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A Novel Serum Protein That Is Selectively Produced by Cytotoxic Lymphocytes

Kazuyuki Ogawa,* Kazuya Tanaka,† Akira Ishii,‡ Yoshiko Nakamura,§ Shigemi Kondo,§ Kazuo Sugamura,† Shoichi Takano,* Masataka Nakamura,‖ and Kinya Nagata**

Cytotoxic lymphocytes such as CTL and NK cells play principal roles in the host defense mechanisms. Monitoring these effector cells in vivo is helpful to understand the immune responses in disorders such as cancer and infectious diseases. In this study, we identified a novel secretory protein, killer-specific secretory protein of 37 kDa (Ksp37), as a Th1-specific protein by a subtractive cloning method between human Th1 and Th2 cells. In peripheral blood leukocytes, Ksp37 expression was limited to Th1-type CD4+ T cells, effector CD8+ T cells, γδ T cells, and CD16+ NK cells. Most of these Ksp37-expressing cells coexpressed perforin, indicating that Ksp37 is selectively and commonly expressed in the lymphocytes that have cytotoxic potential. Ksp37 was released at constant rate from both unstimulated and stimulated PBMCs in vitro and also detected in normal human sera. In healthy individuals, serum Ksp37 levels were significantly higher in children (mean ± SD; 984 ± 365 ng/ml for age 0–9) than in adults (441 ± 135 ng/ml for age 20–99), consistent with reported differences in the absolute counts of blood T and NK cells between children and adults. In patients with infectious mononucleosis, transient elevation of serum Ksp37 levels was observed during the early acute phase of primary EBV infection. These results suggest that Ksp37 may be involved in an essential process of cytotoxic lymphocyte-mediated immunity and that Ksp37 may also have clinical value as a new type of serum indicator for monitoring cytotoxic lymphocytes in vivo. The Journal of Immunology, 2001, 166: 6404–6412.

Lymphocyte-mediated cytotoxicity is the principal mechanism for eliminating a variety of target cells such as tumor cells and cells infected with viruses or other intracellular microbes. It also causes a serious tissue damage in autoimmune diseases. This process is mainly mediated by CTLs and NK cells, which have cytotoxic granules containing various cytotoxic effector molecules such as perforin and granzymes (1). These cells also express Fas ligand (2) and/or TNF-related apoptosis-inducing ligand (TRAIL) (3, 4), through which they can induce apoptosis in Fas- and/or TRAIL receptors-expressing target cells (5, 6). Although CTL response is primarily performed by CD8+ T cells, γδ T cells also elicit cytotoxic activity through similar mechanisms and play a protective role against viral and bacterial infections (7, 8). CD4+ T cells are generally known as Th cells that play a central role in the immune system, principally through secretion of various cytokines. Th cells consist of at least two major subsets with distinct cytokine secretion profiles (9): Th1 cells, which selectively produce IFN-γ and TNF-β, and Th2 cells, which preferentially produce IL-4, IL-5, and IL-13. Upon infection with intracellular pathogens, Th1 cells produce a large amount of IFN-γ, which enhances phagocytic and cytocidal activity of macrophages against microbes and promotes differentiation of CD8+ T cells into effector cytotoxic lymphocytes (9). However, some CD4+ T cells have been shown to elicit cytotoxic activity against target cells through perforin/granzyme (10, 11) and/or Fas ligand (12–14)/TRAIL (15, 16)-dependent mechanisms. Thus, NK cells, CD8+ T cells, γδ T cells, and CD4+ T cells, which belong to different lymphocyte lineages, share the common mechanisms to exert their cytotoxic function.

Monitoring the expansion and decline of these cytotoxic lymphocytes in vivo would be of a great value for understanding ongoing immune conditions in cases such as infection, cancer, autoimmune diseases, and post-transplantation. Here, we report a novel secretory protein, designated as killer-specific secretory protein of 37 kDa (Ksp37), which is commonly and selectively expressed by NK cells, CD8+ T cells, γδ T cells, and CD4+ T cells, in the cytotoxic effector phase. Our results suggest that serum Ksp37 levels reflect the expansion and decline of these cytotoxic lymphocyte populations in vivo.

Materials and Methods

Cells

Origins and culture conditions of cell lines used were previously described (17). PBMCs and cord blood mononuclear cells (CBMCs) were isolated from heparinized peripheral and cord blood of consented subjects, respectively, by density gradient centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). Human Th clones and lines were generated from normal PBMCs and maintained as previously described (17).

Isolation of Th1-specific cDNA fragments and cloning of Ksp37 cDNA

Subtracted Th1 and Th2 cDNA fragments used in this study were previously described (17). The subtracted Th1 cDNA fragments were cloned...
into pBluescript SK— (Strategene, La Jolla, CA) and screened with probes of the subtracted Th1 and Th2 cDNA fragments. Clones selectively hybridized with the Th1 probe were isolated and sequenced as described (17). A Th1 cDNA library was constructed from poly(A)+ RNA of a Th1 line derived from a healthy adult using SuperScript Lambda System (Life Technologies, Rockville, MD), and screened with a probe of the subtracted Th1 cDNA fragment, named T48. Insert cDNAs of the positive clones were excised at MultiNor sites and subcloned into EcoRI/NorI sites of pBluescript SK—.

Expression of Ksp37 in mammalian cells

The longest Ksp37 cDNA (1153 bp) encompassing the whole coding region was excised from a pBluescript SK— clone at HindIII/NorI sites and subcloned into the same sites of pRC/CMV vector (Invitrogen, San Diego, CA), generating pCMV/T48. COS-7 cells were transfected with pCMV/T48 or control vector using FuGENE6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). After 30 h culture, cells and culture supernatants were recovered for analyses of expression of Ksp37.

Preparation of rKsp37

The cDNA encoding a truncated Ksp37 protein lacking both amino- and carboxyl-terminal hydrophobic regions was amplified by PCR using the following primers: 5′-CGAGGATCCGATGACGATGACAAACAGGCC CGGAGACAAAAGCAA-3′ (forward) and 5′-CCCAACAGTCTACAG GCTCTTCTTTGTGTC-3′ (reverse). The PCR product was digested with BamHI and HindIII and subcloned into the same sites of pQE30 vector (Qiagen, Hilden, Germany). The rKsp37 modified by a histidine terminus was generated in Escherichia coli strain M15 and purified with nickel-nitrilotriacetic acid agarose (Qiagen) under 8 M urea-denaturing condition. After removal of urea by dialysis in 30 mM HEPES-NaOH buffer (pH 7.5) containing 0.05% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, the protein was further purified on a Mono S column (Amersham Pharmacia Biotech). The purity of rKsp37 was >95% as assessed by SDS-PAGE.

Monoclonal and polyclonal Abs against Ksp37

DNA-based immunization of mice was performed to generate mAbs to Ksp37 (18). In brief, BALB/c mice were injected with 20–50 μg of pCMV/T48 DNA into each quadriceps muscle four times every 3 wk. The following materials were obtained from BD Biosciences (San Jose, CA): FITC-labeled mAbs to CD4 (SK3), CD8 (SK1), CD14 (MφP9), CD19 (4G7), TCR-y6 (11F2), and HLA-DR (L243); PE-conjugated mAbs to CD4 (SK3), CD8 (SK1), CD56 (MY31), IFN-γ (25723.11), IL-2 (5344.11), and IL-4 (3010.211); peridinin chlorophyll protein (PerCP) or allophycocyanin-conjugated mAb to CD3 (SK7); and appropriate isotype-matched control Abs. FITC-labeled mAbs to CD16 (3G8) and CD45RA (H1/100); PE-conjugated mAbs to CD11b (ICRF44), CD25 (M-A251), CD27 (M-T271), and CD45RO (UCHL1); and control conjugates were purchased from BD Pharmingen (San Diego, CA). FITC- or PE-conjugated anti-perforin mAb (809) was obtained from Ancell (Bayley, MN).

Flow cytometry

Staining of surface Ags was performed according to the manufacturer’s instruction. To detect intracellular Ksp37, cytokines, or perforin, cells were first stained for desirable surface markers, then fixed in 4% formaldehyde in PBS at room temperature for 5 min, and permeabilized in FACS permeabilization solution (BD Biosciences) at room temperature for 10 min. The permeabilized cells were preincubated with 0.5 mg/ml of either normal mouse IgG (for Ksp37 staining) or unlabeled TDA3 (for control staining) at 4°C for >60 min, and then biotinylated TDA3 (2–5 μg/ml) was added to the cell suspensions. After incubation at 4°C for 30 min, cells were washed and further incubated with PE- or RED670-conjugated streptavidin (Life Technologies) together with a PE-labeled mAb to cytokine or perforin for 30 min at 4°C. Stained cells were analyzed on FACSCalibur flow cytometer using CellQuest software (BD Biosciences).

Confocal microscopic analysis

Cells that were fixed and permeabilized as described above were incubated with 2–5 μg/ml of TDA3 or isotype-matched control Ab in the presence of 10% normal goat serum at 4°C for 30 min, washed, and incubated with Alexa 568-labeled goat anti-mouse IgG (Molecular Probes, Eugene, OR) at 4°C for 30 min. After washing, cells were blocked with 10% normal mouse serum at 4°C for 60 min and incubated with FITC-conjugated anti-perforin mAb at 4°C for 30 min. After washing, cells were cytospin onto glass slides, mounted with ProLong antifade reagent (Molecular Probes), and analyzed using Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) with MicroRadiance confocal scanning system (Bio-Rad, Hercules, CA).

Fractionation of leukocytes

Granulocytes and PBMCs were purified from peripheral blood of consented healthy adults by density gradient centrifugation on Mono-Poly Re- solving medium (Dainihon Seiyaku, Osaka, Japan). PBMCs were fractionated into six leukocyte subsets based on their respective surface markers using MACS system (Miltenyi Biotec, Bergisch Gladbach, Germany). Purities of each subset preparation were >90% for CD14+ monocyte, CD19+ B cell, CD16+ NK cell, and CD8+ T cell fractions, and >85% for the CD4+ T cell and TCR-yδ+ T cell fractions as assessed by flow cytometry.

Northern blot and RT-PCR analyses

Northern blot and RT-PCR analyses were performed as described previously (17). Primers used in RT-PCR analysis were as follows: 5′-GAG GCAGAAAGGGAAGGACT-3′ (forward) and 5′-AACGTGAT GAGAAAGGCAGACA-3′ (reverse) for Ksp37, and 5′-GGCCACCAACCTTCTCAATGA-3′ (forward) and 5′-CATTGGCAATTGTTAGTACCGT-3′ (reverse) for β-actin. Amplification was performed by 30 cycles of PCR for Ksp37 and 25 cycles for β-actin.

ELISA

Ksp37-specific sandwich ELISA was performed using TDA3 mAb as a capture Ab, rabbit anti-Ksp37 Ab as a detector Ab, and rKsp37 as a standard protein. The plate-bound rabbit anti-Ksp37 Ab was visualized with an HRP-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA) followed by peroxidase reaction with ABTS (Sigma) as a substrate. Concentrations of IFN-γ and soluble CD8 (sCD8) were determined using the Quantikine human IFN-γ immunoassay (R&D Systems, Minneapolis, MN) and CELLFREE soluble CD8 ELISA kits (Endogen, Woburn, MA), respectively.

Clinical samples

All serum samples were collected from consenting Japanese subjects. Patients with acute EBV infection were diagnosed as having infectious mononucleosis (IM) on the basis of clinical manifestations, an elevation of IgM Ab for viral capsid Ag (VCA), and an absence of Ab for EBV nuclear Ag.
Results

Cloning of a novel protein Ksp37

To isolate genes specific for Th1 but not Th2 cells, a PCR-based cDNA subtraction method was performed (17). After screening of 2100 cDNA fragments from the subtracted Th1 cDNA library, we obtained 16 independent cDNA fragments that were expressed at detectable levels in the original Th1 clone, but not in the Th2 clone, in Northern blot analysis. Search of DDBJ/EMBL/GenBank databases revealed that 2 of the 16 cDNA fragments were novel, clones from chromosome 4, named C0024K08 (map 4p16) with the accession number AB021123. In a homology search of these databases with the basic local alignment search tool (BLAST) program, T48 cDNA sequence was found separately in two regions of a human genomic clone from chromosome 4, named C0024K08 (map 4p16) with the accession number AC005598, indicating that this cDNA derived from two exons.

Hydropathy plot analysis of Ksp37 performed by the algorithm of Kyte and Doolittle (22).

Molecular characterization of Ksp37

The deduced primary structure of Ksp37 showed nine potential O-glycosylation sites as analyzed by the NetOGlyc program (20), but no possible N-glycosylation site (Fig. 1B). As shown in Fig. 1B, homology search of DDBJ/EMBL/GenBank databases by the FASTA program with the deduced amino acid sequence of Ksp37 displayed a 24% identity to HBp17 fibroblast growth factor (FGF)-binding protein, a heparin-binding protein known to be associated with FGF (21), over 203 amino acids (aa 17–219 of Ksp37). No significant homology was found with other known proteins.

Hydropathy plot analysis revealed two strongly hydrophobic regions at both termini of Ksp37 (Fig. 1C). The amino-terminal hydrophobic region, which consists of 14 amino acids, displays a characteristic secretory signal sequence with a predicted cleavage site after the glycine of amino acid position 19 as analyzed by von Heijne’s method (23). The carboxyl-terminal hydrophobic region, which consists of 14 amino acids, seems too short to serve as a transmembrane domain. These findings suggested that Ksp37 might be secreted. Indeed, pCMV/T48-transfected COS-7 cells secreted Ksp37 as a 37-kDa form, while they retained a major 28-kDa species (identical in size with an in vitro transcription/translation product) and a minor 37-kDa form (Fig. 2A).

Characterization of Ksp37 molecule. A, Whole-cell lysates (10^5 cells equivalent) of Ksp37 (K)- and control vector (V)-transfected COS-7 cells were subjected to Western blot analysis with polyclonal anti-Ksp37 Ab as described in Materials and Methods. Immunoprecipitates (IP) by TDA3 (T) and control Ab (C) of the culture supernatant (S) from Ksp37-transfected COS-7 cells were similarly processed. B, Culture supernatants from different Th1 and Th2 cell lines were analyzed for Ksp37 contents by immunoprecipitation and Western blot analysis as described in A.
then modified, possibly by O-glycosylation, and secreted into the extracellular space as a 37-kDa form.

**Expression of Ksp37 in normal peripheral blood leukocytes**

We next examined peripheral blood leukocytes from several healthy adults for their Ksp37 expression. In flow cytometric analysis, living leukocytes were not stained with anti-Ksp37 mAb to characterize Ksp37-expressing lymphocytes

To characterize Ksp37-expressing peripheral blood T cells, we first analyzed their cytokine profiles by stimulating them with PMA and ionomycin in the presence of brefeldin A (BFA). As shown in Fig. 4, nearly all Ksp37-expressing cells showed the ability to produce IFN-γ but not IL-4, demonstrating a typical Th1 phenotype for CD4+ T cell subset and a T cytotoxic 1 phenotype for CD8+ T cell subset. Ksp37-expressing CD4+ and CD8+ T cells lacked the ability to produce IL-2 (Fig. 4).

We next examined surface phenotypes of Ksp37-expressing T cells. In CD4+ T cells, Ksp37 expression was exclusively observed among CD8+ T cells and CD16+ NK cells (Fig. 3A, a and B). In NK cells, Ksp37 was predominantly expressed in the CD56dimCD16bright subset, but not the CD56bright subset (Fig. 3B, g and h). Moreover, another cytotoxic lymphocyte subset, the γδ-type T cells, also expressed Ksp37 (Fig. 3A, b). Ksp37 was not, however, appreciably seen in granulocytes, CD19+ B cells, and CD14+ monocytes (Fig. 3A, b, and B, e and f, respectively). This cell-type specificity of Ksp37 expression was confirmed at the mRNA level in Northern blot analysis (Fig. 3C).
member CD27 (Fig. 5, upper panels). It was reported that repeatedly sensitized, highly differentiated CD4+ T cells highly express integrins CD11b and very late Ag-4 and -5, but lack CD27 expression (24, 25). Therefore, Ksp37-expressing CD4+ T cells appear to correspond to the highly differentiated Th1 cells. Circulating CD8+ T cells can be classified into three major subsets based on the expression of CD45RA and CD27 Ags: CD45RA-CD27+ naive, CD45RA-CD27+ memory, and CD45RA+CD27- effector subsets (26). Effector CD8+ T cells, which have the highest cytotoxic activity among these subsets, highly express integrins such as CD11b and produce a large amount of IFN-γ but little, if any, IL-2 (26, 27). CD45RA-CD27+CD8+ T cells, which form a minor population among circulating CD8+ T cells, also possess the properties similar to the effector subset (26). As shown in Fig. 5 (lower panels), Ksp37 expression was predominantly seen in the CD8+ T cells with a surface phenotype of CD45RA-CD27+CD11b+. Thus, Ksp37 appears to be selectively expressed at the cytotoxic effector stage in CD8+ T cells.

We further investigated Ksp37-expressing lymphocytes for expression levels of perforin, a pivotal molecule in granule-mediated cytotoxicity (27, 28). In adult PBMCs, Ksp37 expression was associated with the expression of perforin in CD16+ NK cells, CD8+ T cells, γδ T cells, and CD4+ T cells, although expression levels of perforin in CD4+ T cells were lower than those in the other three cell types (Fig. 6A, upper panels). When whole blood samples were treated with BFA, which inhibits secretion of newly synthesized proteins, mean fluorescence intensities (MFIs) for Ksp37 increased to 2- to 3-fold higher levels in these cell subsets, whereas those for perforin were not considerably affected (Fig. 6A, middle panels, and B). These findings imply that, unlike perforin, Ksp37 is constitutively synthesized and released by these cells. No change in the cell-type specificity of Ksp37 and perforin expression was observed following BFA treatment. In CBMCs, Ksp37 was coexpressed with perforin in most CD16+ NK cells, whereas cord blood CD4+ and CD8+ T cells expressed neither Ksp37 nor perforin (Fig. 6A, lower panels).

Immunofluorescence confocal microscopic analysis showed that Ksp37 was localized throughout the cytoplasmic space and that Ksp37 was localized throughout the cytoplasmic space and that Ksp37 was localized throughout the cytoplasmic space and that Ksp37 was localized throughout the cytoplasmic space. However, localization of Ksp37 largely differed from that of perforin (Fig. 6C, a). However, localization of Ksp37 largely differed from that of perforin (Fig. 6C, a). However, localization of Ksp37 largely differed from that of perforin (Fig. 6C, a). However, localization of Ksp37 largely differed from that of perforin (Fig. 6C, c).

Secretion profile of Ksp37 in PBMC cultures

The secretion profile of Ksp37 in unstimulated and stimulated PBMC cultures was then examined. Unstimulated PBMCs released Ksp37 at a nearly constant rate with constitutive and constant mRNA levels (Fig. 7, A and B). PHA stimulation induced a slight increase in Ksp37 release within 4 h (Fig. 7A) and transiently down-regulated Ksp37 mRNA expression at day 1 (Fig. 7B). However, after that, the increasing rate of Ksp37 levels was not largely different between unstimulated and stimulated cultures (Fig. 7A). No considerable increase in the cell number was observed for either culture by day 3. Therefore, the secretion rate of Ksp37 per cell was nearly constant during the culture period. The secretion profile of Ksp37 was quite distinct from that of IFN-γ or sCD8, the latter being largely dependent on cell activation (Fig. 7A).

Characterization of serum Ksp37 protein

The above results suggested that Ksp37 might be present in normal sera. Immunoprecipitation and Western blot analysis demonstrated two Ksp37 species of 28 kDa and 37 kDa in PBMC lysates and a single 37-kDa form in normal sera (Fig. 8A). As shown in Fig. 8B, neuraminidase treatment markedly reduced the apparent molecular mass of serum Ksp37 from 37 to 31 kDa, and additional treatment with O-glycosidase caused a further slight decrease in the apparent molecular mass to around 30 kDa, although O-glycosidase treatment alone did not have any effect. As expected, N-glycosidase F showed no effect (Fig. 8B). These results indicate that serum Ksp37 has O-linked sugars that are highly modified by sialic acids.

Serum levels of Ksp37 in healthy individuals

To investigate the circulating level of Ksp37, sera from healthy individuals of various ages were subjected to Ksp37-specific ELISA. Serum Ksp37 levels in children and adolescents (mean ± SD: 984 ± 365 ng/ml for ages 0–9, 658 ± 323 ng/ml for ages 10–19) were significantly higher than those in adults (441 ± 135 ng/ml for ages 20–99) (Fig. 9A), whereas no significant differences were seen between different ages of adults (Fig. 9B). In five healthy adults examined, the serum Ksp37 level in each individual did not considerably change during this study (data not shown).

Change of serum Ksp37 levels in patients with primary EBV infection

Primary EBV infection, which often causes IM in humans, is characterized by a large expansion of activated CD8+ T cells and CD16+ NK cells (29–32). To examine whether serum Ksp37 levels are influenced by primary EBV infection, we measured Ksp37 with serial sera from IM patients. In typical IM cases, which showed a considerable increase in IgM titers to the VCA of EBV, unusually high serum Ksp37 levels were demonstrated in the early acute phase, after which Ksp37 levels rapidly decreased to the normal range in the late acute phase (Fig. 10, a–d). This transient increment of serum Ksp37 level was more remarkable in adults (Fig. 10, c and d) than in children (Fig. 10, a and b), and frequently
preceded the rise of IgM titer to VCA (Fig. 10, a, c, and d). The levels of sCD8, which is known as a useful serum marker for in vivo activation of CD8⁺ T lymphocytes (33), showed similar profiles to those of Ksp37 (Fig. 10, b, c, and d).

Discussion

In this study, we described the cloning of a novel serum protein that is selectively produced by cytotoxic lymphocytes. We initially identified Ksp37 as a T helpers 1-specific secretory protein and then found that it is expressed not only in CD4⁺ T cells, but also in CD8⁺ T cells, NK cells, and γδ T cells. Furthermore, our study indicated that Ksp37 is selectively expressed in lymphocytes that have high cytotoxic potential. Several lines of evidence support this conclusion. In NK cells, Ksp37 is exclusively expressed in the CD56dim CD16 bright population, which has the highest cytotoxic potential among NK cells (34). In CD8⁺ T cells, surface phenotype (CD27⁻CD11b⁻) and cytokine profile (IFN-γ high IL-4 low IL-2 low) of Ksp37-expressing cells well conform to the criteria for the effector CD8⁺ T cell subset that has the highest cytotoxic ability among CD8⁺ T cells (26, 27). A recent report that CD56 expression can define the cytotoxic effector subset among CD8⁺ T cells (35) is also consistent with this notion, because most Ksp37-expressing T cells also expressed CD56 (data not shown). Moreover, the fact that Ksp37 is coexpressed with perforin strongly supports the above-described notion because perforin is a typical marker for cytotoxic lymphocytes (27, 28, 36). Coexpression of Ksp37 with perforin is also the case in γδ T cells and even in CD4⁺ T cells. Ksp37-expressing CD4⁺ T cells are considered to correspond to highly differentiated Th1 cells and to have cytotoxic potential as judged by their surface phenotype (CD27⁻CD11b⁻), cytokine profile (IFN-γ high IL-4 low IL-2 low) (24, 25), and ability to produce perforin. The correlation between Ksp37 expression and cytotoxic potential held true in cord blood lymphocytes. Unlike adult T cells, cord blood T cells, most of which are naive T cells, never expressed Ksp37, whereas cord blood NK cells, which have low cytotoxic activity (37, 38), expressed Ksp37 as well as perforin. These results support the concept that Ksp37 is predominantly expressed in the cytotoxic lymphocytes regardless of cell types.

Cytotoxic CD8⁺ T cells and NK cells secrete various kinds of proteins including cytokines such as IFN-γ, chemokines such as RANTES, soluble membrane proteins such as sCD8 and soluble Fas ligand, and various granule proteins such as perforin and granzymes. Ksp37 seems be distinct from these known secretory proteins in several aspects. Ksp37 is apparently a nonmembrane protein and has no amino acid sequence homology with members of cytokines and chemokines. Moreover, Ksp37 mRNA expression...
was constitutive and transiently down-regulated upon cell activation, whereas most cytokines are up-regulated by cell activation. In addition, an ATTTA nucleotide motif mediating rapid mRNA degradation, which is found in various cytokine mRNA, is not found in the 3′ untranslated region of Ksp37 mRNA. Ksp37 also apparently differs from known granule proteins in its intracellular localization and secretion profile. Granzymes and perforin are once secreted by cytotoxic lymphocytes and released largely through granule exocytosis upon cell activation (39). Also, β-chemokines RANTES and macrophage-inflammatory protein-1α are reported to be colocalized with granzyme A in the same granules and to be released together into extracellular space during activation-induced degranulation processes (40). In contrast, Ksp37 seems to be mostly secreted via a constitutive secretory pathway, independent of cell activation. This is supported by the observations that treatment with BFA, which inhibits the constitutive secretory pathway but not the granule exocytosis pathway (39), caused significant increase in intracellular accumulation of Ksp37 and that Ksp37 was released at similar rate between unstimulated and stimulated PBMC cultures. Here, it should be noted that a small amount of Ksp37 was apparently localized in cytoplasmic granules and seemed to be released by stimulation-induced degranulation. Indeed, PHA stimulation caused a slight increase in Ksp37 release in PBMC cultures within 4 h (Fig. 7A). Interestingly, even in such cases, Ksp37 was localized in the different granules from those including perforin, suggesting a functional difference between Ksp37 and cytotoxic granule proteins. Thus, at present, Ksp37 cannot be categorized into any known group of proteins that are secreted by cytotoxic lymphocytes.

The finding that Ksp37 secretion from cytotoxic lymphocytes is mainly constitutive rather than activation-dependent suggests that serum Ksp37 levels may reflect the total numbers of cytotoxic lymphocytes in vivo, although the expression of Ksp37 in the other tissues than leukocytes remains to be examined. If this is the case, Ksp37 would serve as a unique serum protein as compared with other known serum immune markers including cytokines, soluble forms of CD4, CD8, CD25, HLA-β2-microglobulin (41–45), and Fas ligand (46), and serum granzymes (47). Unlike Ksp37, these soluble molecules are thought to largely reflect the activated state of, rather than the number of, their respective producer cells (Fig. 7A). This interpretation is supported by our clinical data. Several reports showed that absolute counts of blood T and NK cells are 2- to 3-fold higher in healthy children than adult subjects (48–52). These differences are consistent with the difference we observed in serum Ksp37 levels (an ~2-fold difference between children and adults). Moreover, in IM patients, we observed markedly increased levels of serum Ksp37 in the very early phase of primary EBV infection, at which time even the anti-VCA IgM titer, an early viral infection marker, was in the course of rising. Ksp37 levels then rapidly dropped to the normal range within about 2 wk after the initial determination. Recent reports demonstrated that a transient massive expansion of virus-specific CD8+ T cells occurs during the early acute phase of primary EBV infection and that such cells rapidly decline in parallel with viral clearance within 10 days after infection (53). Such reported kinetics of the expansion and decline of virus-specific CD8+ T cells seems to be consistent with the changes of serum Ksp37 levels we observed in this study. The changes of serum Ksp37 levels nearly paralleled those of sCD8

![FIGURE 8. Characterization of serum Ksp37. A, Immunoprecipitates (IP) by TDA3 (T) and control Ab (C) of a PBMC lysate (L) and an adult serum (S) were subjected to Western blot analysis with polyclonal anti-Ksp37 Ab. B, Immunoprecipitates by TDA3 (T) and control Ab (C) of an adult serum were treated as indicated and subjected to Western blot analysis with polyclonal anti-Ksp37 Ab.](Image)

FIGURE 8. Characterization of serum Ksp37. A, Immunoprecipitates (IP) by TDA3 (T) and control Ab (C) of a PBMC lysate (L) and an adult serum (S) were subjected to Western blot analysis with polyclonal anti-Ksp37 Ab. B, Immunoprecipitates by TDA3 (T) and control Ab (C) of an adult serum were treated as indicated and subjected to Western blot analysis with polyclonal anti-Ksp37 Ab.

![FIGURE 9. Levels of serum Ksp37 in healthy individuals. Serum samples were appropriately diluted and supplied to Ksp37 ELISA as described in Materials and Methods. Serum Ksp37 levels in children (ages 0–9), adolescents (ages 10–19), and adults (ages 20–99) are compared in a. Serum Ksp37 levels in different ages in the group of adults shown in a are displayed in b. Each value represents the mean (+ SD) of Ksp37 levels in the indicated age of subjects. The statistical significance of differences was calculated by ANOVA with post hoc Scheffe’s test. Asterisks indicate the statistical significance with the probability of <0.001.](Image)

FIGURE 9. Levels of serum Ksp37 in healthy individuals. Serum samples were appropriately diluted and supplied to Ksp37 ELISA as described in Materials and Methods. Serum Ksp37 levels in children (ages 0–9), adolescents (ages 10–19), and adults (ages 20–99) are compared in a. Serum Ksp37 levels in different ages in the group of adults shown in a are displayed in b. Each value represents the mean (+ SD) of Ksp37 levels in the indicated age of subjects. The statistical significance of differences was calculated by ANOVA with post hoc Scheffe’s test. Asterisks indicate the statistical significance with the probability of <0.001.

![FIGURE 10. Ksp37 levels in serial sera of patients suffering from primary EBV infection. Serum samples were collected at the indicated time points from IM patients aged 14 (a), 6 (b), and 30 (c and d) years, and supplied to ELISA for Ksp37 and sCD8. The IgM titer for VCA was determined by indirect immunofluorescence assay.](Image)

FIGURE 10. Ksp37 levels in serial sera of patients suffering from primary EBV infection. Serum samples were collected at the indicated time points from IM patients aged 14 (a), 6 (b), and 30 (c and d) years, and supplied to ELISA for Ksp37 and sCD8. The IgM titer for VCA was determined by indirect immunofluorescence assay.
levels, but, in some cases, it tended to slightly precede the change of sCD8 levels (Fig. 10, a and b). The latter finding is intriguing in that it raises the possibility that Ksp37 levels may better reflect the expansion of NK cells than sCD8 levels because expansion of NK cells is thought to precede that of cytotoxic CD8+ T cells during a typical viral infection (54). Similar but less prominent results were obtained with patients suffering from primary infection with parvovirus B19 and CMV (data not shown). Thus, Ksp37 may serve as a new type of serum marker to monitor the expansion and decline of cytolytic lymphocytes in vivo.

The biological function of Ksp37 remains to be elucidated. We first predicted that Ksp37 might have a role in the processes of target-cell killing. However, in our unpublished experiments, addition of anti-Ksp37 Abs or Ksp37 from culture supernatant of normal PBMCs against K562 target cells, or on the apoptosis of Jurkat cells by Fas ligand-expressing Ltk− cells. In addition, over-expression of Ksp37 in K562 target cells showed no effect on their killing by normal PBMCs in a standard NK assay. Thus, at present, it seems unlikely that Ksp37 functions as a modulator of cytotoxicity or as a cytotoxic effector molecule by itself. Cytolytic lymphocytes produce various kinds of antimicrobial molecules such as IFN-γ and granulysin (55), and human serum has been reported to possess nonspecific antiviral activity (56). However, Ksp37 had no effect on the growth of E. coli and on the in vitro infectivity of polioviruses, coxsackieviruses, echoviruses, adenoviruses, and herpes simplex viruses (our unpublished data).

Ksp37 shows a 24% identity to HBP17 (FGF-binding protein) in amino acid sequence. HBP17 was originally identified as a heparin-binding protein of 17 kDa in conditioned medium of human epidermoid carcinoma cells (21) and is suggested to be involved in tumor angiogenesis by regulating the release of basic FGF that are stored in the extracellular matrix (57–58). Unlike HBP17, Ksp37 itself showed no direct association with 12S-labeled recombinant human basic FGF and no effect on the exogenous basic FGF-dependent or the spontaneous colony growth of SW-13 cells in soft agar (57) (data not shown). However, the positioning of eight cysteine residues in the signal-truncated form of Ksp37 is completely conserved in HBP17, and calculated isoelectric points (9.15 for Ksp37 and 9.28 for HBP17) and hydrophobicity profiles of these proteins are very similar to each other, suggesting similar conformation. Therefore, like HBP17, Ksp37 could bind to some protein(s) to regulate its activity and thereby mediate an as yet unknown critical function of cytotoxic lymphocytes.

References


