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Molecular cloning and characterization of a novel cystatin-like molecule, CLM, from human bone marrow stromal cells☆,☆☆

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Abstract

The cystatins are physiological cysteine proteinase inhibitors. Here we report the cloning of a novel human cystatin-like molecule (CLM) from human bone marrow stromal cell (BMSC) cDNA library. The putative CLM protein contained 159 residues with a 29-residue signal peptide. CLM protein was highly homologous to family 2 cystatins, especially mouse and human testatin. The CLM gene spanned two exons and was mapped on chromosome 20p11.2, among cystatin superfamily gene clusters. CLM mRNA was barely detected in most tumor cell lines except for breast adenocarcinoma MCF-7 cells and glioblastoma U251 cells, but after LPS or PMA stimulation, CLM expression was increased in myelogenous leukemia cell lines HL-60 and U-937. Northern blot analysis revealed CLM was ubiquitously expressed in normal tissues, which was clearly different from the testis-specific expression pattern of most family 2 cystatins. When overexpressed in 293 cells, GFP-fused CLM targeted extracellularly through secretory pathway by Golgi apparatus. The results indicated that the secreted CLM protein might play roles in hematopoietic differentiation or inflammation.

Keywords: Bone marrow stromal cell; Cystatin; Cysteine proteinase

The cystatins are group of proteins that have the ability to inhibit cysteine proteinases [1]. They are present in a variety of tissues and body fluids and form tight and reversible complexes with cystaine proteinase such as cathepsins B, H, L, and S [2]. Therefore, they serve a protective function to regulate the activities of such endogenous proteinases, which otherwise may cause uncontrolled proteolysis and tissue damage. Based on the molecule complexity, cystatins have been categorized into three classes [3]. Stefins (type I) are generally unglycosylated proteins of about 100 amino acids lacking disulfide bridges. Type II cystatins are about 120 amino acids and have two intra-chain disulfide bonds. Most of them are found both in tissues and body fluids including saliva. Kininogens (type III) are single chain glycoproteins containing three cystatin-like domains. Both type II and type III, but not type I, are considered to be more advanced forms of the cystatin family. All of the characterized cystatins exhibit sequence homologies.

Several groups of proteolytic enzymes are able to degrade components of the extracellular matrix. The cystatins are most likely involved in the regulation of all normal [4] or pathological processes [5] in which these proteinases participate. For example, cystatin member

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☆☆ *Abbreviations:* BMSC, bone marrow stromal cell; CLM, cystatin-like molecule; GFP, green fluorescent protein; LPS, lipopolysaccharide; ORF, open reading frame.

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secreted by adult *Brugia malayi* blocks conventional cysteine proteases as well as asparaginyl endopeptidase involved in the Class II antigen processing pathway in human B cells, and is identified as so-called "immune evasion genes" [6]. Thus, cystatins may influence the intra- and extracellular catabolism of proteins and peptides, regulate proteolytic processing of prohormones and proenzymes [7], protect against penetration of normal tissues by malignant cells or microorganisms [8], and modulate local inflammatory processes such as rheumatoid arthritis and purulent bronchiectasis [9].

Bone marrow stromal cells (BMSCs) are important component of hematopoietic microenvironment, providing the structural and physiological support for hematopoietic cells. In this study we reported the molecular cloning and fundamental characterization of a novel cystatin-like molecule (CLM) from human BMSC. CLM encoded a 159-amino acid protein and was found to be related to a group of genes that encode protein cysteine protease inhibitors known as cystatins, especially mouse and human testatin (cystatin 9) [10,11]. The mRNA expression pattern, inducible expression in BMSC and myeloid leukemia cells, and the extracellular secretion of CLM by overexpression cells were investigated.

Materials and methods

Isolation of CLM cDNA. The full-length CLM cDNA was directly isolated from a human BMSC cDNA library by random sequencing as described previously by us [12]. Briefly, BMSCs were generated from patient's bone marrow aspirates. Red blood cells were depleted with NH_4Cl and the monocytes were plated in 6-well plates (10^6 cells/well) in RPMI 1640 supplemented with 10% FCS, 10% horse serum, 50 μM of 2-mercaptoethanol, 10 mM Hepes, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 24 h of culturing, floating cells were removed and an adherent stromal cell layer was established. On day 14, the stromal cells were activated with 10 μg/ml LPS (Sigma) and 100 ng/ml PMA (Sigma) for 8 h and collected for cDNA library construction. A plasmid cDNA library of pCMV·SPORT6.0 vector (Invitrogen) was constructed using the Superscript plasmid system (Invitrogen) for cDNA synthesis. Plasmid cloning was performed according to manufacturer's instructions (Invitrogen). The full-length cDNA of clone HNB7C1 was found to potentially encode a protein with a cystatin domain sharing homology with the known cystatins, hence designated as CLM (cystatin-like molecule). The full-length sequence is available in the GenBank database with Accession No. AF494536.

RT-PCR and Northern blot analysis of CLM mRNA expression. The following cell lines used for RT-PCR were: U251, PC-3, HeLa, MCF-7, A549, LoVo, K-562, THP-1, MOLT-4, Reh, U-937, HL-60, and Jurkat. Standard procedures were used for cell culture. For stimulation, U-937 and HL-60 cells were treated with 5 μg/ml PMA and 2 μg/ml LPS, respectively, for 12–48 h. Human BMSCs obtained as mentioned above were treated with 1 μg/ml LPS or 10 nmol/L PMA (Sigma), respectively, for 8 h.

Total cellular RNA was isolated using Trizol reagent (Invitrogen) according to manufacturer's instructions and first-strand cDNA was prepared with AMV reverse transcriptase (Promega) using an oligo$(dT)_{15}$ primer. The synthesis of cDNA was checked by RT-PCR using β-actin primers. RT-PCR with the primers 5′-CAGAATTCA TCATGTCG AGTCCGCAGAG-3′ (forward) and 5′-AGAAGCTTT TCCCTTTGTCCCTCGGAATG-3′ (backward) specific for CLM ORF was subjected to denaturing (95 °C for 15 s), annealing (58.8 °C for 30 s), and extension (72 °C for 30 s) for 35 cycles using a Perkin–Elmer GeneAmp PCR System 9600.

Human adult multiple tissue Northern blots (Clontech) were used to detect mRNA expression by Northern blot analysis. The RT-PCR products mentioned above were cloned into a T/A vector for sequencing and labeled by random priming with [α-^{32}P]dCTP (50 μCi, 3000 Ci/mmol, Amersham Pharmacia) as a probe. Unincorporated nucleotides were removed by spin column chromatography (Roche Molecular Biochemicals). Hybridization was performed in ExpressHyb hybridization solution (Clontech) according to manufacturer's instructions. After stringently washing at 50 °C for 20 min in 0.1× SSC and 0.1% SDS, the filters were subjected to autoradiography.

Western blot and dot blot analyses of CLM protein expression. The ORF of CLM was amplified by PCR using forward (5′-GCGAA TTCATGTCGAGTCCGCAGAGG-3′) and reverse (5′-GCGGTA CCG CCTTCCCTTTGTCCCTCGG-3′) primers from BMSC cDNA as a template. The fragment was fused with GFP code region and cloned into the expression vector pcDNA3.1/Myc-His(−)A. The recombinant CLM–GFP construct (named as pCLM–GFP) or the GFP construct (pGFP) was transfected using LipofectAMINE reagent (Invitrogen) into 293 cells.

The crude extracts of 293 cells transfected with pCLM–GFP and its mock control were separated by SDS–PAGE and were transferred onto nitrocellulose membranes. The culture media of un-transfected 293 cells and pCLM–GFP or pGFP transfected 293 cells were directly dotted on nitrocellulose membranes. After incubated with anti-GFP Ab (first Ab) and HRP-conjugated anti-rabbit IgG (second Ab) (Santa Cruz), the membranes were detected using LumiGlo reagent (Cell Signaling) according to manufacturer's instructions.

Confocal microscopy analysis of CLM protein. Transiently transfected 293 cells growing on glass coverslips placed in 6-well plates were incubated with 0.5 μM BODIPY 558/568 BFA derivatives (Molecular Probes) for 15–30 min at room temperature in the dark. Samples were washed briefly in PBS prior to observation, mounted onto a microscope slide, and observed by fluorescence confocal microscopy (Carl Zeiss).

Results

Identification and sequence analysis of CLM

The CLM cDNA directly isolated from human BMSC cDNA library was composed of 1597 bp and contained an open reading frame (ORF) of 480 bp. A putative polyadenylation signal was located 15 bases upstream of the poly(A) stretch. CLM cDNA potentially encoded a 159-aa protein with a calculated molecular weight of 18.2 kDa and an isoelectric point of 8.18. The novel protein showed strong hydrophobicity in the N-terminal, and a signal peptide was predicted with a putative cleavage site between Cys30 and Ser31, which represented a secretory protein. There was no glycosylation site found by structure analysis and motif analysis revealed a cystatin domain on 38th–125th amino acid.

The novel protein displayed similarity to family 2 cystatins, such as cystatin C, D, S, SA, and SN, which function as cysteine protease inhibitors. As shown in

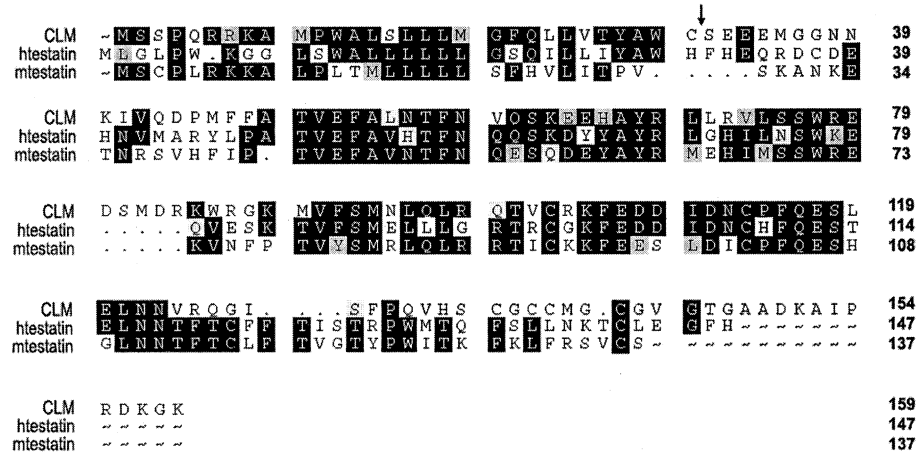

Fig. 1. Multiple alignment of CLM with closely related cystatin family members. Alignment was performed with the GCG package and minimally adjusted manually. Identical residues are boxed in black and similar residues are in gray. The putative cleavage site of signal peptide was shown by arrow. CLM sequence has been deposited in the GenBank database with Accession No. AF494536.

Fig. 1, it shared 46.2% identity and 61.4% similarity with mouse testatin (cystatin 9) [10], and 46.4% identity and 52.9% similarity with human testatin [11], both of which are specifically expressed in testis and are thought to have functions in reproduction. So the novel molecule was designated as cystatin-like molecule (CLM).

The human CLM cDNA corresponded to Unigene cluster HS.319742 located on human chromosome 20p11.2. The CLM gene was located approximately 30 kb distal to the CST3 gene [13], along with several other cystatins (Fig. 2), including cystatin SA (CST2) [14], cystatin C, cystatin D precursor (CST5) [1], testatin(CST9) [11], and CST11 [15]. The CLM gene contained 2 exons and 1 intron (Fig. 2), and first intron/exon junction was exactly conserved with CST3 [16], CST5, CST8 [17], and CST11. Conserved intron/exon structure and relatively low amino acid sequence homology characterize the family 2 cystatins and many vertebrate protein families involved in extracellular and

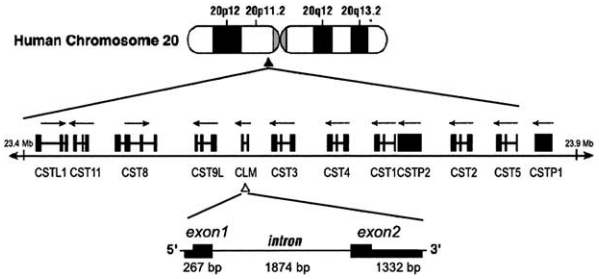

Fig. 2. Chromosome location of human CLM gene. Filled triangle indicated location of the CST family 2 gene cluster on chromosome 20. The organization of the CST gene cluster from 23.4 to 23.9 Mb forms the short-arm telomere and the expanded view of CLM gene structure is shown. Arrows indicated direction of transcription. Boxes beneath arrows represent diagramed intron/exon structures (not to scale). Narrow and wide portions of black boxes represent untranslated and translated regions of the mRNA, respectively.

often immune-related activities [18]. The cluster arrangement of CLM gene with other family 2 cystatin genes indicated that there have been multiple gene duplications of the cystatin family genes during evolution from a common ancestral gene.

Expression pattern of CLM mRNA

The expression of CLM mRNA was analyzed by RT-PCR and Northern blot. Low levels of CLM expression were seen in breast adenocarcinoma MCF-7 cells and glioblastoma U251 cells (Fig. 3A). No expression was detected in PC-3, HeLa, A549, LoVo, K-562, MOLT-4, THP-1, or Reh cells. Thirty-five cycles for CLM and 25 cycles for β-actin were performed, indicating the low level of expression of CLM in cells.

On Clontech human MTN blots, CLM was expressed as a strong 2.1 kb message (Fig. 3B). CLM mRNA could be detected in heart, placenta, lung, liver, skeletal muscle, and pancreas, but not in brain. An additional transcript of about 1.35 kb was also seen faintly in placenta. So CLM was different from its homologues, mouse and human testatin, which had a testis-specific expression pattern.

Inducible expression pattern of CLM mRNA

The expression of CLM mRNA was examined in resting and stimulated human BMSC and myeloid leukemia cell lines. RT-PCR demonstrated that CLM was expressed in human un-stimulated BMSC, which confirmed its derivation. The expression level showed no significant change after BMSC was stimulated with LPS or PMA for 8 h (Fig. 4A). In HL-60 (promyelocytic leukemia) cells, CLM expression was increased after LPS stimulation (Fig. 4B). In PMA-stimulated U-937 (myelomonocytic leukemia) cells, CLM expression was

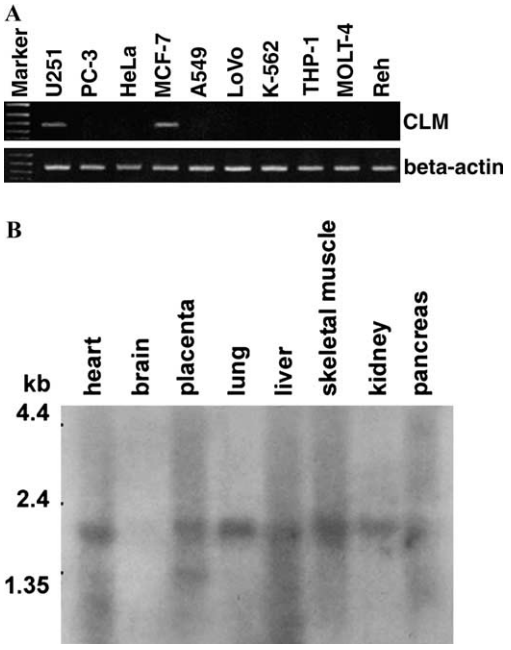

Fig. 3. Expression pattern analysis of CLM mRNA by RT-PCR and Northern blot. (A) RT-PCR with CLM-specific primers on various cell lines. All cells were similarly positive for β-actin (lower panel). Identity of the CLM PCR product was confirmed by sequencing. (B) Northern blot analysis for tissue distribution of CLM. Blots were analyzed with a probe corresponding to CLM ORF. A major band of 2.1 kb and a minor band of 1.35 kb were seen on Clontech blots.

also increased (Fig. 4C). This inducible expression pattern in HL-60 and U-937 cells suggested that CLM might play a role in the development of hematopoietic system.

Secretion of CLM protein in CLM-transfected 293 cells

The expression of GFP-fused CLM protein in transfected 293 cells was assayed by Western blot analysis with anti-GFP antibody. Two hundred and ninty-three cells were lysed and subjected to Western blot. An about 45 kDa protein was detected in pCLM–GFP transfected 293 cells under reduced condition (Fig. 5A), indicating the integrity of the fusion protein. Subtracting the 27 kDa for GFP, the molecular weight was in agreement with that calculated from the deduced amino acid sequence of CLM (18.2 kDa). Lysates of cells transfected only with pGFP showed a single band corresponding to the size of 27 kDa. The results confirmed that GFP remained conjugated to CLM and was indicative of the location of the CLM moiety.

The cytolocalization and secretion of GFP-fused CLM protein was monitored by fluorescence confocal microscopy. Twenty four hours after transfection with pCLM–GFP, GFP fluorescence signal displayed a cytoplasmic distribution in the transfected cells, especially in the perinuclear region, and co-localized with BODIPY 558/568 BFA signal for Golgi apparatus, showing as yellow in overlaid image (data not shown). After 72 h of transfection, the overall cytoplasmic fluorescence was greatly enhanced, developed peripherally and basally, and continued to be detected in the Golgi region (Figs. 5B–D). In the cells transfected with pGFP, cells showed fluorescence in both cytoplasm and nucleus (Fig. 5F), which did not co-localize with Golgi apparatus (Fig. 5G). These results may suggest the targeting of synthesized precursor CLM through Golgi and the secretory pathway, at least in CLM-overexpressed cells.

Therefore, the culture media of these cells were examined by dot blot analysis. Forty eight hours after transfection, the culture media of pCLM–GFP transfected cells contained substantial amount of CLM–GFP fusion protein, while those of pGFP transfected cells did not reveal the presence of GFP (Fig. 5H). This result indicated active expression and extracellular secretion of CLM–GFP fusion protein by transfected 293 cells.

Taken together, the findings suggested that much or most of the CLM synthesized by cultured cells was targeted extracellularly via the secretory pathway.

Discussion

Cystatins are natural and specific inhibitors of endogenous mammalian lysosomal cysteine proteinases and exogenous microbial cysteine proteinases. Cystatins

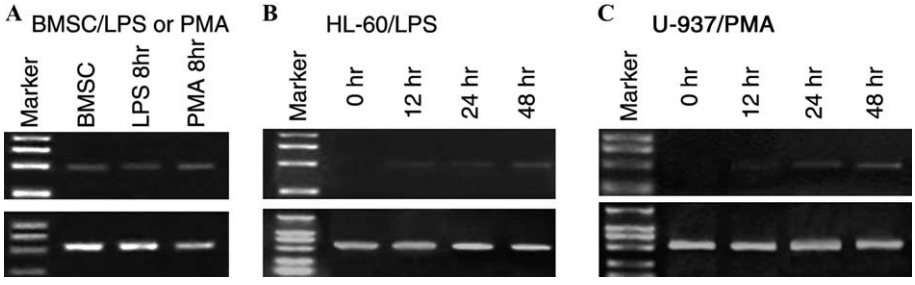

Fig. 4. Inducible expression pattern analysis of CLM mRNA by RT-PCR. RT-PCR with CLM- and β-actin-specific primers on the following cells: (A) human BMSC and LPS or PMA-stimulated BMSC, (B) promyelocytic leukemia HL-60 cells were stimulated with 2 μg/ml LPS for 48 h, and (C) myelomonocytic leukemia U-937 cells were stimulated with 5 μg/ml PMA for 48 h.

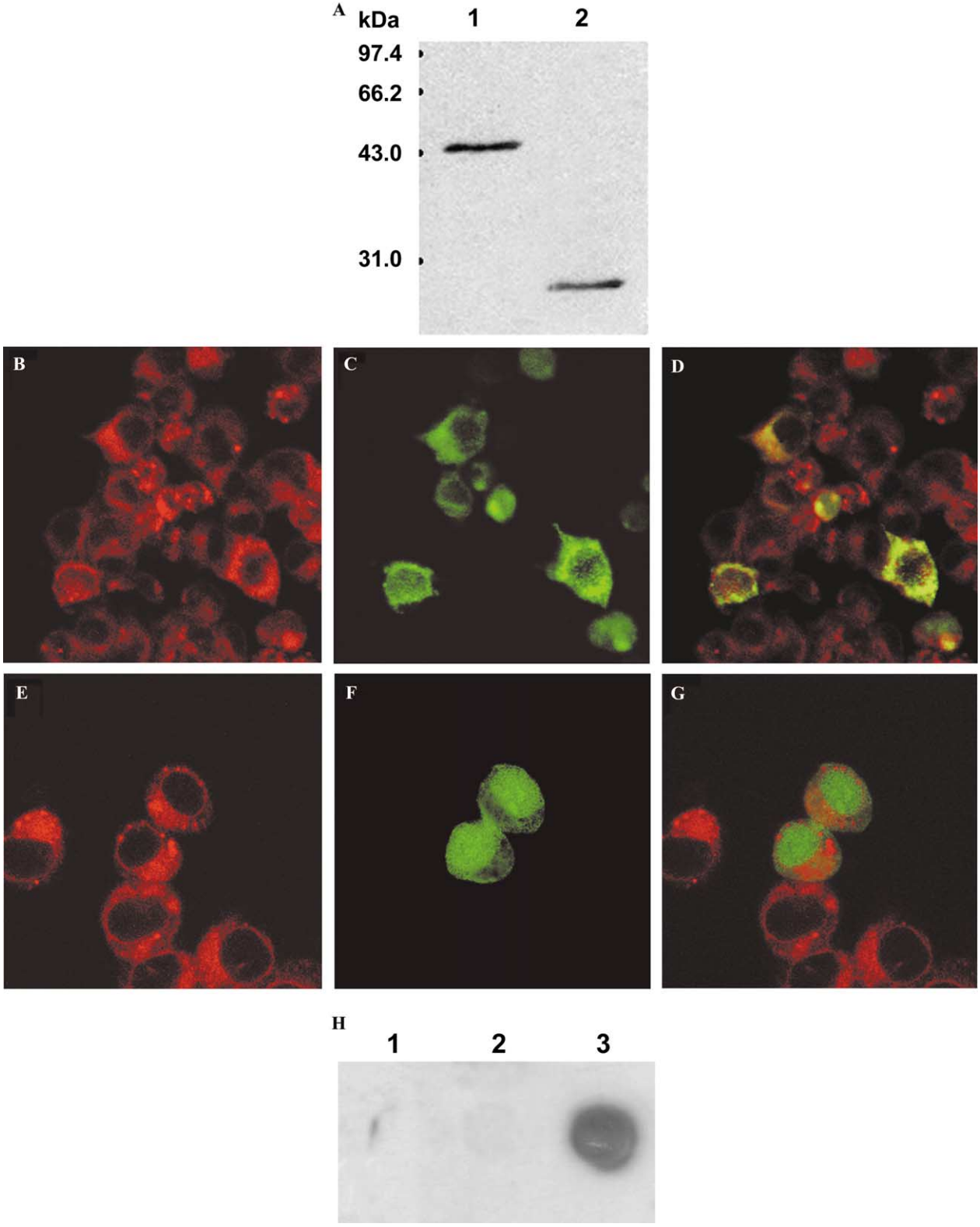

Fig. 5. Expression and detection of CLM–GFP fusion protein in transfected 293 cells. (A) Lysates of pCLM–GFP (lane 1) and pGFP (lane 2) transfected 293 cells were resolved by SDS–PAGE and Western blot were immunolabeled with anti-GFP antibody. Two-color confocal microscopy analysis of Golgi apparatus (red) and CLM–GFP or GFP (green) was performed at 72 h after transfection with pCLM–GFP (B–D) and pGFP (E–G). Confocal micrographs showing 293 cells expressing precusor CLM–GFP fusion protein (C) and GFP protein (F), with the Golgi apparatus stained in vivo (B and E), and the respective fluorescence signals overlaid (D and G). Co-localization of the green fluorescence with the Golgi apparatus was seen (yellow color) in D but not in G. (H) The culture media of un-transfected 293 cells (1), pGFP transfected cells (2), and pCLM–GFP transfected cells (3) were dot-blotted onto nitrocellulose membrane and detected with anti-GFP antibody.

were shown to provide regulatory and protective functions against uncontrolled proteolysis in several disease processes. Currently, cystatin C is the most frequently investigated family member and is involved in processes such as tumor invasion and metastasis, inflammatory processes, and some neurological diseases. In such diseases the emphasis is placed on the fine balance and regulation of both the cysteine proteases and their inhibitors, with an imbalance resulting in irreversible damages of pathological state. Accordingly, the cystatin superfamily members, especially cystatin C, have been studied as a possible index of tumor growth and as a marker of the effectiveness of antitumor therapy [19].

We reported here the cloning of a family 2 cystatin-related cystatin-like molecule, CLM, from human BMSC. The CLM protein represented a secreted protein with an N-terminal signal peptide; besides, CLM also contained a cystatin domain, which is typical among cysteine superfamily. But there are obvious differences between CLM and other cystatin members. CLM lacked some conserved residues forming the inhibitory sites of cystatin protein, such as the Q-X-V-X-G and the P-W in the first and second hairpin loops. Also it contained only two of the four conserved Cys in the C-terminal required for the formation of two disulfide bridges, suggesting that the binding specificity of CLM to its unknown target is different from those of other cystatins. Human family 2 cystatins contain no Cys residue except in signal sequences or the four consensus Cys residues near the C-terminal. In contrast, CLM has Cys residues at positions 30, 136, 138, 139, and 142, although it is unclear whether these Cys residues form additional disulfide bridges. This unique C-terminal structure, together with the result that CLM expression was not restricted to testis as its homologues, mouse and human testatin, might suggest its different specificity of the physiological role from that of classic cystatins.

The predicted CLM protein contained a signal peptide as other family 2 cystatins. The cytolocalization of CLM protein also confirmed its secreted pathway. Evidence was reported that members of the cystatin superfamily function both intracellularly and extracellularly to regulate numerous processes dependent on endogenous cysteine proteinase activity. For example, it has been found that in tumor tissue and plasma cystatin C expression relative to cathepsin B expression was decreased in breast cancer, lymphosarcoma, and colon carcinoma [20,21], which could be associated with the looseness of cancerous interstitial tissue and might be crucial in cancer invasion and metastasis. Intra- and extracellular cystatins also play roles in immune defense systems, including the inhibition of virus replication within cell and targeting proteolytic enzymes that are essential virulence factors for prokaryotic and eukaryotic parasites during all stages of the infectious process [22–25]. Therefore, before and after the CLM protein was secreted to extracellular space, the interaction with many related molecules may also be happen.

Many myeloid leukemia cells are able to differentiate into monocytes/macrophages. During the process these cells gradually acquire the ability of antigen-presentation characterized by the increasing lysosomal proteases. In addition, the differentiation also leads to induction of these protease inhibitors for prevention from excessive proteolysis. In the present study, we analyzed the expression of the novel cystatin-like molecule in resting and stimulated leukemia cell line, U-937, which can be induced to monocyte/macrophage differentiation in the presence of PMA. The upregulated expression of CLM in PMA-treated U-937 cells suggested that CLM might be a candidate to control the cysteine protease activity as a novel functional inhibitor in hematopoietic differentiation.

LPS can arise strong pro-inflammatory responses that can eventually cause a fatal sepsis syndrome. The signaling pathway of LPS-mediated activation results in changes of gene expression encoding mediators in inflammation and immune responses. These mediators include activators of inflammation such as chemokines, heat shock proteins, and proteases, and also anti-inflammation factors such as growth factors and protease inhibitors for tissue repair. The secretion of family 2 cystatin member, cystatin C, which has been found to be constitutively secreted by isolated human monocytes and mouse peritoneal macrophages as well as some hematopoietic tumor cell lines such as J774, was down-regulated by in vitro treatment of resident mouse peritoneal macrophages with LPS, IFN-γ, or thioglycollate [26]. Here we showed that CLM expression was upregulated in LPS-stimulated HL-60 cells, suggesting that CLM might be implicated in inhibiting cysteine protease activity in the secreting form for avoiding inflammatory tissue injury. Future studies are required to elucidate the significance of functions of the family 2 cystatin-related molecules including CLM in the hematopoietic differentiation and inflammation.

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