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Human Neutrophil Cathepsin G: In Vivo Synthesis of Anti-HNCG Antibody, Inhibition of the Activity of HNCGs and Mechanism of the Inhibitions*

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ABSTRACT

Human neutrophil cathepsin-G, which has been known as one of the active enzymes causing inflammatory diseases, was purified by two steps procedure involving one size exclusion (Ultorogel AcA54) and one ion exchange (CM-Sephadex) chromatography. Purified HNCGs were cross-reacted with Anti-HNCathepsin-G antibodies which were radised in rabbits and purified by cathepsin-G labeled Sepharose 4B affinity chromatography. HNCGs were effectively inhibited by NSAIDs including phenylbutazone, sulindac, oxyphenbutazone, salicylic acid and salicyluric acid. IC_{50}s of these drugs for inhibition of Cathepsin G were 0.3-0.8 mM. Other NSAIDs inicluding aspirin showed little or no inhibition effect on the activity of Cathepsin G. These results strongly indicated that NSAIDs which showed inhibition effect on the activity of HNCGs possibly be at least a part of mechanism of action which might be related to direct inhibition of cathepsin G at the tissue destruction sites beside of their known mechanism of action as an anticyclo-oxygenase in treatment of inflammatory diseases. Lipid soluble component of Korean Red Ginseng which was known as an anti-inflammatory agent inhibited HNCGs strongly, but no other fractions did inhibited HNCGs. Antibiotics including novobiosin and rifamycin showed some inhibition effect on HNCGs, i. e.., IC_{50} of these drugs were 2.6 mM and 1.5 mM respectively, and other antibiotics including penicillin G showed no or negligible inhibition effect on the activity of HNCGs. However, tetracyclines inhibited HNCGs very effectively at the concentration of therapeutic range. The inhibition effect of the activity of HNCGs by tetracycline are not related to the N-dimethyl radical on the 4 position of the tetracycline molecule. Furthermore, N-dedimethylated tetracyclines may have beneficial effect for long term treatment of chronic inflammatory diseases without developing any drug resistance to microorganisms.

Key Words: Neutrophil, Cathepsin G, NSAIDs, Antibiotics

INTRODUCTION

Human neutrophil elastases and cathepsin G have been Known as the causative enzymes in inflammatory diseases including rheumatoid arthritis (Glynn 1972) and emphysema (Mittman, 1972; Galdston *et al.*, 1973; Cohen, 1983). In rheumatoid arthritis, which is perhaps the most widely studied of these diseases, high density of PMNs are in the joint space. Released enzymes from neutrophils are usually regulated by the plasma proteinase inhibitors, alpha-1-antitrypsin and alpha-2-macroglobulin (Janoff, 1972c; Cohen, 1975, Starkey, 1975; Janoff, 1983). Over released enzymes or/and under production of inhibitors or chronic immunological stimulation to

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increase the free enzymes in the specific sites may cause the inflammatory diseases. The mechanism of antiinflammtory drugs like aspirin, salicylate and indomethacin are known as inhibitors of cyclooxygenase in synthesis of prostaglandins, and there are some speculations involving proteolytic enzymes in the tissue destruction (Flower *et al.*, 1985). Recently, Lentini *et al.*, and we reported that some NSAIDs inhibited human neutrophil elastase and/or cathepsin G (Lentini *et al.*, 1987; Bae *et al.*, 1990), and we also reported that some antibiotics could modulate the activity of human heutrophil elastases (Ghim *et al.*, 1989). Furthermore, Han reported that Korean Red Ginseng also had the anti-inflammatory effect (Han *et al.*, 1972). Therefore we attempt to investigate any possible relationship between inhibition of Cathepsin G activity and its involvement to the antiinflammatory effects. In this report we describe the inhibition effect of NSAIDs, antibiotics and Korean Red Ginseng fractions on the activity of HNCGs and possible mechanism of action of specific drug on the activity of the HNCGs.

MATERIALS AND METHODS

Materials

L-benzoyl-DL-Phenylalanine-P-Naphthylamine (BPNE), N-Succinyl-Ala-Ala-Pro-Phe-P-Nitroanilline (SAPNA), Fast Garnet GBC Base, Brij 35, N-Succinyl-L-Ala-L-Ala-L-Ala-P-Nitroanilide (SANA), Non-Steroidal-Anti-Inflammatory Drugs (NSAIDs) and antibiotics were purchased from SIGMA Chemical Co., Ultrogel AcA54 was purchased from LKB. Korean Red Ginseng fractions including ethanol soluble fraction, lipid soluble fraction, water soluble fraction and Crude Red Ginseng extract were generously supplied from Korean Ginsang and Tobacco Research Institute, Taejun, Korea. Other chemicals were of highest quality obtainable.

Cathepsin G assay and inhibition test

Microplate assay: Proper amount (10-100 ug) of purified Cathepsin Gs were pre-incubated in 250 ul of 100 mM of Tris-Cl, pH 7.4, containing 150 mM of NaCl and 5 mM of $CaCl_2$ with or without inhibitor for 20 minutes, then substrate, SAPNA, was added to the medium to give a final concentration of 0.6 mM and incubated for 5, 10, 20, 30, 60 minutes and the quantity of nitroaniline was monitored by absorbance at 410 nm using a Titertek Multiskan Spectrophotometer and the activity was calculated by the statistic analysis.

In Vivo synthesis and purification of anti-Cathepsin G antibodies: Purified HNCGs were mixed with same volume of Freunds complete adjuvants and were injected in the peritoneal cavity at every other day for eight days, then two booster injections were performed every week. After three days, blood was collected and incubated at 23°C for one hour, then placed at 4°C for overnight and centrifuged it at 4000 rpm for 10 minutes at 4°C. Immunoglobulins were precipitated from the supernatant with 33% of ammonium sulfate. The crude precipitation was dissolved in the minimum amount of borate buffered saline, pH 8.2 and dialyzed in the same BBS buffer at 4°C for over night, then the Immunoglobulins were kept at −20°C until use it.

Purification of IgG by DEAE-cellulose 25 ion-exchanger chromatography: Curde Immunoglobulin prepared from immunized rabbits were equilibrated in 10 mM phosphate buffer, pH 8.0, and the 2 ml of the sample was loaded on the DEAE-cellulose ion exchange column and performed linear salt gradient chromatograph from 10 mM phosphate buffer, pH 8.0 to 300 mM phosphate buffer, pH 5.4. The protein was monitored by recording the absorbance at 280nm. The fractions which showed the precipitation characteristics against HNCGs were collected and dialyzed against borate buffer containing 0.5 M NaCl and kept in deep freezer until use it.

Purification of anti-Cathepsin G antibody by Cathepsin G bound Sepharose 4B affinity chromatography: Preparation of Cathepsin-G labeled Sepharose 4B affinity gel: Cathepsin Gs were applied to CNBr-activated Sepharose 4B gel beads at the ratio 100ug: 1ml of swelled beads. After the mixture was Incubated in glycine buffer, pH 8.2, containing 0.5 M NaCl, the Cathepsin G bound beads were washed 4 times repeatedly with 0.1 M borate buffer, pH 8.2 (containing 0.5 M NaCl) and 0.1 M acetate buffer, pH 4.0 (containing 0.5 M NaCl) to remove the residual ligands. Then The sample was incubated in 0.1 mM DiPF at room temperature for 60 minutes to

inactivate the bound enzyme activity.

Cathepsin G (Sepharose 4B) Affinity chromatography: The HNCGs affinity gel column was prepared with Cathepsin G bound Sepharose 4B beads. The column was washed with to 3-5 column volume of borate buffer, then purified lgGs were infused in this Cathepsin G bound Sepharose 4B column and the column was eluted with borate buffer, pH 8.2 (containing NaCl) and 0.1M glycine buffer, pH 2.8 (containing 0.5M NaCl) sequentially. The protein peaks were monitored by spectrophotometer at the wave length of 280nm. The protein peak which has specificity against Cathepsin G was collected and dialyzed against 5mM borate buffer for over night and lyophilized it and kept at refrigerator until use it.

Synthesis of de-dimethylaminotetracycline (DDATC) and test for the antibiotic activity: The dimethylamino group of the C_4 position of 'A' ring of the tetracycline molecule was chemically removed by modified method of Golub's (details of the method will be published separately). The modified chemical structure was identified by NMR spectroscopy comparing with the original tetracycline. Antibiotic activity was identified by testing minimal inhibitory concentration of the growth of *U. urealyticum, S. aureus* and *E coli.*

RESULTS

Purified-anti-cathepsin G antibody

In Vivo synthesized anti-Cathepsin G antibodies were purified effectively by a three steps procedure involving one ammonium sulfate precipitation, one DEAE-Cellulose ion exchange chromatography (Fig. 1) and one Cathepsin G bound Sepharose 4B affinity chromatography (Fig. 2). This antibody was cross reacted against human neutrophil cathepsin G when applied to the Ouchterlony immunodiffusion test.

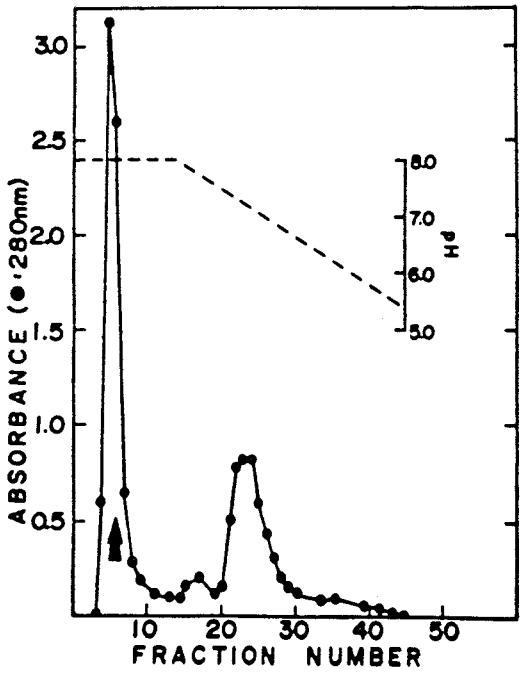


Fig. 1. Purification of anti-HNCG antibody by DEAE-cellulose.
The column(1 × 20cm) was equilibrated with 0.01 M phosphate buffer (pH 8.0). After sample (rabbit immunoglobulins) was applied to the top of the column, three column volume of phosphate buffer was eluted then pH and linear concentration gradient (from 0.01 M phisphate, pH 8.0 to 0.4 M phasphate, pH 5.4) was started (---). Flow rate was 7drops/sec and 3ml of eluent was collelcted in each tube. (—): protein profile (280 nm)

Inhibition of cathepsin G by different agents

We tested the inhibition effects of three different groups of drug including NSAIDs, antibiotics and extract & fractions of Korean Red Ginseng on the activity of Cathepsin G. Effects of inhibition of Cathepsin G by NSAIDs showed in Table 1. Phenylbutazone, oxyphenbutazone, sulindac, salicylic acid and salicyluric acid inhibited HNCGs effectively at the concentration of therapeutic range (rheumatoid arthritis), i.e., IC_{50}s of those drugs at our specific experimental condition were less than 1mM, and inhibitions of HNCGs by indomethacin, acetylsalicylic acid, ketoprofen, zomepirac sodium, ibuprofen, tolmetin, naproxen and phenacetin were neglingible.

Inhibitions of cathepsin G by Korean Red Ginseng showed in Fingure 3. Inhibitions of human neutrophil Cathepsin G by lipid soluble fraction of Korean Ginseng extract was impres-

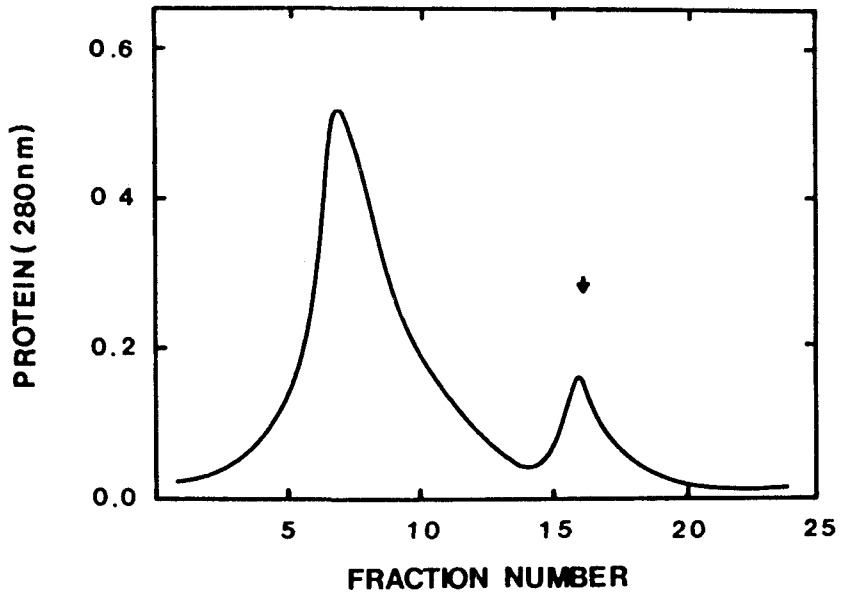


Fig. 2. Purification of anti-HNCG antibody by sepharose 4B affinity chromatography.
Cathepsin G labled Sepharose 4B was packed in the column(0.3×5 cm) and the column was equilibrated with 0.1M borate buffer, pH 8.0, containing 0.5 M NaCl. After sample was applied, the column was washed with three column volume of same buffer, then elution was started with 0.1M glycine, pH 2.2, containing 0.5M NaCl. Flow rate was 1drop/14 second.

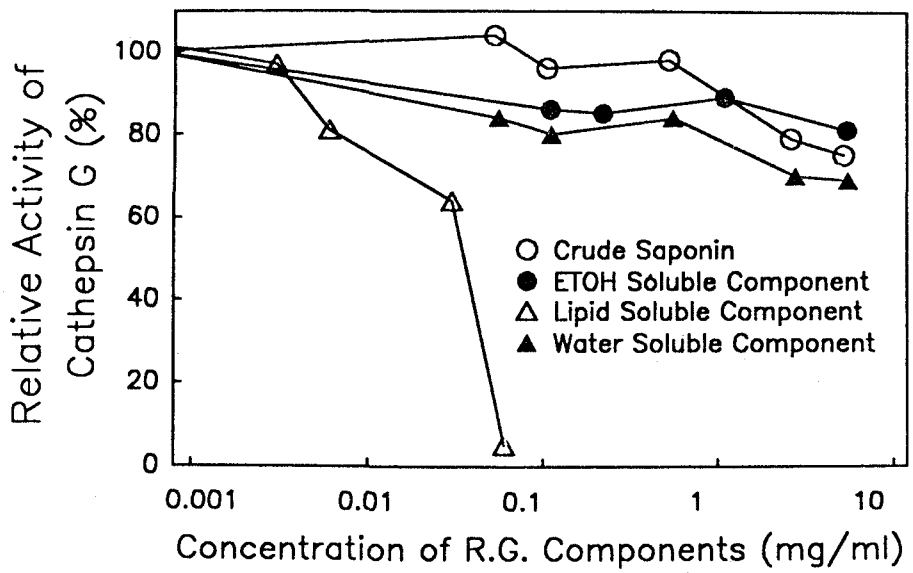


Fig. 3. Inhibition of HNCGs By Korean Red Ginseng components.

sive since the enzyme activity was inhibited more than 95% at the concentration of less than 0.1mg/ml. Inhibition effects of crude saponin, ethanol soluble fraction and water soluble component of Ginseng extract on the activity of HNCGs were negligible even at the concentration of 10mg/ml.

To test antibiotic effect on the microorganisms, equivalent minimal inhibitory concentrations of tetracycline derivatives against *S. aureus, U. urealyticum*, and *E. coli* were measured and the results are in Table 2. The derivatives which were modified or removed the dimethylamino radical from the 4 position of tetracycline were loosed the antibiotic activities completely.

Effects of Inhibition of the activity of Cathepsin G by antibiotics are in Table 3. Antibiotics tetracycline, oxytetracycline and demeclocycline inhibited HNCGs effectively. Their IC_{50}s, at the specific our experimental condition, were less than 1mM. Chlortetracycline, rifamycin and novobiosin also inhibited HNCGs reasonably well. Their IC_{50}s were 1.1, 1.5 and 2.6 respectively. Rest of antibiotics including cefamandole, nystatin, nalidixic acid, amphotericin B, penicillin G, cloxacillin, cefzoline, cefaperazone, cycloserine, chloramphenicol, erythromycin, kanamycin, spectinomycin, neomycin, gentamycin, amikacin and vancomycin showed very little or no effect on the activity of human neutrophil cathepsin G.

Effects of tetracyclines on the activity of HNCGs showed in Figure 4. De-dimethylaminotetracycline (Fig. 5), methiodided tetracycline and tetracycline (Fig. 5) inhibited HNCGs effectively. Especially, inhibition of HNCGs by dedimethylaminotetracycline was most effective

Table 1. IC_{50} of NSAIDs on the activity of HNCGs

Drug	IC_{50}(mM)
Phenylbutazone	0.3
Oxyphenbutazone	0.5
Sulindac	0.4
Salicylic Acid	0.7
Salicyluric acid	0.8

IC_{50}(concentration of inhibitor giving 50% inhibition) was determined from a plot of % inhibition versus log inhibitor concentration.

Table 3. IC_{50} of antibiotics on the activity of HNCGs

Antibiotics	IC_{50}(mM)
Cefamandole	9.6
Tetracycline	0.9
Chlortetracycline	1.1
Oxytetracycline	0.9
Demeclocycline	0.4
Novobiocin	2.6
Rifamycin	1.5

IC_{50}(concentration of inhibitor giving 50% inhibition) was determined from a plot of % inhibition versus log inhibitor concentration.

Table 2. Equivalent Minimal Inhibitory Concentration of tetracycline derivatives on *U. urealyticum. S. aureus* and *E. coli*

Tetracycline derivatives	MIC(ug/ml)		
	U. urealyticum	*S. aureus*	*E. coli*
Tetracycline	0.87	0.78	1.56
Tetracycline methiodide	100	50	100
De-dimethylamino-tetracycline	>100	>100	>100

S. aureus and *E. coli* were incubated in 2ml of brain heart infusion broth for 24 hours at 37°C, and 0.1 ml of these microorganisms were incubated in 5ml of same medium for 6 hours, then adjust the number of microorganisms to 10^3/ml and utilized for testing minimum inhibitory concentration of drugs. *U. urealyticum* was incubated in 2ml of 10-B broth at 37°C for 24 hours, then number of the microorganism was adjusted to 10^{-1}-10^{-6} by serial dilution and final concentration of the number of microorganism was adjust to 10^3CCU/ml by calculating the color changing unit(CCU). MICs were tested by serial dilution method.

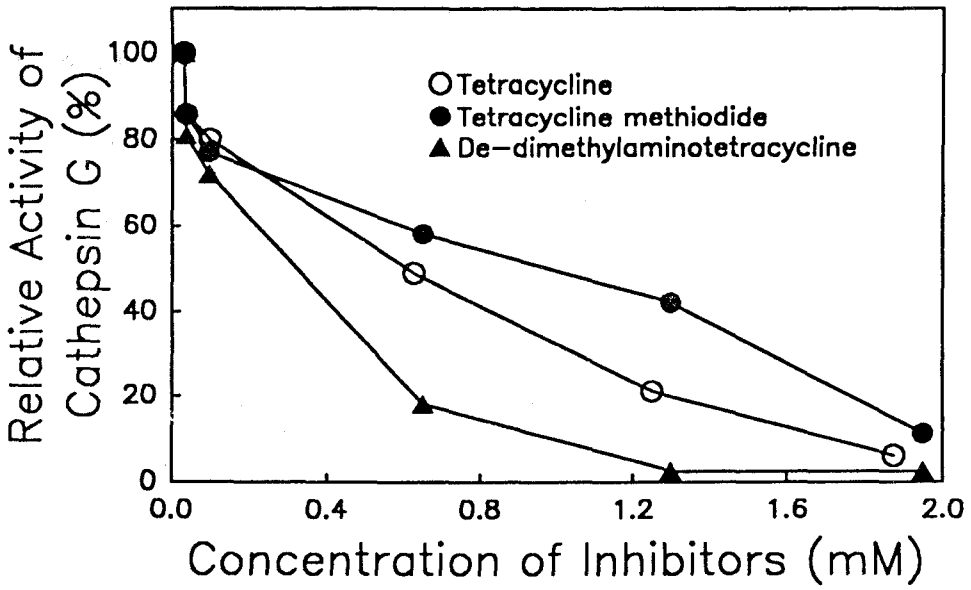
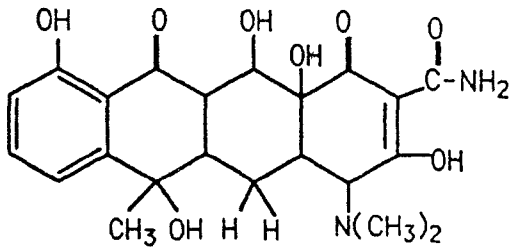
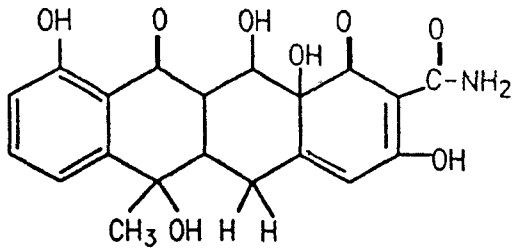


Fig. 4. Inhibition of HNCGs by tetracyclines.



tetracycline



De-dimethylaminotetracycline

Fig. 5. Structure of tetracycline and De-dimethylamino-tetracycline.

as seen in the Figure 4, the activity of HNCGs was inhibited up 80% at the concentration of 0.6 mM. Tetracycline and tetracycline methiodide also inhibited the activity of HNCGs as much as almost half of the control activity at the concentration of 0.6 mM.

DUSCUSSION

It has been known that mass of blood rushing accompanied infiltration of mass of fluid and high density of neutrophils to the inflammatory sites. One of the effects of dilatation of blood vessels and infitration of fluid caused by the prostaglandins is effectively blocked by inhibition of fatty acid cyclooxygenases with NSAIDs including indomethacin and aspirin. On the other hand, it has been known that migration of the neutrophil to the specific site is caused by the chemotactic factors like microorganism or chemicals invaded to the local site and the migrated neutrophils attack the foreign materials by phagocytic process or by secreting enzymes and/or disintegration processes of the neutrophils to liberate proteolytic and oxidative enzymes. At nor-

mal condition, secreted proteases are formed complex rapidly with antiprotease inhibitors which are circulated in the systemic blood. However, in the case of over secretion of proteases or individual who is genetically defected not to produce enough antiproteases in the systemic circulation, the secreted residual proteases can cause host tissue destruction at the secreted site. At this point of view, neutrophil proteases like elastase and cathepsin G are well known as causative enzymes in tissue destructive inflammatory diseases and the enzyme involvement on the tissue destruction is direct hydrolysis of intact host tissue (Glynn, 1972; Janoff, 1972a; Starkey, 1980; Cohen, 1983). When we considered the causes of inflamation, one of the logical approaches for treatment of the inflammatory diseases would be inhibition of the neutrophil proteases, including elastase and cathepsin G. We attempt to investigate the inhibition effects of NSAIDs and Korean Red Ginseng since they are known as a class of drug to treat rheumatoid arthritis and /or inflammation. Futhermore, we attempted to investigate whether the antibiotics have another direct effect at the infected site as an agents having antiprotease activity to protect the host tissue or not. In our experiment, we found that there are two different classes of NSAIDs, i.e., one group of NSAIDs (phenylbutazone, oxyphenbutazone, sulindac, salycyluric acid and salicylic acid) which inhibited HNCGs effectively at the concentration of μM range, and the other group of NSAIDs which showed negligible or no inhibition effect on the activity of HNCGs. From this result, we cordially suggest that NSAIDs which showed effective inhibition effect on the activity of HNCGs possibly have an additional mechanism of action of those specific drugs on the treatment of inflammatory disease beside of the known mechanism of action as theinhibitors of fatty acid cyclooxygenase activity to block the synthesis of prostaglandins. This similar inhibition effects also showed on the activity of neutrophil elastase (Ghim *et al.*, 1989). The mechanism of inhibition effects of NSAIDs on the activity of HNCGs were not much clear yet. However, we speculated that the inhibition effect may not be related to the main structure of the drugs rather related to the side chain, especially, carboxyl radical and hydroxy radical. Because, structurally different drugs; salicylic aicd and phenylbutazone which have intact carboxyl radical, showed similar inhibition effects on the activity of HNCGs, however, aspirin which has intact carboxyl radical and the hydroxyl radical was masked with acetyl radical, did not show the inhibition effect on the activity of HNCGs, even though the main structure is similar to salicylic acid. Still we have to work on the details on the mechanism of action of NSAIDs on the activity of the HNCGs.

Another agent like Korean Red Ginseng (KRG) have been known as an antiinflammatory agent (Han *et al.*, 1972) and we attempted to investigate the mechanism of action of KRG on the antiinflammatory effect, i.e., to find out how the Korean Red Ginseng affects to the activity of HNCGs. We tested four different preparations of KRG, i.e., crude extract, ethanol soluble fraction, lipid soluble fraction and water soluble fraction. Lipid soluble fraction inhibited the activity of HNCGs very effectively (Fig. 3). Still we do not know the chemical components of the lipid soluble fraction, however, this fraction inhibited cathepsin G almost 97% of the original activity at the concentration of 80 μg/ml. On the other hand, other fractions showed no effect or the inhibition effects were negligible at the concentration of 10mg/ml. It is not clear yet the reasons why crude extract did not showed the inhibition effect even though it contained the lipid soluble components in the fraction. We speculated, however, that the concentration of the lipid soluble components in the crude extract could not be enough to show the inhibition effect, or the concentration of the other three components might be much higher than the lipid soluble components and the lipid soluble components might have less possibility to have molecular interaction with the molecules of HNCGs.

Antibiotics are common agents for treatment of the infections of microorganisms. The mechanisms of action of different class of antibiotics are unique. For instance, inhibition of cell wall synthesis or inhibition of protein synthesis or misleading of mRNA codon or detergent effect on the membrane. However, the properties of inhibition of the activity of HNCGs were not corresponded to the mechanism of action of different class of antibiotics, i.e., the antiprotease activity was mixed results comparing the activity of specific class of the antibiotics with the

effect of same drug to the HNCGs. From this results we suggested that some antibiotics which showed the anti-cathepsin G activity must have antiinflammatory effect on the infected sites, especially, tetracyclines showed strong inhibition effect on the activity of the HNCGs. The inhibition effect of tetracyclines might be related the chelating property of tetracyclines. Similar effect was shown in human neutrophil elastase, i.e., it was inhibited by the chelating agents and tetracyclines (Kang, 1985; Ghim *et al.*, 1989). It was another interesting point to investigate whether the active site for the antibiotic effect is the same site for the inhibition of the activity of the HNCGs or not. To investigate this question, we synthesized dedimethylaminotetracycline (Fig. 5) and the intermediate substance, tetracycline methiodide, and examined the inhibition effect of these compounds on the activity of HNCGs. Both of the tetracycline and de-dimethylaminotetracycline which was removed the active antibiotic site and not shown any antibiotic effect (Table 2), inhibited the activity of HNCGs effectively. Futhermore, tetracylcline methiodide, an intermediate compound, did show strong inhibition effect on the activity of the HNCGs (Fig. 4). This experimental result was the solid evidence that the active site for the antibiotic effect was not the same active site for the inhibition of the activity of the HNCGs.

These results strongly indicated that NSAIDs which showed inhibition effect on the activity of HNCGs possibly be at least a part of mechanism of action which might be related to direct inhibition of cathepsin G at the tissue destruction sites beside of their known mechanism of action as an anticyclooxygenase in treatment of inflammatory diseases. Antibiotics like tetracyclines, which showed strong anti-Cathepsin G activity, might have antiinflammatory activity in part at the sites infected by microorganisms beside of the direct antibiotic effects against the microorganisms. Furthermore, N-dedimethylated tetracyclines may have beneficial effect for long term treatment of chronic inflammatory diseases without developing any drug resistance to microorganisms.

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=국문초록=

사람 호중구 Cathepsin G: Anti-HNCG Ab의 In Vivo 합성, HNCG의 활성도 억제와 그 기전에 관한 연구

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배성준 · 김우미 · 김기찬 · 장명웅 · 강구일

염증성 질환의 원인 인자중 하나로 알려진 사람 호중구 Cathepsin G를 두단계의 크로마토그라피를 거쳐 분리하였다. 이 순수 분리된 효소를 이용하여 토끼에서 항체를 In Vivo 합성하고 그 혈액으로부터 순수 항체를 분리하였다. NSAIDs 약제중 phenylbutazone, sulindac, oxyphenbutazone, salicilic acid등은 이 효소를 강력하게 억제하였으며 IC_{50}은 0.3~0.8 mM 이었다. 고려인삼의 지용성분획도 tetracycline, novobiosin, rifamycin이 Cathepsin G의 효소 활성도에 대해서 강력한 억제 작용을 나타내었으나 다른 항생제는 그 작용이 무시할 수 있을 정도였다. 그러나 tetracycline계열의 항생제의 경우 실제 치료 효과를 나타내는 혈중농도에서 강한 억제 작용을 보였다. 특히 항균 작용과 관계하는 tetracycline의 4번 위치의 N-dimethy radical을 제거한 tetracycline은 감염균의 약제 저항성을 피할 수 있을 것으로 생각되므로 만성 염증성질환의 장기 치료에 이용될 수 있는 새로운 약제로써 제시한다.