様式（５）

研究内容報告書

報告番号	甲医第　　６５４　　号	氏名	大日　輝記

寄生虫感染時の免疫応答には大きく分けて２つの型がある。蠕虫感染では Th2 型の免疫応答が優位となり、血清総 IgE 量の上昇、好酸球血症、肥満細胞増多などが特徴的に観察される。いっぽう、原虫感染においては、Th1 型の免疫応答が主導となり細胞性殺寄生虫反応などによって寄生虫の排除にはたらく。申請者らは、リーシュマニア症の感染モデルにおいて、感染感受性マウスに対しリソソーム内カテプシン B 選択的阻害剤である CA074 を投与することで免疫応答が Th2 型から Th1 型へと変化し感染抵抗性になることを発見した。申請者らはさらに、卵白アルブミン免疫マウスにおいても同様に CA074 の投与で抗体産生などの免疫応答が Th2 型から Th1 型へと変化することを確認し、この現象がより普遍性を持つものであることを証明した。これらのことから、ある種の寄生虫感染に特徴的な免疫応答が抗原プロセッシングに関わる宿主リソソーム内酵素の種類によって選択的に誘導される可能性が考えられた。

いっぽう申請者らは、リーシュマニア感染マウスに対しリソソーム内カテプシン D 阻害剤であるペプスタチン A を投与した場合に Th1 型・ Th2 型双方の免疫応答が低下することを発見した。 この現象も卵白アルブミン免疫マウスにおいて同様に観察された。これが T リンパ球に対する直接的・非特異的機能抑制ではないことを確認し、さらに詳細な解析により、宿主細胞内で抗原提示分子の輸送・調節にはたらくインバリアント鎖の分解抑制によるものであることを解明した。

更に申請者らは、マウス・ラットの腸管内寄生性線虫である *Nippostrongylus brasiliensis* が、カテプシン B 等を阻害するシステインプロテアーゼインヒビターを分泌することを発見し、この分子を新規にクローニングしニッポシスタチンと名付けた。ニッポシスタチンは試験管内でリソソームによる卵白アルブミンのプロセッシングを阻害した。ニッポシスタチンは卵白アルブミン免疫マウスへの生体内投与で脾細胞の抗原特異的増殖反応及びサイトカイン分泌を抑制し、抗原特異的 IgE 産生も低下させた。申請者らは *N. brasiliensis* が宿主内でこの分子を分泌することで抗原プロセッシングを変化させ、宿主免疫を調節している可能性を示唆した。

以上申請者らは、寄生虫感染における宿主の免疫応答がリソソーム酵素の種類と機能によって異なるだけでなく、宿主リソソーム酵素が寄生虫による免疫回避・修飾機構の標的となりうることを明らかにした。

様式（6）

業　績　目　録

報告番号	甲医第　　654　　号	氏名	大日　輝記

学位論文

題目：

Molecular cloning of a cystatin from parasitic intestinal nematode, *Nippostrongylus brasiliensis.*

(腸管内寄生性線虫 *Nippostrongylus brasiliensis* 由来シスタチンの分子クローニング)

著者：

大日輝記、前川洋一、石井一成、姫野國祐

平成 13 年 2 月公刊　The Journal of Medical Investigation 第 48 巻第 1, 2 号に掲載予定

参考論文

1．

題目：

Switch of $CD4^+$ T cell differentiation from Th2 to Th1 by treatment with cathepsin B inhibitor in experimental leishmaniasis.

著者

Yoichi Maekawa, Kunisuke Himeno, Hiroyuki Ishikawa, Hajime Hisaeda, Tohru Sakai, Teruki Dainichi, Tetsuji Asao, Robert A. Good, and Nobuhiko Katunuma

平成 10 年 9 月　J Immunol　第 161 巻第 5 号

2120 ページから 2127 ページに発表済

概要：

抗原分解に関与するプロテアーゼについては十分に明らかでない。リソソーム酵素のカテプシン B 特異的阻害剤である CA074 をリーシュマニア感染感受性の BALB/c マウスに投与したところ感染抵抗性となり免疫応答が Th2 型から Th1 型へと変化した。CA074 は試験管内でも抗原分解を阻害した。これらのことはカテプシン B 阻害剤が CD4 陽性細胞を Th2 から Th1 に移行させうることを示しており、抗原分解が Th 分化の方向性を左右することが示唆された。

様式（6）

2．

題目：

Lysosomal cathepsin B plays an important role in antigen processing, while cathepsin D is involved in degradation of the invariant chain in ovalbumin-immunized mice.

著者：

T. Zhang, Y. Maekawa, J. Hanba, T. Dainichi, B. F. Nashed, H. Hisaeda, T. Sakai, T. Asao, K. Himeno, R. A. Good & N. Katunuma

平成 12 年 5 月　Immunology　第 100 巻第 1 号

13 ページから 20 ページに発表済

概要：

我々は OVA 免疫マウスで CA074 が BALB/c マウスの特異的免疫応答を Th2 型から Th1 型へと変化させ、カテプシン D 特異的阻害剤であるペプスタチン A が Th1, Th2 両方の免疫応答を抑制することを観察した。試験管内で CA074 は OVA の分解を阻害し、ペプスタチン A は不変鎖の分解を阻害した。したがってカテプシン B が Th2 の誘導に関与し、、カテプシン D が Th1, Th2 両方の抗原特異的免疫応答に不可欠であることが示唆された。

その他の学術論文

なし

学会発表

題目：

線虫由来システインプロテアーゼインヒビターの分子クローニングと抗原プロセッシングへの影響

発表者：

大日輝記、前川洋一、石井一成、張天塹、Baher Fawzy Nashed、姫野國祐

第 30 回日本免疫学会総会・学術集会　平成 12 年 11 月 16 日発表

概要：

我々は *N. brasiliensis* の分泌抗原が試験管内でカテプシン L の活性を阻害することを発見し、*N. brasiliensis* の cDNA からシステインプロテアーゼインヒビターの遺伝子をクローニングした。このことから *N. brasiliensis* が宿主の体内でシステインプロテアーゼインヒビターを分泌することで宿主の抗原プロセッシングを変化させ、宿主免疫の方向付けに影響している可能性が示唆された。

様式（6）

その他

1．

題目：

線虫由来プロテアーゼ阻害物質の抗原提示への影響

著者：

大日輝記、姫野國祐

平成13年4月公刊　臨床免疫　第35巻第4号に掲載予定

2．

題目：

細胞内寄生性原虫の免疫回避機構

著者：

大日輝記、姫野國祐

平成11年10月　医学のあゆみ　第191巻第1号

119ページから123ページに発表済

所属学会

1．日本免疫学会　　平成8年　入会

2．日本寄生虫学会　平成12年　入会

様式（7）

論 文 内 容 要 旨

題　目　Molecular cloning of a cystatin from parasitic intestinal nematode, *Nippostrongylus brasiliensis.*
(腸管内寄生性線虫 *Nippostrongylus brasiliensis* 由来シスタチンの分子クローニング)

著　者　大日輝記、前川洋一、石井一成、姫野國祐

平成 13 年 2 月公刊　The Journal of Medical Investigation
第 48 巻第 1, 2 号に掲載予定

内容要旨

シスタチンスーパーファミリーはシステインプロテアーゼに対して阻害活性を有するタンパク群で、構造と局在によりファミリー1（ステフィンファミリー）、2（シスタチンファミリー）、3（キニノーゲンファミリー）の3つに分けられる。このうちファミリー2に属するシスタチンは分子量 13-15kDa の分泌型タンパクで、線形動物では寄生性線虫のフィラリアの一部や自由生活線虫である *Caenorhabditis elegans* での発現が報告されている。*Nippostrongylus brasiliensis* はヒト糞線虫に類似した生活史をもち、ラット、マウスを宿主とする腸管内寄生性線虫である。今回我々は、*N. brasiliensis* の分泌・抽出抗原中より新規シスタチンをクローニングし、ニッポシスタチンと名付けた。

我々はこれまでに報告された線虫由来シスタチンで保存された DNA 配列をもとにプライマーを設計し RT-PCR 法と 3' RACE, 5' RACE 法を用いて *N. brasiliensis* より 435 bp の cDNA をクローニングした。この cDNA がコードするタンパクは 144 アミノ酸からなり、N 末端にシグナルペプチドを有する分泌型タンパクであった。またシスタチンスーパーファミリーに共通した配列である Gln-Val-Val-Ala-Gly (QVAAG)を持ち、*C. elegans* やその他の線虫由来シスタチンに対し1次構造で高い類似性を示した。これらのことから我々はこのタンパクをシスタチンファミリーに属するタンパクであると考え、ニッポシスタチンと名付けた。ニッポシスタチンは *N. brasiliensis* の感染性第3期幼虫および成虫の両方で mRNA の発現が確認された。

我々はニッポシスタチン組み替えタンパクを作成しさらに解析を進めた。イムノ

ブロット法により、ニッポシスタチンは 14-kDa の成熟型として成虫の分泌・抽出抗原中に認められた。組換えニッポシスタチンはカテプシン L、カテプシン B といったシステインプロテアーゼの酵素活性を著明に阻害したが、カテプシン D のようなアスパラギン酸プロテアーゼは阻害しなかった。さらに成虫の分泌・抽出抗原もシステインプロテアーゼの酵素活性を阻害することが確認された。

ニッポシスタチンの一次構造はこれまでに報告されたシスタチンのなかでも *C. elegans* 由来シスタチンに最も近く、このことは分類学的にも矛盾しない。いっぽう、シスタチンが免疫担当細胞の機能に影響することが卵白シスタチンやフィラリア由来シスタチンの系で報告されている。我々はシステインプロテアーゼによる抗原分解が、Th1, Th2 と呼ばれるヘルパー T リンパ球の機能的分化に大きく影響することをすでに報告している。システインプロテアーゼは感染宿主において抗原分解や抗原提示など抗原特異的免疫応答に不可欠な部分に機能することが知られている。したがって、*N. brasiliensis* がニッポシスタチンの分泌を介して宿主免疫応答を変化させ、宿主からの攻撃を回避していることは十分に考えられる。

Molecular cloning of a cystatin from parasitic intestinal nematode, *Nippostrongylus brasiliensis*.

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Abstract : A novel member of the cystatin family, nippocystatin (NbCys), was identified from excretory-secretory (ES)-products of a nematode *Nippostrongylus brasiliensis*, and the cDNA was cloned and sequenced. The mRNA of NbCys was confirmed to be expressed in both larvae and adults of the parasite. NbCys was translated as a proform with a single domain for secretion and was detected as a 14-kDa mature form in ES-products of the adult worm. Recombinant protein of NbCys profoundly inhibited the activity of cysteine proteases such as cathepsin L and B, but not that of cathepsin D, an aspartic protease. Furthermore, the ES-products had also been confirmed to inhibit cysteine proteases. Taken together, NbCys may play a role in evasion of *N. brasiliensis* from host defense systems, since cysteine proteases are known to participate in immune systems of infected hosts.
J. Med. Invest. 48 : ●-●, 2001

Key words : *Cystatin, Nippostrongylus brasiliensis, cloning*

INTRODUCTION

Natural cysteine protease inhibitors are part of the cystatin superfamily, which is subdivided into families 1, 2 and 3, or the stefin family, cystatin family and kininogen family, respectively (1). There is high sequence homology among all of these families, particularly with respect to a highly conserved, proposed reactive site consisting of 5 amino acids, Gln-Val-Val-Ala-Gly (QVVAG) (2). Members of family 2, the cystatin family, are secretion-type proteins that have a single domain with a molecular weight of 13-15-kDa. In nematodes, members of this family have been identified from *Caenorhabditis elegans* (3) and some species of filaria (4-7).

Nippostrongylus brasiliensis, from the order Rhabditida, generally produce acute infections in rodents, and have life cycles similar to trichostrongyle parasites that infect the small intestines of humans and livestock (8). Although *N. brasiliensis* is supposed to express cystatin, there is no report of cystatin derived from this nematode.

We here describe a newly defined, 14-kDa nippocystatin (NbCys) belonging to the cystatin family 2 and derived from *N. brasiliensis*. This was a secretion-type cystatin, and its recombinant protein selectively inhibited cysteine proteases. Cysteine protease is indispensable for antigen-specific immunity because of its function in antigen processing and presentation (9,10). Therefore, this parasite may require this protease inhibitor to evade the host defense system via modulation of the host immune responses.

MATERIALS AND METHODS

1. Parasites

The strain of *Nippostrongylus brasiliensis* was provided by Dr. M. Yamada, Kyoto Prefectural University of Medicine, Kyoto, Japan (11). Parasites were maintained by serial passage in SD rats. Excretory-secretory (ES)-products of adult *N. brasiliensis* were collected by the protocol described below. Adult worms of *N. brasiliensis*, collected from the small intestine of rats that had been infected 7 d previously with 4,000 infective-stage larvae per animal, were sterilized by

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repeated washing with PBS containing penicillin and streptomycin. Worms were cultured with PBS at 37℃ for 6 hr. Culture supernatant was collected and concentrated using Centricon Plus 20 PL-10 centrifuge (Millipore, Bedford, MA).

2. cDNA cloning of NbCys

Total RNA was isolated from adult worms of *N. brasiliensis*, prepared by the method above, using Trizol reagent (Life Technologies, Rockville, MD). A fragment of NbCys cDNA was obtained by RT-PCR. The total RNA was reverse-transcribed using hexanucleotide random primers with Superscript II reverse transcriptase (Life Technologies). Then the cDNA was amplified with Taq DNA polymerase (Takara Shuzo, Shiga, Japan). The thermocycle conditions were 35 cycles of 94℃ for 30 sec, 52℃ for 30 sec and 72℃ for 30 sec. The sense and antisense primer sequences were 5'-TCATCTCAAGTTGTCGCTGGT-3' and 5'-AAATTTTCCCATGGTTTCTCCCA-3', the designs of which were based on conserved sequences among previously defined cystatin from other nematodes, *Onchocerca volvulus* (4), *Brugia malayi* (7), *Acanthocheilonema viteae* (6) and *Caenorhabditis elegans* (3). Amplified DNAs were resolved by 2-3% agarose gel electrophoresis and stained with ethidium bromide. The DNAs were extracted using Qiaex II gel extraction kit (Qiagen, Hilden, Germany) and then subcloned into pGEM-T easy vector (Promega, Madison, WI). Plasmids were extracted using a Qiaprep spin miniprep kit (Qiagen). The DNA sequences were defined using ABI PRISM BigDye terminator cycle sequencing ready reaction kit (Perkin Elmer, Norwalk, CT) and ABI PRISM 377 DNA sequencer (Perkin Elmer). On the basis of the nucleotide sequence of this cDNA fragment, specific primers were then synthesized for 3'- and 5'-RACE. For 3'-RACE, total RNA from adult *N. brasiliensis* was reverse-transcribed using Not I primer-adapter with dT-polymer (Life Technologies). The adapter-added cDNA was amplified with Taq DNA polymerase. The thermocycle conditions were 35 cycles of 94℃ for 1 min, 64℃ for 1 min and 72℃ for 1 min. The sense primer sequence was 5'-GCAAGCGAACTTACGGCGACGA-3', and the antisense primer was the adapter primer. For 5'-RACE, total RNA from adult *N. brasiliensis* was reverse-transcribed using the gene specific primer, 5'-TGGCAGTTCGTCGCCGTAAGTTCG-3'. The dC-polymer was added to the resultant RT-product with terminal deoxynucleotidyltransferase (TdT) and was amplified with Taq DNA polymerase. The thermocycle conditions were 30 cycles of 94℃ for 1 min, 54℃ for 1 min and 72℃ for 1 min. The sense primer was the adapter primer with dG-polymer (Life Technologies) and the antisense primer was 5'-TCGCCGTAAGTTCGCTTGCAG-3'. The DNA sequences of the RACE-PCR products were defined as written above.

3. Evaluation of cystatin mRNA expression

Total RNA from adult worm and from third stage larvae were extracted with Trizol reagent and were reverse-transcribed using hexanucleotide random primers with Superscript II. Then the cDNAs were amplified with Taq DNA polymerase. The thermocycle conditions were 35 cycles of 94℃ for 1 min, 54℃ for 1 min and 72℃ for 1 min. The sense and antisense primer sequences were 5'-ATGCCATCTGCGTTCGTTCTG-3' and 5'-TTAAACCTGCTCTCCTGGAGC-3'. The amplified DNAs were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized with UV light.

4. Expression of rNbCys

The NbCys cDNA was subcloned into the expression plasmid vector pET and expressed in *E. coli* strain BL21 as a protein fused to a leader sequence of influenza virus HA epitope and six histidines. rNbCys was purified from *E. coli* lysate by affinity chromatography using TALON resin (Clontech Laboratories Inc., Palo Alto, CA). The purity of rNbCys was determined by SDS-PAGE and staining with Coomassie brilliant blue (CBB). Purified rNbCys was washed and dialyzed with PBS.

5. Measurement of protease activities and their inhibition

The activity of cysteine proteases was measured as follows. Protease was incubated with or without NbCys in 0.1 M sodium acetate buffer [pH 5.5] containing 1 mM EDTA, 8 mM cysteine and 20 μM Z-Phe-Arg-MCA (Peptide Institute, Osaka, Japan) as substrate at 37℃ for 15 min. The reaction was stopped by addition of 100 mM monochloroacetate [pH4.3]. The amount of product was monitored fluorometrically with excitation at 370 nm and emission at 460 nm in a fluorescence spectrometer (Hitachi, Ibaraki, Japan). Activity of cathepsin D was measured in a Folin-Lowry reaction, as reported previously (12).

RESULTS

The cystatins identified from parasitic nematodes are all from species of the superfamily Filarioidea,

namely filaria. Although cystatin has also been identified in *C. elegans* by analysis of genomic sequence, this nematode is free living. All of the cysteine protease inhibitors from nematodes reported to date belong to the cystatin family. To obtain a homologue of cystatin from *N. brasiliensis*, we amplified cDNA by PCR using primers of a consensus sequence found among nematode cystatins. A PCR product of 174 bp was amplified as expected.

On the basis of the sequence of this cloned cDNA fragment, the full length of the cDNA was cloned by using 3'-RACE and 5'-RACE (Fig. 1). The length of the open reading frame was 435 bp and the protein consisted of 144 amino acids. An amino acid alignment of the protein with cystatins from other nematodes using the BLASTP program with the sequence databases at NCBI revealed the following similarities : two cystatins from *C. elegans* (72% and 63%), onchocystatin from *Onchocerca volvulus* (55%), *Acanthocheilonema viteae* cystatin (33%), *and Brugia malayi* cystatin (30%). The amino acid sequence of NbCys showed a lesser similarity to rat cystatin C (27%). This cloned protein had a stretch of 5 amino acids, QVVAG, which is highly conserved in the cystatin superfamily, and had a hydrophobic signal peptide like other members of cystatin family 2 (Fig. 2). We concluded that this newly defined molecule belongs to family 2, and named this molecule nippocystatin (NbCys).

We evaluated whether NbCys mRNA is expressed in infective third stage larvae as well as in adult worms by RT-PCR. The open reading frame of NbCys cDNA was amplified as a 435 bp band from total cDNA of both larvae and adult worm (Fig. 3). These findings indicate that NbCys was expressed at the mRNA level at both developmental stages.

```
        10         20         30         40         50         60
CTTTATGGGCGGGTGCGTTCAGTAAACATGCCATCTGCGTTCGTTCTGCGTATTGCTTTA
                              M  P  S  A  F  V  L  R  I  A  L

        70         80         90         100        110        120
GCATCGGTTGTGGTTACCAGCACAGTATCATCAATGGTTGGCGGTTTCACTCCTCAGGAT
 A  S  V  V  V  T  S  T  V  S  S  M  V  C  G  F  T  P  Q  D

        130        140        150        160        170        180
GTGTCTGACCCAGAGTACATGACACGAGCATGGAAGGCTGCGAAGGGAATTAATGATGAC
 V  S  D  P  E  Y  M  T  R  A  W  K  A  A  K  G  I  N  D  D

        190        200        210        220        230        240
GCGTCCAATGAGGGACCATATCATATGATTCCAGTCAAGATTTTAAACGCGAAGACTCAA
                                                  PCR >>>>>>>>>
 A  S  N  E  G  P  Y  H  M  I  P  V  K  I  L  N  A  K  T  Q

        250        260        270        280        290        300
GTGGTCGCCGGAGTGAACCATGTTTTCGAAGTTCTTTTCGGAGAATCTAGCTGCAAGAAA
>>>>>>>>>>>>
 V  V  A  G  V  N  H  V  F  E  V  L  F  G  E  S  S  C  K  K

        310        320        330        340        350        360
GGCGACCTATCTGCAAGCGAACTTACGGCGACGAACTGCCAATTGAAGGAGGGAGGAAGG
          <<<<<<<<<<<<<<<<<<<<<<< 5'RACE
     3'RACE >>>>>>>>>>>>>>>>>>>>>>>>
 G  D  L  S  A  S  E  L  T  A  T  N  C  Q  L  K  E  G  G  R

        370        380        390        400        410        420
AAAGTGATCTACGAAGTGCATCTATGGGAGAAACCGTGGGAGAACTTTGAGCAGTTCAAT
                              <<<<<<<<<<<<<<<<<<<<<<<<< PCR
 K  V  I  Y  E  V  H  L  W  E  K  P  W  E  N  F  E  Q  F  N

        430        440        450        460        470        480
GTGAAGAAAGTTCGAACGCTTGCTCCAGGAGAGCAGGTTTAATAGACCGGCATACATATT
 V  K  K  V  R  T  L  A  P  G  E  Q  V  *

        490        500        510        520        530        540
TTAAATTCTACTCGCCAGTTATTCCTTTGTTGTGCTACTTTGAACACATGCAGTTACCGT

        550        560        570        580        590        600
TGTATCGGTCAACGTTTCAAGTGCGAACTGTACATTAGAGAATTCGTCTTATCTACTTAT

        610        620        630        640        650        660
CCGCACACATAAATTCGTTTCGTGTTTTGTACGCGTGAATGTCAAAGGGAAGATACAGGA

        670        680        690        700        710
TTGAAAAATTGTGAAAAAAATAAATATGAGGATTCCCGGGAAAAAAAAAA
```

Fig. 1. Sequence of nippocystatin cDNA. Conserved sequences of the cystatin superfamily are shaded. Gene-specific primers of PCR for cDNA cloning of nippocystatin are marked by (>>>) for the 5' primer and by (<<<) for the 3' primer. The signal peptide sequence predicted using the program SignalP v1.1 is underlined (13). The sequence data of nippocystatin are available from GenBank under accession number AB050883.

```
NbCys       1 ----------------------------MPSAFVLRIALASVVVTSTVSSMVCGFTPQDV  32
                                           .  *     .      .  **   .*
CeCys         ----------------------------M-KAILVFALIAISIISVNAGMMTCGSVEQDA
OvCys       MLTIKDGTLLIHLLLFSVVALVQLQGAKSARAKNPSKMESKTGENQDRPVLLCGWEDRDP

Cys.C,rat     ---------------------------------VLAVAWAGTSRPPPRLLCAPQEADA

NbCys      33 SDPEYMTRAWKAAKGINDDASNEGPYHMIPVKILNAKTQVVAGVNHVFEVLFGESSCKKG  92
              .. *.   .... ...*.......*.  *.* ..... *****.  . .  .*....* ***.
CeCys         SQKEYSDKAWKAVKGINDQASNNGPYYYAPIKVTKASTQVVAGISTKLEVLVGESNCKKG
OvCys         KDEEILELLPSILMKVNEQSNDE--YHLMPIKLLKVSSQVVAGVKYKMDVQVARSQCKKS

Cys.C,rat     SEEGVQRALDFAVSEYNKGSNDA--YHSRAIQVVRARKQLVAGINYYLDVEMGRTTCTKS

NbCys      93 DLSASELTATNCQLKEGGRKVIYEVHLWEKPWENFEQFNVKKVRTLAPGEQV          144
               . . ..*  ... . ..    ..*  ********... . . . .  ..
CeCys         ELQAHEITSSNCQIKDGGSRALYQVTIWEKPWENFEQFTVEKIRDVTADEQF
OvCys         SNEKVDLTKCKKLEGHPEKVMTLEV--WEKPWENFMRVEILGTKEVIK----

Cys.C,rat     QTNLT-NCP-FHDQPHLMRKALCSFQIYSVPWKGTHTLTKSSCKNA------
```

Fig.2. Alignment of the amino acid sequence translated from cDNA of nippocystatin (NbCys), *C. elegans* cystatin (CeCys), onchocystatin from *O. volvulus* (OvCys) and rat cystatin C (Cys.C, rat). Conserved sequences of the cystatin superfamily are shaded. Among three sequences of cystatin from nematodes, completely conserved amino acids are marked by an asterisk (*), and the partially conserved amino acids are marked by a period (.).

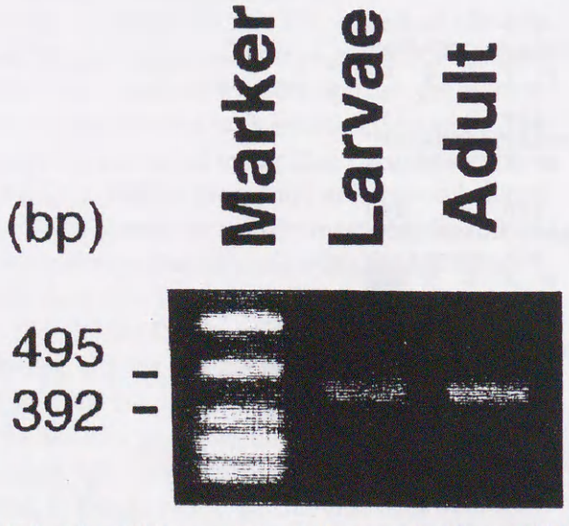

Fig.3. The mRNA expression of nippocystatin. The total RNA was extracted from adult and third stage larvae and the level of nippocystatin mRNA was determined by RT-PCR. The PCR products were resolved by 2% agarose gel electrophoresis, stained with ethidium bromide and visualized with UV light as a band with a size of 435 bp.

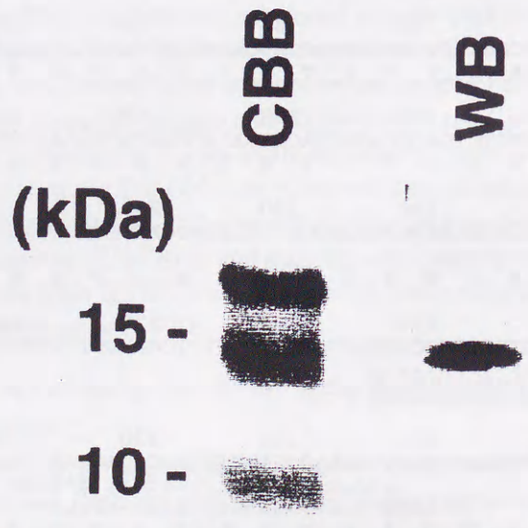

Fig.4. Nippocystatin contained in ES-products. ES-products were separated by SDS-PAGE [15%] and stained with CBB (left). Nippocystatin was detected by immunoblot analysis with rat anti-rNbCys antiserum (right).

Since the cDNA of NbCys encoded a hydrophobic signal peptide with 22 amino acids, NbCys was expected to be secreted as a 14-kDa mature form without a signal sequence. To confirm that NbCys is expressed as a mature form, we analyzed ES-products by immunoblotting with anti-recombinant NbCys (rNbCys) antiserum (Fig. 4). We detected NbCys as a 14-kDa band in ES-products, suggesting that NbCys is actually expressed as a protein of mature form.

To investigate the function of NbCys as a cystatin, we used rNbCys to examine the ability to inhibit several cathepsins (Fig. 5). rNbCys strongly inhibited the enzymatic activity of two cysteine proteases, cathepsin L and cathepsin B. Cathepsin L was inhibited at a lower concentration of rNbCys than cathepsin B. On the other hand, aspartic protease cathepsin D was

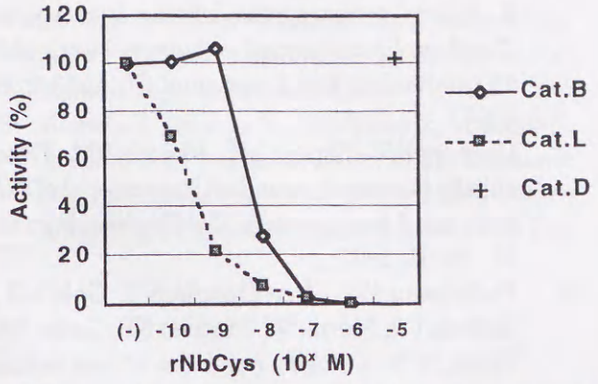

Fig.5. The inhibition of proteases by rNbCys. Purified cathepsin B (rhombus), cathepsin L (square) and cathepsin D (cross) were incubated with each of the substrates in the presence of various concentrations of rNbCys. Incubation of a cathepsin without rNbCys corresponds to 100% activity of the enzyme.

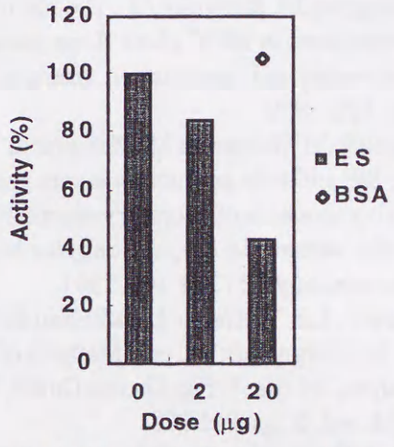

Fig.6. The inhibition of a cysteine protease by ES-products from *N. brasiliensis*. Purified cathepsin L was incubated with the synthetic substrate Z-Phe-Arg-MCA in the presence of various amounts of ES-products from *N. brasiliensis* or 20μg of BSA. Protease activity was assessed by subtraction of ES-derived activity. Incubation of cathepsin without ES-products corresponds to 100% activity of the enzyme.

not inhibited by rNbCys even at extremely high concentrations. These findings indicate that NbCys has the ability to inhibit cysteine proteases, like other members of the cystatin family.

To evaluate whether ES-products from *N. brasiliensis* actually have a component to inhibit cysteine proteases, we examined the ability of ES-products to inhibit cathepsin L as an index. When cathepsin L was incubated with ES-products, cathepsin L activity was inhibited in a dose-dependent manner (Fig. 6). This inhibitory activity in ES-products was not due to the large amount of protein, since an equivalent amount of BSA did not suppress the cathepsin L activity. NbCys was expressed in ES-products as a mature form (Fig. 3). These findings strongly suggest that the cysteine protease inhibitory component in ES-products is NbCys.

DISCUSSION

We newly cloned a cystatin from ES-products of intestinal nematode, *N. brasiliensis*. It consisted of 144 amino acids with a MW of 14-kDa and was named NbCys. NbCys was expressed in both third stage larvae and adult worm. Further, we synthesized its recombinant protein and confirmed that this protein exclusively inhibited cysteine proteases selectively. Immunoblot analysis showed that ES-products from *N. brasiliensis* contained NbCys. Furthermore, ES-products were confirmed to inhibit cysteine protease activity *in vitro*. These findings suggest that NbCys is secreted as a mature form and acts in the host.

Onchocystatin from the filarial parasite, *Onchocerca volvulus*, is the first cystatin to be identified in nematode (4). Onchocystatin belongs to cystatin family 2, having signal peptide and being expressed in third stage larvae and adults, suggesting that this protein is a secretion-type and has an extrasomatic function. Analysis of the genomic sequence in the free living nematode, *C. elegans*, indicated that this nematode has a cystatin encoded by two genes (3). The amino acid sequence of NbCys showed greater similarity to the sequence of two cystatins from *C. elegans* than to that of onchocystatin. These similarities do not contradict taxonomical findings, since both *N. brasiliensis* and *C. elegans* belong to the order Rhabditida, while *O. volvulus* belongs to the order Spiruria.

Recently there have been several reports that cystatin modulates immune responses. For example, chicken cystatin up-regulates NO-release from IFN-γ-activated mouse peritoneal macrophages via cytokine synthesis (14, 15). Cystatins from parasites have also been reported to show immunomodulatory effects. Recombinant cystatin from *Acanthocheilonema viteae* down-regulates T-lymphocyte proliferation and enhances IL-10 production *in vitro* (6). Furthermore, Garraud *et al.* reported that recombinant cystatin of *Onchocerca volvulus* induces either polyclonal or antigen-specific IgE and IgG4 antibodies *in vitro* in an IL-4-and/or IL-13-dependent manner (5). Thus, it is quite conceivable that NbCys also has immunomodulatory functions.

We previously reported that treatment of mice with CA074, a synthetic and specific inhibitor for cathepsin B, changes the immune response from a Th2- to Th1-type in BALB/c mice infected with *Leishmania major* (16) or immunized with ovalbumin (17). That is, treatment with CA074 suppresses IL-4-production

and augments IFN-γ-production. In general, host defense against helminth infection is mediated by Th2-type responses with IgE production, eosinophilia and mastocytosis (8). That NbCys has a similar effect as CA074 is quite reasonable since *N. brasiliensis* can defeat the host immunity by impairing the Th2-type responses.

ES-products of *N. brasiliensis* are well known as antigens that evoke an immune response in hosts infected with *N. brasiliensis* (11). Some functional proteins that induce the production of specific antibodies in infected rats have been isolated from ES-products of *N. brasiliensis* (18, 19). At present, however, although these products have been regarded as immunodominant antigens, their immunomodulatory effects have not been elucidated, (18, 19). Our results suggest that NbCys is a novel candidate for the immunomodulatory effector secreted by *N. brasiliensis*.

ACKNOWLEDGEMENTS

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様式（１０）

論　文　審　査　の　結　果　の　要　旨

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審査委員	主　査　松　本　　満 副　査　木　戸　　博 副　査　高　濱　洋　介		

題　目　Molecular cloning of a cystatin from parasitic intestinal nematode, *Nippostrongylus brasiliensis.*
(腸管内寄生性線虫 *Nippostrongylus brasiliensis* 由来シスタチンの分子クローニング)

著　者　大日輝記、前川洋一、石井一成、姫野國祐

平成 13 年 2 月公刊　The Journal of Medical Investigation
第 48 巻第 1, 2 号に掲載予定（主任教授　姫野國祐）

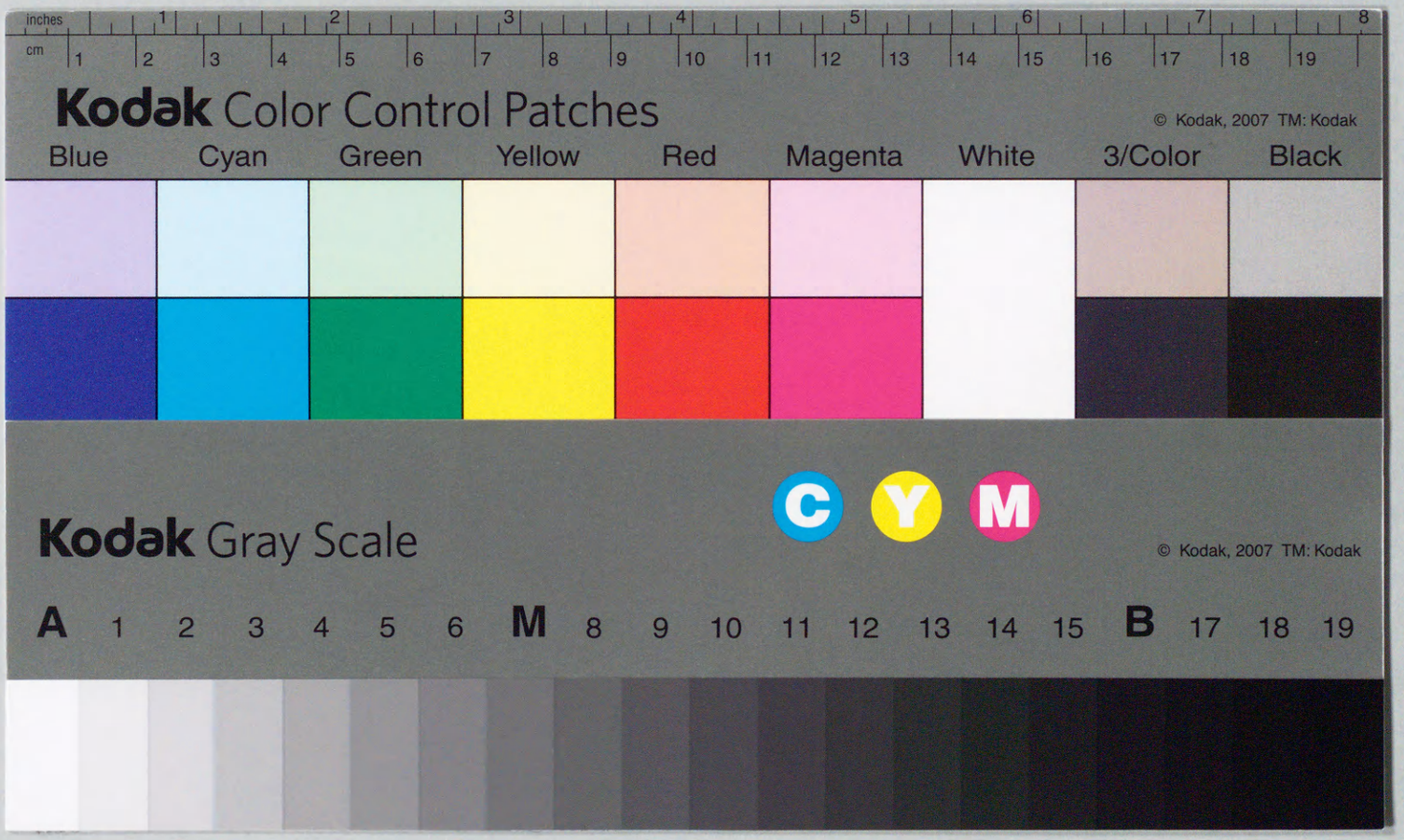
要　旨　シスタチンスーパーファミリーはシステインプロテアーゼに対して阻害活性を有するタンパク群で、構造と局在によりファミリー１（ステフィンファミリー）、２（シスタチンファミリー）、３（キニノーゲンファミリー）の３つに分けられる。このうちファミリー２に属するシスタチンは分子量 13-15kDa の分泌型タンパクで、線形動物では寄生性線虫のフィラリアの一部や自由生活線虫である *Caenorhabditis elegans (C. elegans)* での発現が報告されている。*Nippostrongylus brasiliensis (N. brasiliensis)* はヒト糞線虫に類似した生活史をもち、ラット、マウスを宿主とする腸管内寄生性線虫である。線虫に対する宿主の免疫応答機構を研究する目的で、申請者らは、*N. brasiliensis* の分泌・抽出抗原中より新規シスタチンをクローニングし、その性状を解析した。

申請者らはこれまでに報告された線虫由来シスタチンで保存された DNA 配列をもとにプライマーを設計し RT-PCR 法と 3' RACE, 5' RACE 法を用いて *N. brasiliensis* より 435 bp の cDNA をクローニングした。この cDNA がコードするタンパクは 144 アミノ酸からなり、N 末端にシグ

ナルペプチドを有する分泌型タンパクであった。またシスタチンスーパーファミリーに共通した配列である Gln-Val-Val-Ala-Gly (QVVAG)を持ち、*C. elegans* やその他の線虫由来シスタチンと一次構造上、高い類似性を示した。ニッポシスタチンの一次構造はこれまでに報告されたシスタチンのなかでも *C. elegans* 由来シスタチンに最も近く、このことは分類学的にも矛盾しない。これらのことから申請者らはこのタンパクをシスタチンファミリーに属するタンパクであると考え、ニッポシスタチンと名付けた。ニッポシスタチンは *N. brasiliensis* の感染性第３期幼虫および成虫の両方で mRNA の発現が確認された。

申請者らはニッポシスタチン組換えタンパクを作成し、その性状を解析した。イムノブロット法により、ニッポシスタチンは 14-kDa の成熟型として成虫の分泌・抽出抗原中に認められた。組換えニッポシスタチンはカテプシン L、カテプシン B といったシステインプロテアーゼの酵素活性を著明に阻害したが、カテプシン D のようなアスパラギン酸プロテアーゼは阻害しなかった。さらに成虫の分泌・抽出抗原もシステインプロテアーゼの酵素活性を阻害することが確認された。

本研究は寄生虫による免疫回避機構のなかでも宿主による特異的免疫誘導に欠かすことのできない抗原プロセッシングの阻害物質に焦点をしぼってその存在を明らかにしたもので、独創的な着眼により行われたものである。本研究から感染体がプロテアーゼ阻害により免疫応答を低下または変化させていることが推測され、感染免疫の研究に新たな道を開くものと考えられる。抗原プロセッシングおよび抗原提示を標的とした免疫修飾機構の解明は未開拓の分野であるが、新興・再興感染症の制圧やアレルギー疾患の予防・治療などにも応用可能であり、今後さらなる展開も期待され、学位授与に値すると判定した。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