with normal rats: cathepsin L, cathepsin B, dipeptidyl aminopeptidase I, dipeptidyl aminopeptidase II, cathepsin H. BVA(5㎕/kg) significantly decreased these enzyme activities of dipeptidyl aminopeptidase I, dipeptidyl aminopeptidase II, cathepsin B, cathepsin L in CIA<Table Ⅱ>.

Table Ⅰ. Comparison of Cytoplasmic Protease Activity in Synovial Fluid from CIA, Normal and BVA Group[a]

Protease type	Enzyme activity (nmol/h per mg protein)		
	BVA(5㎕/kg)	Normal	CIA
Alanyl aminopeptidase	1.3±0.06*	1.2±0.1	12.2±0.5
Arginyl aminopeptidase	1.4±0.09*	0.8±0.05	15.1±1.5
Leucyl aminopeptidase	0.3±0.02*	0.08±0.01	2.89±0.1
Pyroglutamyl aminopeptidase	0.1±0.01	0.007±0.001	0.13±0.01
α-Glutamyl aminopeptidase	0.8±0.05	0.07±0.01	0.88±0.10
Dipeptidyl aminopeptidase Ⅲ	0.01±0.001*	0.009±0.001	0.16±0.01
Dipeptidyl aminopeptidase Ⅳ	0.8±0.06*	0.8±0.1	6.9±0.9
Tripeptidyl aminopeptidase	0.2±0.02*	0.2±0.03	2.4±0.1
Proline endopeptidase	0.4±0.04*	0.2±0.02	3.8±0.2
Macropain	0.007±0.001*	0.03±0.002	0.07±0.02

[a]Details of protease assays in synovial fluid from CIA, normal and BVA rats are given under Materials and Methods. Enzyme activities are expressed as nmol substrate hydrolysed/h per mg soluble protein. Values given are mean±SD(n=12).

The significance of differences in activity of individual protease types in CIA and BVA(5㎕/kg) group was assessed via one way student's T-test (*P<0.05).

Table II. Comparison of Lysosomal Protease Activity in Synovial Fluid from CIA, Normal and BVA Group[a]

Protease type	Enzyme activity (nmol/h per mg protein)		
	BVA(5㎕/kg)	Normal	CIA
Dipeptidyl aminopeptidase I	0.24±0.01*	0.03±0.001	1.16±0.2
Dipeptidyl aminopeptidase II	0.31±0.01*	0.04±0.01	1.32±0.2
Cathepsin B	0.12±0.01*	0.02±0.01	0.74±0.1
Cathepsin H	1.61±0.2	0.31±0.02	2.83±0.3
Cathepsin L	0.31±0.02*	0.07±0.01	2.21±0.22

[a]Details of protease assays in synovial fluid from CIA, normal and BVA rats are given under Materials and Methods. Enzyme activities are expressed as nmol substrate hydrolysed/h per mg soluble protein. Values given are mean±SD (n=12).

The significance of differences in activity of individual protease types in CIA and BVA(5㎕/kg) group was assessed via one way student's T-test (*P<0.05).

3. Effect of BVA on matrix protease activity in synovial fluid

In this regard, it is of note that extracellular matrix degrading (e.g. collagenase, tissue elastase) and leukocyte associated proteases (leukocyte elastase, cathepsin G) showed significantly increased levels in CIA versus normal synovial fluid (in agreement with previously reported studies[12]). Synovial fluid is thought to be produced via dialysis of plasma across the synovial membrane. The activity levels for some proteases in normal/CIA synovial fluid reported above are broadly comparable with those for corresponding enzymes in plasma. Also, BVA(5㎕/kg) decreased these enzyme activities of collagenase, tissue elastase and leukocyte associated elastase, cathepsin G in CIA <Table III>.

Table III. Comparison of Matrix Protease Activity in Synovial Fluid from CIA, Normal and BVA Group[a]

Protease type	Enzyme activity (nmol/h per mg protein)		
	BVA(5㎕/kg)	Normal	CIA
Collagenase	1.54±0.3*	0.13±0.02	3.32±0.4
Elastase (tissue)	0.07±0.01*	0.004±0.001	0.15±0.02
Elastase (leukocyte)	0.005±0.001*	0.014±0.001	0.08±0.001
Cathepsin G	0.42±0.4*	0.01±0.002	0.71±0.1

[a]Details of protease assays in synovial fluid from CIA, normal and BVA rats are given under Materials and Methods. Enzyme activities are expressed as nmol substrate hydrolysed/h per mg soluble protein. Values given are mean±SD (n=12).

The significance of differences in activity of individual protease types in CIA and BVA(5㎕/kg) group was assessed via one way student's T-test (*P<0.05).

4. Effect of BVA on cytoplasmic and lysosomal protease activity in plasma

Other protease types (e.g. leucyl aminopeptidase, proline endopeptidase) showed substantially higher levels of activity in synovial fluid compared with plasma<Table Ⅳ, Ⅴ>. Comparison of the levels of plasma proteases in CIA, BVA(5㎕/kg) and normal cases is of interest, since some protease types (particularly dipeptidyl aminopeptidase Ⅳ, proline endopeptidase) may be involved in antigen processing and the immune response[13,14]. Comparison of plasma protease activities in CIA, BVA(5㎕/kg) and normal cases is shown in <Table Ⅳ, Ⅴ>. Levels of plasma proteases (including dipeptidyl aminopeptidase Ⅳ and proline endopeptidase) in CIA, BVA(5㎕/kg) and normal plasma samples were not significantly different. These data, therefore, suggest that altered activity of plasma proteases (particularly those enzymes putatively involved in the immune response) is not a contributory factor in the pathogenesis of CIA.In the work described above, proteolytic enzyme activities are determined using various fluorogenic aminoacyl-7-amido-4-methyl-coumarin derivatives(i.e. non-physiological substrates). It is, therefore, of interest to compare the relative levels of endogenous proteins (presumably the physiological targets of synovial fluid proteases in vivo) in synovial fluid samples.

5. Effect of BVA on protein carbonyl levels in synovial fluid and plasma

The potential role of ROS in joint(particularly inflammatory) disease has been discussed previously[5] ; increased levels of ROS and increased levels of lipid peroxidation in syno-

Table Ⅳ. Comparison of Cytoplasmic Protease Activity in Plasma from CIA, Normal and BVA Group[a]

Protease type	Enzyme activity (nmol/h per mg protein)		
	BVA (5㎕/kg)	Normal	CIA
Alanyl aminopeptidase	8.9±0.7	8.9±0.8	9.0±0.8
Arginyl aminopeptidase	4.2±0.5	3.5±0.4	4.5±0.5
Leucyl aminopeptidase	0.1±0.02	0.09±0.01	0.2±0.01
Pyroglutamyl aminopeptidase	Nil	Nil	Nil
Dipeptidyl aminopeptidase III	0.4±0.03	0.5±0.04	0.04±0.01
Dipeptidyl aminopeptidase IV	8.1±0.7	7.8±0.6	8.7±0.6
Tripeptidyl aminopeptidase	0.9±0.06	1.2±0.06	0.7±0.1
Proline endopeptidase	0.3±0.04	0.2±0.01	0.2±0.01

[a]Details of protease assays in synovial fluid from CIA, normal and BVA rats are given under Materials and Methods. Enzyme activities are expressed as nmol substrate hydrolysed/h per mg soluble protein. Values given are mean±SD (n=12).

The significance of differences in activity of individual protease types in CIA and BVA(5㎕/kg) group was assessed via one way student's T-test (*P<0.05).

Table V. Comparison of Lysosomal Protease Activity in Plasma from CIA, Normal and BVA Group[a]

Protease type	Enzyme activity (nmol/h per mg protein)		
	BVA(5㎕/kg)	Normal	CIA
Dipeptidyl aminopeptidase I	0.42±0.04	0.34±0.04	0.43±0.05
Dipeptidyl aminopeptidase II	0.38±0.04	0.45±0.04	0.42±0.02
Cathepsin B	0.18±0.02	0.21±0.01	0.12±0.1
Cathepsin H	0.80±0.7	0.76±0.06	0.84±0.08
Cathepsin L	0.56±0.04	0.47±0.03	0.54±0.05
Cathepsin D+	0.42±0.03	0.32±0.04	0.42±0.04

[a]Details of protease assays in synovial fluid from CIA, normal and BVA rats are given under Materials and Methods. Enzyme activities are expressed as nmol substrate hydrolysed/h per mg soluble protein, with the exception of cathepsin D+ which is expressed in enzyme units/mg protein (where 1 unit=increase in absorbance of 0.001/h at 280 nm, with 1 cm pathlength cell at 37C). Values given are mean±SD (n=12).

The significance of differences in activity of individual protease types in CIA and BVA(5㎕/kg) groups was assessed via one way student's T-test (*P<0.05).

Table VI. Comparison of Protein Carbonyl Levels in Synovial Fluid and Plasma from CIA, Normal and BVA Group[a]

Tissue	Protein carbonyl content (nmol/ mg protein)
Synovial fluid	
Normal	1.2±0.2
CIA	3.4±0.4
BVA(5㎕/kg)	0.5±0.05*
Plasma	
Normal	0.5±0.05
CIA	1.23±0.2
BVA(5㎕/kg)	0.67±0.07*

[a]Details for the determination of protein carbonyl levels in synovial fluid and plasma from normal, CIA and BVA rats are given under Materials and Methods. Values given are mean±SD (n=14).

The significance of differences in measured parameter in CIA and BVA(5㎕/kg) group was assessed via one way student's T-test (*P<0.05).

vial tissues, and decreased levels of antioxidants in synovial fluid (and plasma) have been described in RA[15]. In the present study, the level of ROS induced oxidative damage to synovial fluid proteins(quantified asprotein carbonyl derivative) in CIA cases was approximately three times than in normal cases. However, BVA(5㎕/kg) significantly decreased level of ROS induced oxidative damage to synovial fluid proteins(quantified as protein carbonyl derivative) as shown in Table VI.

V. Summary

We have compared the levels of proteolytic enzyme activities and free radical-induced protein damage in synovial fluid from CIA, normal and BVA cases. Many protease types showed significantly increased activity in CIA, compared with normal rats.

However, BVA significantly reduced the cytoplasmic enzyme activities of alanyl amino-

peptidase, arginyl aminopeptidase, leucyl aminopeptidase, dipeptidyl aminopeptidase Ⅲ, dipeptidyl aminopeptidase Ⅳ, tripeptidyl aminopeptidase, and proline endopeptidase, macropain to almost about 1/10 each. For the lysosomal proteases, synovial fluid samples from CIA rats, BVA significantly reduced the enzyme activities of dipeptidyl aminopeptidase I, dipeptidyl aminopeptidase II, cathepsin B and cathepsin L. In extracellular matrix degrading (collagenase, tissue elastase) and leukocyte associated proteases (leukocyte elastase, cathepsin G), BVA decreased these enzyme activities of collagenase, tissue elastase, leukocyte associated elastase and cathepsin G in CIA. In cytoplasmic and lysosomal protease activities in plasma from CIA, BVA and normal plasma samples were not significantly different, suggesting that altered activity of plasma proteases (particularly those enzymes putatively involved in the immune response) is not a contributory factor in the pathogenesis of CIA.

In addition, the level of free radical induced damage to synovial fluid proteins in CIA cases was approximately three fold higher, compared with normal cases. BVA significantly decreased the level of ROS induced oxidative damage to synovial fluid proteins (quantified as protein carbonyl derivative). When levels of proteolytic enzyme activities and free radical-induced protein damage in synovial fluid from CIA and BVA cases were compared, many types of proteases showed significantly decreased activity in BVA rats, compared with CIA rats.

Also, the level of free radical induced damage to synovial fluid proteins was approximately one-sixth over of that in BVA, compared with CIA group. Therefore, it was concluded that BVA inhibited production of the proteolytic enzyme and the level of free radical induced damage to synovial fluid proteins was significantly reduced in BVA rats. Also, both proteolytic enzymes and free radicals are likely to be of equal potential importance as damaging agents in the pathogenesis of inflammatory joint disease, and that the design of novel therapeutic strategies for patients with the latter disorder should include both protease inhibitory and free radical scavenging elements.

In addition, the protease inhibitory element should be designed to inhibit the action of a broad range of protease mechanistic types(i.e. cysteine-, metallo- and serine-proteinases and peptidases).

Ⅵ. Discussion

Bee venom therapy has been used in oriental medicine to relieve pain and to treat inflammatory diseases such as RA. However, the mechanism by which the BVA modify the clinical status of RA are not well understood. In previous other reports, individual components of bee venom have only been tested to

verify the anti-inflammatory effect of bee venom treatment on RA. For example, Adolapin(20㎍/kg) and purified MCD peptide(1 ㎎/kg)have anti-inflammatory activity[16] although these substances are present in very small quantities in whole bee venom. Saini et al[17] reported that melittin is a major component of bee venom and that it binds to secretory phospholipase A_2(PLA_2) and inhibits its enzymatic activity. Because PLA_2 is a major inflammatory contact-detonating factor that causes arachidonic acid release, whose activity is enhanced in RA. Besides, in some investigation on the effect of whole bee venom administration, it was reported that whole bee venom successfully suppressed induction of arthritis, in animal experiments[18~20]. Also, in clinical reports, some investigators reported that whole bee venom administration was effective treatment to relieve symptoms of RA or osteoarthritis[21,22]. In previous our serial papers, BVA inhibited production of IL-1β and TNF-α from macrophages in response to in-vivo stimulation with bacterial lipopolysaccharides when BVA was administered into mice once a day for 7 days, suggesting that bee venom administered by i.p injection into the patients inhibit cytokine production from both T cells and macrophages and potent effects on RA. Also, we examined the influence of BVA on cellular immune responses by using rat CIA(collagen induced arthritis), an experimental model for RA. It was clearly demonstrated that BVA strongly inhibits T-cell activation including blastogenesis and cytokine production in response to antigenic stimulation in vitro. Furthermore, macrophage activation was also suppressed by the BVA.

RA is characterized by cartilage destruction and bone erosion, resulting from mechanical wear or following joint tissue inflammation, respectively. Although the precise mechanisms responsible for the differential pathogenesis of RA remain unknown, the action of proteolytic enzymes (from joint tissues and inflammatory cells) has been implicated in joint tissue destruction, based on the following experimental evidence: (i) in vitro, degradation of major (collagen, proteoglycan) and minor components of the extracellular matrix of cartilage and bone by individual proteases[23,24]; (ii) increased activity of proteolytic enzymes in joint tissues (synovial lining/cartilage/bone) or synovial fluid samples (the mechanical properties of which are known to degenerate in joint disease[25] from patients with RA); (iii) a beneficial effect on the course of disease of experimentally induced joint degeneration in animal model systems following administration of protease inhibitors[26]. Much of the previous research into the role of proteases in degenerative joint disease has focused on individual enzymes or small groups of related enzymes (particularly collagenase type; metalloproteases and cysteine type; cathepsins[27,28]). It is difficult to determine the relative contribution of different protease types to the overall process of degeneration, because of the different experimental approach used by different research groups; in addition, the potential role of some

protease types in the pathogenesis of degenerative joint disease remains to be determined. It has been well-known that oxygen radicals and their derivatives from inflammatory cells are implicated in the tissue damage occurring during inflammation. Somerfield et al[29] investigated the effect of melittin and other BV peptides on O_2^- production by human peripheral blood leukocytes. The result was that melittin inhibited O_2^- production both pre- and poststimulation in contrast to other BV fractions which were without effect. So, in the present investigation, we have undertaken the systematic investigation, using the same rats series and assay methodology, of a wide range of protease types in synovial fluid samples from CIA cases, in an attempt to determine the relative contribution of these enzymes to the degeneration process characteristic of each disorder and to evaluate inhibitory effect of BVA on other proteolytic enzymes activity and free radical-induced protein damage(determined as protein carbonyl derivative). The results were that BVA significantly inhibited production of the proteolytic enzyme<Table I ~ III>, and the level of free radical induced damage to synovial fluid proteins was significantly reduced in BVA rats<Table VI>. In conclusion, BVA is estimated as effective treatment inhibiting protease activities and removing ROS. Since activation of proteolytic enzymes and free radicals are likely to be of equal potential importance as protein damaging agents in the pathogenesis of RA, the development of novel therapeutic strategies for the latter disorder should include both protease inhibitory and free radical scavenging elements. In this point, BVA might be a useful tool for the treatment of RA.

One of the inevitable side effects of bee venom therapy is a potential allergic reaction that has an incidence of about 3% in the general population[30]. But it has also been demonstrated that long-term bee venom treatment induces T-cell hypo-responsiveness to the allergen and modulation of cytokine secretion in humans[31]. Thus hyposensitization induced by bee venom treatment is currently recognized as a successful long-term therapy for bee venom allergy in humans[32]. Since the side effects of long-term bee venom therapy are negligible, it may be one of the safer therapeutic approachs for the treatment of arthritis. However, It would be incredible if the treatment as powerful as this did not have serious toxicity, but further studies will be necessary to answer precisely this question. To develop novel strategies for treatment of CIA rats based on administration of protease inhibitors on a rational basis, it is necessary to know the relative contribution of protease types present in joint tissues to the degeneration process. However, biochemical and metabolic analysis of the constituents of bee venom have to be performed in further delineating its mechanisms of action in arthritis.

Ⅶ. References

1. Kim JY, Koh HK, Kim YS, Park YB, Kim CH, Kang SK. Reviews of recent research on bee venom therapy. The Journal of Korean Acupuncture & Moxibustion Society. 1997;14(2):47-71.
2. Testa V, Capasso F, Muffulli M, Sgambato A, Ames PR. Proteases and antiproteases in cartilage homeostasis. Clinical Orthopaedics & Related Research. 1994;308:79-84.
3. Einhorn TA, Majeska RJ. Neutral proteases in regenerating bone. Clinical Orthopaedics & Related Research. 1991 ; 262:286-97.
4. Murphy G, Hembry RM. Proteinases in rheumatoid arthritis. Journal of rheumatology. 1992;19:61-4.
5. Halliwell B, Gutteridge JM. Role of free radicals and catalytic metal ions in human disease: an overview. Methods in Enzymology. 1990;186:1-84.
6. Arnett FC, Edworthy SM, Bloch DA, Mc-Shane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis & Rheumatism. 1988:31: 315-24.
7. Pennington RJ. Proteinases of muscle. In : Barrett AJ Editor. Proteinases in m ammalian cells and tissues. Amsterdam: Elsevier North Holland. 1977: 515-45.
8. Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. Methods in Enzymology. 1990; 186:464-78.
9. Davies KJ. Protein damage and degradation by oxygen radicals: general aspects. Journal of Biological Chemistry. 1987;262(20):9895-901.
10. Fricke H, Hart EJ. Radiation dosimetry. In : Affix FH, Roesch WC Editors. Radiation dosimetry. New York : Academic Press. 1966:167.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RI. Protein measurement with Folin phenol reagent. Journal of Biological Chemistry. 1951;193:265-75.
12. Breedveld FC, Lafeber GJ, Seigert CH, Vleeming LJ, Cats A. Elastase and collagenase activities in synovial fluid of patients with arthritis. Journal of Rheumatology. 1987;14(5):1008-12.
13. Marguet D, David F, Vivier I, Bernard A M, Naquet P, Pierres M. Dipeptidyl peptidase IV (CD26): expression, structure and functions in lymphocytes. Adv Neuroimmunol. 1993;3:209-15.
14. Aoyagi T, Wada T, Daskalov HP, Kojima F, Nagai M, Harada S, Fujiwara M, Makino M, Umezawa H. Dissociation between serine proteinases and proline related enzymes in spleen of MRL mouse as a model of systemic lupus erythematodes. Biochemistry International. 1987 ;14(3):435-41. 15. Fairburn K, Grootveld M, Ward RJ, Abiuka C, Kus M, Williams RB, Winyard PG, Blake DR. α

-Tocopherol, lipids and lipoproteins in knee joint synovial fluid and serum from patients with inflammatory joint disease. Clinical Science. 1992;83(6):657-64.

16. Shkenderov S, Koburova K. Adolapin: a newly isolated analgetic and anti-inflam matory polypeptide from bee venom. To xicon. 1982;20:317-21.
17. Saini SS, peterson JW, Chopra AK, Mell itin binds to secretory phopholipase A_2 and inhibits its enzyme activity. Bioc-hem Biophys Res Commun. 1997;238: 436-42.
18. Hadjiipetrou-Kourounakis L, Yiangou M. Bee venom and adjuvant induced disea-se. J Rheumatol. 1984;11:720.
19. Hadjiipetrou-Kourounakis L, Yiangou M. Bee venom and adjuvant induced disea-se and interleukin production. J Rheu-matol. 1988;15:1126-8.
20. Eiseman JL, von Bredow J, Alvares AP. Effect of honeybee(Apis mellifera) ve-nom on the course of adjuvant-induced arthritis and depression of drug meta-bolism in the rat. Biochem Pharmacol. 1982;31(6):1139-46.
21. Kwon YB, Kim JH, Yoon JH, Lee JD, Han HJ, Mar WC, Beitz AJ, Lee JH. The analgesic efficacy of bee venom acupuncture for knee osteoarthritis : a comparative study with needle acupunc-ture. Am J Chin Med. 2001;29(2):187-99
22. Steigerwaldt F, Mathies H, Damrau F. Standardized bee venom (SBV) therapy of arthritis. Controlled study of 50 cas-es with 84 percent benefit. Ind Med Surg. 1966 Dec;35(12):1045-9.
23. Maciewicz RA, Wotton SF, Etherington DJ, Duance VC. Susceptibility of carti-lage collagens types II, IX and XI to degeneration by the cysteine proteina-ses, cathepsins B and L. FEBS Letters. 1990;269(1):189-93.
24. Morrison RI, Barrett AJ, Dingle JT, Pr-ior D. Cathepsins B and D : Action on human cartilage proteoglycans. Biochi-mica et Biophysica Acta Bi. 1973;302 (2):411-9.
25. Gysen P, Malaise M, Gaspar S, Fran-chimont P. Measurement of proteogly-cans, elastase, collagenase and protein in synovial fluid in inflammatory and degenerative arthropathies. Clinical Rh-eumatology. 1985;4(1):39-50.
26. Van Noorden CJ, Smith RE, Rasnick D. Cysteine proteinase activity in arthritic rat knee joints and the effects of a se-lective systemic inhibitor, Z-Phe-AlaCH 2F. Journal of Rheumatology. 1988;15 (10):1525-35.
27. Barrett AJ. J Saklatrala. Proteinases in joint disease. In: Kelley WN, Harris EP, Ruddy S, Sledge B Editors. Textbook of rheumatology. 1. Philadelphia: Saunders. 1985:182-92.
28. Brinckerhoff CE. Joint destruction in arthritis: metalloproteinases in the spotli ght. Arthritis & Rheumatism. 1991;34 (9):1073-5.

29. Somerfield SD, Stach JL, Mraz C, Gervais F, Skamene E. Bee venom melittin blocks neutrophil O2- production. Inflammation. 1986;10(2):175-82.
30. Golden DB, Epidemiology of allergy to insect venoms and stings. Allergy Proc. 1989;10:103-7.
31. Kammerer R, Chvatchko Y, Kettner A, Dufour N, Corradin G, Spertini F. Modulation of T-cell response to phospholipase A_2-derived peptides by conventional bee venom immunotherapy. J Allergy Clin Immunol. 1997;100:96-103
32. Valetine MD. Insect venom allergy: diagnosis and treatment. J Allergy Clin Immunol. 1992;82:1189-95.