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원저

류마티스 관절염 실험용쥐의 활액에서 단백분해효소의 활성 및 항산화에 대한 녹용약침의 효과

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Abstract

Effect of Cervi Pantotrichum Cornu Herbal acupuncture on protease activities, antioxidant in Rheumatoid arthritis rats

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류마티스 관절염의 쥐의 활액에서 단백분해효소, 산화제와 유리기에 대한 녹용약침의 비특이적 면역억제효과를 연구하였다. 일련의 실험표본으로서 여러 가지 세포질, 리소좀, 기질 단백분해효소의 제 활성을 RA대조군과 녹용약침군의 활액에서 카르보닐기 유도로 생성되는 유리기-유발 단백질손상과 항산화를 비교하였다. 전반적으로 단백분해효소활성이 정상군과 비교하여 RA대조군에서 유의성 있게 증가하였다. 세포질 단백분해효소들은 정상군과 RA군의 차이에서는 유의성이 없었다. 녹용약침처리 (100μg/kg)결과 세포질, 리소좀, 기질 단백분해효소생성을 억제하였으며, RA군과 녹용약침군 또는 정상군 사이에 활액 또는 세포질 항산화에서 유의성 있는 차이가 없음에도 불구하고, RA군 활액의 단백질손상을 유발하는 유리기는 녹용약침군과 정상군에 비교하여 약 2배 정도 높았다. 이상의 결과에서 단백분해효소와 유리기는 RA유발시 단백질손상을 유도하는 물질로 밝혀졌으며, 따라서 단백분해효소 저해와 유리기소거능을 갖는 치료법개발이 새로운 RA예방치료법으로 제시되었다. 나아가서 여러가지 기질특이성을 갖는 활액내 단백분해효소류(cysteine, serine, metallo proteinases와 peptidases)에 대한 효과적인 저해제개발이 필요한 것으로 보인다. 따라서 본 녹용약침은 이와같은 새로운 개념의 2가지(유리기제거, 단백분해활성) 관절염치료요소를 충족하는 약리활성을 포함하는 훌륭한 제제로 평가된다.

Key Words : CPH, RA, protease activities, antioxidant

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Ⅰ. Introduction

Cervi Pantotrichum Cornu(CPC), which is pilose antler of deer has been used invigorate the kidney-yang, replenish vital essence and blood and strengthen muscle and bones in Korean traditional medicine for a long time. Also extract of CPC by water boiling methods has been widely used in the treatment of some immune-related diseases, especially rheumatoid arthritis(RA) and satisfactory results are obtained[1)2)]. However, little is known about the mode of action of this herbal medication 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 [3,4)], and that altered activity of these enzymes is responsible for cartilage destruction and bone erosion associated with degenerative disorders such as RA. In clinical practice, RA is the most commonly encountered of the many forms of degenerative joint disease, with the former characterised by localised degeneration of articular cartilage mainly in weight-bearing joints, it is a systemic inflammatory disorder characterised by inflammatory cell infiltration of proliferated synovial linings, and subsequent tissue erosion. Although increased protease activity has been implicated in the pathogenesis of RA, differences in mechanism associated with these disorders remains to be elucidated.

RA has been classified as several different names in Korean oriental medicine. Those are Bi(痺), RoukjulPoong(歷節風), BaekHoRoukJul Poong(白虎歷節風), and TongPoong(痛風)[5)].

Much of the previous research in this area has focused upon relatively few proteolytic enzyme types, particularly the metalloproteases collagenase, elastase and stro-melysin, as well as serine proteases such as plasmin and kallikrein, and the cysteine type lysosmal cathepsins B, L and N [6,7,8)]. 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.

In addition, ROS have been implicated in the pathogenesis of degenerative joint disease [9)]. ROS are highly reactive transient chemical species with the potential to initiate cellular damage(to proteins, lipids, etc.) in joint tissues. 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 endogenous ROS scavenging proteins, enzymes and chemical compounds [10)].

The present study was investigated to see changes in protease activities determined in

(i) above with corresponding protein carbonyl (a marker of free radical induced tissue damage) and antioxidant levels in synovial fluid from RA rats, and hence to evaluate the relative contribution of proteolytic enzymes and ROS to the pathogenesis of each disorder. The present results show that treatment with CPH can inhibit the protease production and ROS production.

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 a 12 h light/dark cycle) and were used at 7 weeks of age.

Sliced CPC was purchased from Kyungju Oriental Medical Hospital(Kyungju, Korea) and extracted by water boiling method. The extraction of CPC was tableted after filtrated and lyophilizationed. Each tablets contained 100μg of the extract. For herbal acupunture into rats, randomly selected tablets were ground and suspended in normal saline at a concentration of 50μg/10μl. For this experiment CPH used as an i.p injection grade for human.

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, St. Louis, MO) extracted from bovine articular cartilage was dissolved overnight at 4℃ in 0.1mol/liter acetic acid at 2.0mg/ml, 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 0.1ml of the cold emulsion into the base of the tail.

2) CPC herbal acupucture

CPC herbal acupunctured on Shinsu(B23, shensu) and both knee joints daily to Lewis rats at dosages of 100μg/kg rat being day before the initiation of the arthritis for 7days and for 7days after the initiation of the arthritis. RA and normal groups were given the inje-ction of saline and phosphase-buffered saline on Shinsu(B23, shensu) and both knee joints at dosages of 100μg/kg for 14days.

3) Sample collection

Synovial fluid samples were obtained from rats. The diagnosis of RA was based on clinical criteria described previously [11]. 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(0.05ml synovial fluid/plasma) was incubated with the appropriate assay medium (total volume 0.3ml) at 37℃(10-120min), and the reaction terminated by addition of 0.6 ml of ethanol. The fluorescence of the liberated aminoacyl 7-amino-4-methylcoumarin(AMC) was measured by reference to a tetraphe-nylbutadiene fluorescence standard block(λ_{ex}380nm, λ_{em}440nm). 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(2.5mmol/l) were prepared in 10% ethanol. Assays were carried out for the following enzyme types: alanyl aminopeptidase: 50mmol/l Tris- acetate buffer pH 7.5 at 37℃, 5mmol/l $CaCl_2$, 1mmol/l DTT, 0.25mmol/l Ala-AMC; arginyl aminopeptidase: 50mmol/l K_2HPO_4/KH_2PO buf-fer pH 6.5, 0.15mol/l NaCl, 1mmol/l DTT, 0.25mmol/l Arg-AMC; leucyl aminopeptidase: 50mmol/l glycine/NaOH buffer pH 9.5 at 37℃, 5mmol/l MCl_2, 1mmol/l DTT, 2mmol/l Leu -AMC; pyroglutamyl amino-peptidase; 50mmol/l gly-cine/NaOH buffer pH 8.5 at 37℃, 2mmol/l DTT, 0.25mmol/l pyroglutamyl AMC; a-glu-tamyl aminopeptidase: 50mmol/l Tris- acetate buffer pH 7.5 at 37℃, 5mmol/l $CaCl_2$, 1mmol/l DTT, 0.25mmol/la-Glu-AMC; dipeptidyl ami-nopeptidase I: 50mmol/l CH_3 $COOH/CH_3COONa$ buffer pH 5.5, 2mmol/l DTT, 0.25mmol/l Gly-A rg-AMC; dipeptidyl amino-peptidase II: 50 CH_3COOH/ CH_3COONa buffer pH 5.5, 2mmol/l DTT, 0.25mmol/l Lys-Ala -AMC; dipeptidyl aminopeptidase III: 50mmol/l gly-cine/NaOH buffer pH 9 at 37℃, 2mmol/l DTT, 0.25mmol/l Arg-Arg-AMC; dipeptidyl amino p-eptidase IV; 50mmol/l Trizma base; acetate buffer pH 7.5 at 37℃, 2mmol/l DTT, 0.25mmol/l Gly-Pro-AMC; tripeptidyl amino p-eptidase: 50mmol/l Tris-acetate buffer pH 7.5 at 37℃ 2mmol/l DTT, 0.25mmol/l Ala-Ala-Phe-AMC; proline endopeptidase: 50m mol/l Tris-acetate buffer pH 7.5 at 37℃, 2mmol/l DTT, 0.25 CBZ-Gly-Pro-AMC; high Mr multicataltic proteinase(Macropain) 50 mmol/l Triza base; acetate buffer pH 7.5 at 3 7℃, 5mmol/l CaCl2, 1mmol/l DTT, 0.25mmol/l Glu-Gly-Gly-Phe-AMC; cathepsin B or cathepsin B+L: 50mmol/l CH_3COOH /CH_3 COONa buffer pH 5.5, 2mmol/l DTT, 0.25 mmol/l CBZ-Phe-Arg-AMC(cathepsin B+L) or 0.25mmol/l CBZ-Arg-Arg-AMC(cathepsin B only); cathepsin H: 50mmol/l KH_2PO_4/ K_2HPO_4 buffer pH 6.0, 1mmol/l DTT, 0.5 mmol/l puromycin, 0.25mmol/l Arg-AMC; collagenase: 50mmol/l Tris-acetate buffer pH 7.5, 1mmol/l DTT, 5mmol/l CaCl2, 0.25mmol/l Suc-Gly-Pro-Leu-Gly-Pro-AMC; tissue el-astase: 50mmol/l Tris-acetate buffer pH 7.5 at 37℃, 1mmol/l DTT, 0.25mmol/l Suc-Ala-Ala-AMC; cathepsin G: 50mmol/l Tris-acetate buffer pH 7.5 at 37℃, 2mmol/l DTT, 0.25 mmol/l Suc-Ala-Ala -Phe-AMC. Using the assay procedures described above, it is po-ssible to accurately quantitate the activity levels of specific individual cytoplasmic, ly-

sosomal 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[12]: 50mmol/l CH3COOH/CH3 COONa buffer pH 3.5, 1mmol/l DTT, 3mg/ml acid–denatured haemoglobin substrate(total assay volume 0.5ml). 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

The protein carbonyl assay used was based on the method of Levine et al.[13]. Streptomycin sulphate(10%, w/v, in 50mmol/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 15min and then centrifuged at 11000×g for 15min. 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, followed by cent-rifugation at 3000×g for 15min. The supernatants were discarded, and the pellet from one sample aliquot reconstituted in 0.5ml of 10mmol/l 2,4–dinitrophenylhydrazine in 2mol/l hydrochloric acid, and the pellet from the second aliquot in 0.5ml of 2mol/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 5min(the supernatants are discarded). The pellets were then washed three times with 1ml of ethanol/ethyl acetate(1:1, v/v) to remove unbound reagent, standing the samples for 10min before centrifugation and discarding the supernatant each time. The protein pellet was redissolved in 1ml of 6mol/l guanidine solution in 20mmol/l potassium phosphate adjusted to pH 2.3 with trifluoroacetatic acid and incubated at 37℃ for 15min. For each sample the spectrum was read between 360 and 400 nm 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 in a separate experiment as follows. Samples of synovial fluid obtained from a normal 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 20 mmol/l sodium formate as a scavenger of OH^- radicals) for subsequent generation of su-

peroxide(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[14].

Quantification of free radical dosage rate (equivalent to 99 krad/h) was determined by standard dosimetric techniques[15]. 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) Determination of total antioxidant status

Stock solutions of H_2O_2(0.5 mol/l in PBS), 2,2'-azinobis-3 ethylbenzothiazoline-6 -sulphonic acid(ABTS, 5mmol/l in PBS), metmyoglobin(70 umol/l in PBS), and Trolux(2.5 mmol/l in PBS) were prepared as described previously[16]. For determination of total antioxidant status, 10ul of sample(synovial fluid /plasma) were added to 0.49ml PBS, 36ul MetMb(final conc. 2.5umol/l), and 300ul of ABS(final conc. 150umol/l) in a 1-cm pathlength spectrometer cuvette(1-ml ca-pacity), and the reaction initiated by addition of 167 ul of H_2O_2(final conc. 75umol/l). The absorbancy at 734nm is monitored using a Pye-Unicam SP8-100 spectrometer at 30℃. A quantitative relation exists between the absorbancy at 734nm after 6min incubation, and the total antioxidant status of the sample, determined relative to appropriate Trolux an-tioxidant standards(0-2mmol/l) and expressed in terms ofmmol/l Trolux equivalent [16].

7) 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.[17] using bovine serum albumin as standard.

8) Analytical methods

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

9) 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 CPH on cytoplasmic protease activities in synovial fluid from RA

For the cytoplasmic proteases, the highest levels of activity(in relative terms) in normal samples was observed in alanyl aminopeptidase, dipeptidyl aminopeptidase IV, arginyl aminopeptidase and other proteases. For RA samples, high activities were alanyl aminopeptidase, arginyl aminopeptidase, dipeptidyl aminopeptidase IV, other proteases.

In terms of absolute activity level comparison, the activity of arginyl aminopeptidase

19-fold), leucyl aminopeptidase(30-fold), pyroglutamyl aminopeptidase(20-fold), tripeptidyl aminopeptidase(12-fold) and proline endopeptidase(20-fold) were significantly increased in RA samples compared to normal samples(alanyl- and alpha-glutamyl aminopeptidase activities were also significantly different in RA/normal samples). However, CPH significantly reduced these enzyme activities to about 1/10 each(Table I).

Table I. Comparison of Cytoplasmic Protease Activities in Synovial Fluid from RA and Normal Rats, and Effect of CPHa

Protease type	Enzyme activity (nmol/h per mg protein)		
	CPH (100μg/kg)	Normal	RA
Alanyl aminopeptidase	1.6±0.02*	1.2±0.1	12.2±0.5
Arginyl aminopeptidase	0.7±0.06*	0.8±0.05	15.1±1.5
Leucyl aminopeptidase	0.2±0.05*	0.08±0.01	2.89±0.1
Pyroglutamyl aminopeptidase	0.1±0.01	0.007±0.001	0.13±0.01
Dipeptidyl aminopeptidase IV	0.7±0.03*	0.8±0.1	6.9±0.9
Tripeptidyl aminopeptidase	0.2±0.06*	0.2±0.03	2.4±0.1
Proline endopeptidase	0.5±0.03*	0.2±0.02	3.8±0.2
Macropain	0.06±0.001	0.03±0.002	0.07±0.02

aDetails of protease assays in synovial fluid from RA, normal and CPH 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 1cm pathlength cell at 37℃). Values given are mean±SD (n=12).

The significance of differences in activity of individual protease types in RA and CPH groups was assessed via one way student T-test (*$P<0.05$).

2. Effect of CPH on Lysosomal Protease Activities in synovial fluid from RA

For the lysosomal proteases, synovial fluid samples from RA rats, the relative levels of activity were dipeptidyl aminopeptidase I (15%), dipeptidyl aminopeptidase II(14%) and cathepsin B(10%). It is apparent from this data that the relative activity levels for corresponding enzyme types in and RA samples is broadly similar. In terms of comparison of absolute levels of enzyme activity, all of the lysosomal proteases were significantly increased in RA samples, compared with normal rats(fold increase in parentheses): cathepsin B(6.6), dipeptidyl aminopeptidase I(4.9), dipeptidyl aminopeptidase II(3.6), cathepsin H(1.8). For cathepsin D, the mean activity level was higher in normal rats than in RA samples, although this difference was not significant.

CPH significantly reducued the enzyme activities of cathepsin B, dipeptidyl aminopeptidase I and dipeptidyl aminopeptidase II(Table II).

Table II. Comparison of Lysosomal Protease Activities in Synovial Fluid from RA and Normal Rats, and Effect of CPHa

Protease type	Enzyme activity (nmol/h per mg protein)		
	CPH (100μg/kg)	Normal	RA
Dipeptidyl aminopeptidase I	0.13±0.02*	0.03±0.001	1.16±0.2
Dipeptidyl aminopeptidase II	0.07±0.02*	0.04±0.01	1.32±0.2
Cathepsin B	0.18±0.02*	0.02±0.01	0.74±0.1

aDetails of protease assays in synovial fluid from RA, normal and CPH 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 RA and CPH groups was assessed via one way student T-test (*P<0.05).

3. Effect of CPH on Matrix Protease Activities in synovial fluid from RA

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 inc - reased levels in RA versus normal synovial fluid(2-3-fold and 3-6-fold, respectively, in ag r - eement with previously reported studies [18]). Synovial fluid is thought to be produced via dialysis of plasma across the synovial memb - rane(with subsequent supplementation of the mu - copolysaccharide hy - aluronic acid). The activity levels for some proteases in normal/RA synovial fluid reported above are broadly comparable with those for corresponding enzymes in plasma(e.g. alanyl aminopeptidase, dipeptidyl aminopeptidase IV). Also, CPH decreased these enzyme activities of collagenase, tissue elastase and leukocyte associated elastase in RA(Table Ⅲ).

Table Ⅲ. Comparison of Matrix Protease Activities in Synovial Fluid from RA and Normal Rats, and Effect of CPHa

Protease type	Enzyme activity (nmol/h per mg protein)		
	CPH (100μg/kg)	Normal	RA
Collagenase	1.49±0.03*	0.13±0.02	3.32±0.4
Elastase(tissue)	0.07±0.04*	0.004±0.001	0.15±0.02
Elastase(leukocyte)	0.011±0.001*	0.014±0.001	0.08±0.001

aDetails of protease assays in synovial fluid from RA, normal and CPH 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 RA and CPH groups was assessed via one way student T-test (*P<0.05).

4. Effect of CPH on Cytoplasmic and Lys - osomal Protease Activities in plasma from RA

Other protease types(e.g. leucyl amino - peptidase, proline endopeptidase) showed su - bstantially higher levels of activity in synovial fluid compared to plasma(Table Ⅳ, Ⅴ).

Table Ⅳ. Comparison of Cytoplasmic Protease Activities in Plasma from RA and Normal Rats, and Effect of CPHa

Protease type	Enzyme activity(nmol/h per mg protein)		
	CPH (100μg/kg)	Normal	RA
Leucyl aminopeptidase	0.09±0.07	0.09±0.01	0.2±0.01
Dipeptidyl aminopeptidase IV	8.0±0.3	7.8±0.6	8.7±0.6
Tripeptidyl aminopeptidase	1.0±0.03	1.2±0.06	0.7±0.1
Proline endopeptidase	0.25±0.03	0.2±0.01	0.2±0.01

aDetails of protease assays in synovial fluid from RA, normal and CPH 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 1cm pathlength cell at 37℃). Values given are mean±SD (n=12).

Table Ⅴ. Comparison of Lysosomal Protease Activities in Plasma from RA and Normal Rats, and Effect of CPH[a]

Protease type	Enzyme activity (nmol/h per mg protein)		
	CPH (100μg/kg)	Normal	RA
Dipeptidyl aminopeptidase II	0.40±0.01	0.45±0.04	0.42±0.02
Cathepsin B	0.20±0.03	0.21±0.01	0.12±0.1
Cathepsin H	0.79±0.05	0.76±0.06	0.84±0.08

[a]Details of protease assays in synovial fluid from RA, normal and CPH 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 1cm pathlength cell at 37℃). Values given are mean±SD (n=12).

Comparison of the levels of plasma proteases in RA, CPH and normal cases is of interest, since some protease types(particularly dipeptidyl aminopeptidase IV, proline endopeptidase and macropain) may be involved in antigen processing and the immune response[19,20]. Comparison of plasma protease activities in RA, CPH and normal cases is shown in Table Ⅳ,Ⅴ, from which it is apparent that:(i) the levels of activity for corresponding proteases in plasma from normal cases is substantially less than in tissues such as kidney, brain, muscle, etc.[21,22]; levels of plasma proteases(including dipeptidyl aminopeptidase IV and proline endopeptidase) in RA, CPH 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 RA.

In the work described above, proteolytic enzyme activities are determined using various fluorogenic aminoacyl-7-amido-4-methylcoumarin 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 CPH on Protein carbonyl and Total antioxidant levels in synovial fluid and plasma from normal and RA

The potential role of ROS in joint(particularly inflammatory) disease has been discussed previously[8]; increased levels of ROS(demonstrated via ESR spectrometry) and increased levels of lipid peroxidation(demonstrated colorimetrically via reaction with thiobarbituric acid) in synovial tissues, and decreased levels of antioxidants(particularly a-tocopherol) in synovial fluid(and plasma) have been described in RA[23]. In the present study, the level of ROS induced oxidative damage to synovial fluid proteins(quantified as protein carbonyl derivative) in RA cases was approximately three times that in normal cases. However, CPH significantly decreased the level of ROS induced oxidative damage to synovial fluid proteins(quantified as protein carbonyl derivative) as shown in Table Ⅵ.

For comparative purposes, exposure of synovial fluid from a normal case to OH^- or O^{--} generated via $Co^{60}\delta$ irradiation in vitro resulted in the following protein carbonyl levels(nmol/mg protein): no irradiation(1.16);

sample irradiated with 300 krad OH^-(3.90) or O_2^{--}(1.69); sample irradiated 1800 krad OH^-(12.8) or O_2^{--}(7.1). However, increased protein damage induced by ROS could not be rationalised in terms of compromised anti-oxidanttotal) capacity, since the latter was not significantly altered in RA synovial fluid(or plasma) compared to CPH or normal rats Table VI).

Table VI. Comparison of Protein Carbonyl and Total Antioxidant Levels in Synovial Fluid and Plasma from Normal, RA and Effect of CPHa

Tissue	Protein carbonyl content (nmol/ mg protein)	Total antioxidant level (mmol/l trolox equiv.)
Synovial fluid		
Normal	1.2±0.2	0.46±0.06
RA	4.1±0.3	0.45±0.04
CPH(100 μg/kg)	0.6±0.03*	0.53±0.03
Plasma		
Normal	0.5±0.05	0.72±0.07
RA	1.23±0.2	0.65±0.07
CPH(100 μg/kg)	0.60±0.04*	0.70±0.07

[a]Details for the determination of protein carbonyl and total antioxidant levels in synovial fluid and plasma from normal, RA and CPH rats are given under Materials and Methods. Values given are mean±SD(n=12). The significance of differences in measured parameter between normal, RA and CPH groups was assessed via one way student T-test (*$P<0.05$).

Ⅳ. Discussion

CPH is widely used in chronic management and treatment of RA, particularly, in Korea. However, the mechanism by which the CPH modify the clinical status of RA are not well understood.

Previously, our CPH inhibited production of IL-1β and TNF-α from macrophages in response to in vivo stimulation with bacterial lipopolysaccharides when the extract was administered into mice once a day for 7 days, suggesting that the CPH administered orally into the patients inhibit cytokine production from both T cells and macrophages and potent effects on RA. However, in this study, we examined the influence of CPC extract on protease activities, oxidant and free radical damages by using rat CIA(collagen induced arthritis), an experimental model for RA. The present results clearly demonstrated that the extract strongly inhibits protease activities and free radical damages. It was observed that the CPH injection has significant reductive effects on the development of CIA in rats at dosages of 100-150μg/kg/week.

RA is characterised 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/or 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 [24,25];(ii) increased activity of proteolytic

enzymes in joint tissues(synovial lining/artilage bone) or synovial fluid samples(the mechanical properties of which are known to degenerate in joint disease[26] from patients with RA[6];(iii) a beneficial effect on the course of disease of experimentally induced joint dege-neration in animal model systems following administration of protease inhibitors[27].

To develop novel strategies for treatment of RA 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. 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 [5,6,7]. 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. In the present investigation we have, therefore, undertaken the systematic investigation, using the same rats series and assay methodology, of a wide range of protease types in synovial fluid samples from RA cases, in an attempt to determine the relative contribution of these enzymes to the degeneration process characteristic of each disorder(Table Ⅰ-Ⅲ).

CPH might be a useful tool for the treatment of RA. It would be incredible if the drugs as powerful as this did not have seriuos toxicity, but further studies will be necessary to answer this question. The recommended dose of CPH in the management and treatment of rat RA will be 100μg/kg, which is two-fifth of human therapeutic dose. However, biochemical and metabolic analysis of the constituents of CPH have to be analysed in further delineating its mechanisms of action in arthritis.

V. Summary

We have compared(using the same series of experimental tissue samples) the levels of proteolytic enzyme activities and free radical-induced protein damage in synovial fluid from RA and CPH cases. Many protease types showed significantly increased(typically by a factor of approximately 2-3-fold) activity in RA, compared to normal rats. However, CPH significantly reduced the cytoplasmic enzyme activities of arginyl aminopeptidase, leucyl aminopeptidase, pyroglutamyl aminopeptidase, tripeptidyl aminopeptidase, and proline endopeptidase to almost about 1/10 each. For the lysosomal proteases, synovial fluid samples from RA rats, CPH significantly reduced the enzyme activities of cathepsin B, dipeptidyl aminopeptidase I and dipeptidyl aminopeptidase II. In extracellular matrix degrading(collagenase, tissue elastase) and leukocyte as-

sociated proteases(leukocyte elastase, cathepsin G), CPH decreased these enzyme activities of collagenase, tissue elastase and leukocyte associated elastase in RA. In cytoplasmic and lysosomal protease activities in plasma from RA, CPH 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 RA. In addition, the level of free radical induced damage to synovial fluid proteins was approximately twice that in RA, compared with CPH. CPH significantly decreased the level of ROS induced oxidative damage to synovial fluid proteins(quantified as protein carbonyl derivative). Therefore we conclude that 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). However, increased protein damage induced by ROS could not be rationalised in terms of compromised antioxidant total capacity, since the latter was not significantly altered in RA synovial fluid or plasma compared with CPH.

VI. References

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