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Detection of Expressed IL-32 in Human Stomach Cancer Using ELISA and Immunostaining

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Interleukin (IL)-32 is a recently identified proinflammatory cytokine that is one of the IL-18 inducible genes, and plays an important role in autoimmune and inflammatory diseases. We produced antibodies against IL-32 and studied the expression of IL-32 in human stomach cancer. We detected IL-32 secreted from K-562 cells which were stably transfected with IL-32 and in the sera of stomach cancer patients by a sandwich ELISA using a monoclonal antibody KU32-52 and a polyclonal antibody. In order to optimize a sandwich immunoassay, recombinant IL-32α was added, followed by the addition of a biotinylated KU32-52 into microtiter plate wells precoated with a goat anti-IL-32 antibody. The bound biotinylated KU32-52 was probed with a streptavidin conjugated to HRP. This sandwich ELISA was highly specific and had a minimal detection limit of 80 pg/ml (mean±SD of zero calibrator) and measuring up to 3,000 pg/ml. This ELISA showed no cross-reaction with other cytokines such as hIL-1α, hIL-1β, hIL-2, hIL-6, hIL-8, hIL-10, hIL-18, and hTNF-α. Intra-assay coefficients of variation were 18.5% to 4.6% (n=10), and inter-assay coefficients were 23% to 9% (n=10). The average IL-32 level in the sera of 16 stomach cancer patients (189 pg/ml) was higher than that of 12 healthy control men (109 pg/ml). Our results indicate that serum IL-32 level can be detected by using an established ELISA, and that this immunoassay and mAb KU32-09 specific for immunohistochemistry can be used in the detection of expressed and secreted IL-32 in stomach cancer patients.

Keywords: IL-32, ELISA, IHC, monoclonal antibody, stomach cancer

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IL-32 is a multifunctional cytokine produced by T lymphocytes, natural killer cells, monocytes, and epithelial cells [12, 19]. The gene encoding IL-32 is located on human chromosome 16p13.3 and organized into eight exons [4]. There are six splice variants (IL-32α, IL-32β, IL-32γ, IL-32δ, IL-32ε, and IL-32ξ) [9], and IL-32α is the most abundant transcript [25]. It is still controversial whether IL-32 acts on cells from outside or inside, because the newly identified isoforms of IL-32 lack the putative signal peptide [9], and the amounts of IL-32 secreted from the cells are small, compared with those in the cytosol [12]. IL-32 exhibits several properties typical of proinflammatory cytokines through nuclear factor-κB and p38 mitogen-activated protein kinases (MAPKs). IL-32 has been implicated in inflammatory disorders such as rheumatoid arthritis (RA) [3, 11, 26], *Mycobacterium tuberculosis* infection [14, 20], inflammatory bowel disease (IBD) [25], and Crohn's disease (CD) [25]; however, the pathophysiological role of IL-32 in gastric cancer remains unclear. Proinflammatory cytokines induce NO production, iNOS expression, and invasiveness of adenocarcinoma cells [18]. As described above, harmful effects on the host are also induced by chronic exposure to proinflammatory cytokines, and therefore, the exposure time must be limited by an appropriate mechanism [5, 10].

IL-18 has been studied for its biological effects on the broad spectrum of Th1- or Th2-related autoimmune diseases [17, 21, 22]. IL-1 and IL-18 belong to the IL-1 ligand family because they share structural similarity and require caspase-1 for processing [2]. IL-18 also uses a signaling pathway similar to that of IL-1 family members. They recruit IL-1 receptor-associated kinases (IRAKs), form IRAK complexes with the tumor necrosis factor receptor-associated factor-6, and activate the cascade of inhibitor of kappa B/

nuclear factor-kappa B (Ik-Bα/NF-κB) [13]. Members of the IL-1 ligand family play important roles in the development and pathogenesis of autoimmune and inflammatory diseases [8]. Blocking IL-18 in animal disease models reduced disease severity, including arthritis [1, 23], inflammatory bowel disease [27], graft versus host disease [16], ischemia-reperfusion injury [24], and spontaneous atherosclerosis [15]. Therefore, IL-18-inducible genes might contribute to autoimmune and inflammatory diseases. IL-32, an inducer of TNF-α, was identified as one of the IL-18 inducible genes in IL-18 responsible cells [12].

It has recently been reported that IL-32 is expressed in rheumatoid arthritis and in the epithelial cells of IBD and CD patients. However, no study on the effects of IL-32 on cancers has yet been carried out. Research on a new cytokine has generally been hampered by the lack of well-characterized, readily available antibodies that could be used for immunochemistry on fixed tissues, Western blotting, ELISA, and other purposes. We, therefore, produced antibodies reactive to IL-32, and the IL-32 level could be detected by using an established sandwich ELISA and mAb KU32-09 specific for immunohistochemistry in the sera and tissues of stomach cancer patients. In this study, we investigated expressed or secreted IL-32 in stomach cancer.

MATERIALS AND METHODS

Reagents and Antibodies

K-562 lymphoblast cell line was obtained from ATCC (Bethesda, MD, U.S.A). RPMI-1640 and fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT); Polyethylene glycol 1500 (PEG 1500) and hypoxanthine aminopterin thymidine (HAT) were purchased from Sigma (St. Louis, MO, U.S.A). Other reagents used in this study were of analytical grade and commercially obtained. Goat anti-IL-32 antibody was purchased from R&D Systems, Inc. (Minneapolis, MN, U.S.A). Streptavidin-peroxidase and anti-mouse IgG HRP conjugate were obtained from Sigma. Human recombinant hIL-1α was from Glaxo Inc. (Research Triangle, NC, U.S.A.), hIL-2 from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel), and recombinant hIL-18 was prepared as previously described [6]. Human recombinant IL-1β, hIL-6, hIL-8, hIL-10, and hTNF-α were from R&D Systems.

IL-32 Cloning and Expression of Recombinant Proteins

Total RNA was isolated from the human NK cells (ATCC) with TRIZOL-Reagent (Sigma, St. Louis, MO, U.S.A.). A pair of sense primer, 5'-CTGTCCCGAGTCTGGACTTT-3', and reverse primer, 5'-GCAAAGGTG GTGTCAGTATC-3', was used for RT-PCR. Two μg of total RNA was converted to first-strand cDNA, with superscript II RT (Invitrogen, San Diego, CA, U.S.A.) and then 30 cycles of PCR reaction was performed at 94°C for 45 s, 70°C for 2 min, and 59°C for 1 min. The PCR products were ligated into pGEMT-Easy (Promega, Madison, WI, U.S.A.) for DNA sequencing, and IL-32 inserts were then transferred into pPROEX/HTa (Invitrogen) as previously described [12].

Recombinant IL-32α and IL-32β proteins were expressed in *E. coli* and purified with a TALON affinity column (Invitrogen) using a 6× His-tag at the N-terminus of recombinant proteins as previously described [12]. The TALON affinity-purified proteins were subjected to size exclusion chromatography (Superdex 75, A KTAFPLC) and then digested with TEV (Tobacco Etch Virus; Invitrogen) for 16 h at 4°C in order to remove the 6× His-tag fusion protein as previously described [12]. The TEV-cleaved recombinant proteins were dialyzed in phosphate buffer (20 mM, pH 9.0), and then identified using SDS-PAGE.

Preparation and Purification of Antibodies

A 5-week-old Balb/c female mouse was immunized with 20 μg of IL-32α recombinant protein emulsified in Freund's complete adjuvant (Sigma). On days 14 and 21, the mouse received intravenous and intraperitoneal injections with the antigen emulsified in Freund's incomplete adjuvant (IFA) (Sigma). After three injections, the mouse was sacrificed, the spleen was aseptically removed, and splenocytes were prepared for fusion. Briefly, 1×10^7 splenocytes and 1×10^6 of an NS-1 mouse myeloma cell line (ATCC) were fused using polyethylene glycol 1500 (Roche Applied Science, Indianapolis, IN, U.S.A.). Fused cells were resuspended at 1×10^6 cells/ml in hybridoma selection RPMI media containing 10% FBS and HAT. Hybridomas were screened with IL-32α-coated plates for direct ELISA. Subsequently, mAb classes and subclasses were determined by using an Immuno-Type™ mouse mAb isotyping kit (BD Bioscience, San Diego, CA, U.S.A.) according to the manufacturer's instructions.

Two hybridomas (each 5×10^6 cells), KU32-09 (IgG1) and KU32-52 (IgG1) clones, were intraperitoneally injected into an 8~9-week-old Balb/c female mouse preinjected with 0.5 ml of IFA one week prior. From one week after injection, the ascitic fluids were removed with an 18G sterile needle (Green Cross Inc., Seoul, Korea). Supernatants were collected by centrifugation, and proteins were purified using protein A/G Sepharose (Bio-Rad), and the antibodies were eluted with 0.1 M glycine-HCl (pH 2.7).

Immunohistochemistry (IHC)

The tissue samples of stomach from 29 individual patients were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. Three-μm-thick sections from the paraffin blocks were used for IHC of IL-32 with the EnVision horseradish peroxidase detection system (DAKO). IHC steps were carried out at room temperature (RT). After deparaffinization, the sections in 10 mM sodium citrate buffer (pH 6.0) were placed into a pressure cooker at full power for 4 min, followed by treatment with 3% hydrogen peroxide for 10 min. The primary antibody KU32-09 (IgG1, 0.1 μg/ml) was incubated for 30 min with the tissue sections, and exposed to EnVision reagent (DAKO) for 30 min. The slides were then sequentially incubated with the chromogen reagent for 5 min, counterstained with Meyer's hematoxylin, and mounted. Negative control staining was performed by using mouse IgG1 isotype antibody. An Olympus microscope (BX50 model) equipped with a digital camera was used to prepare microphotographs with magnifications of 200× or 400×.

Optimization of ELISA System Using Monoclonal and Polyclonal Antibodies

mAb KU32-52 was biotinylated by a commercial kit using BIOTIN-LC-SULFO-NHS-ESTER (BioVeris, Gaithersburg, MD, U.S.A.). Briefly, 0.5 ml of KU32-52 (0.7 mg/ml) solution was mixed with Biotin (26 μg), reacted for 1 h at RT, and then the mixture loaded onto Sephadex G-10 pre-equilibrated with PBS. The biotinylated KU32-52 (0.5 μg/ml)

was aliquoted and stored at −70°C until use. Serially diluted IL-32α, β proteins, or other cytokines were added to wells precoated with goat anti-IL-32 antibody at a concentration of 4 μg/ml and incubated overnight at 4°C. Plates were washed three times with PBST, and biotinylated-KU32-52 (0.2 μg/ml) diluted in 3% skimmed milk was then added, followed by 1 h of incubation. Plates were then washed three times with PBST and further incubated with streptavidin-HRP (0.3 μg/ml; Upstate) diluted in 3% skimmed milk. After the plates were washed as above, SureBlue TMB peroxidase substrate (KPL, Inc. Gaithersburg, MD, U.S.A.) was added and 50 μl of 2.5 N sulfuric acid was added to the wells to stop the enzyme reaction. The enzyme activity was detected at 450 nm using an ELISA reader (Photoread, Berthold, Germany). All values represent mean± standard deviation of at least three independent experiments.

Analytical Validation of ELISA

The sensitivity of the ELISA was calculated as the mean absorbance value of duplicate measurements of 0 standard plus three times the SD. The concentration in pg/ml was calculated from the standard curve. The intra-assay variance was determined with duplicate samples at different levels (high, medium, and low) of IL-32 to determine the precision of the assay. The samples were assayed in 10 replicates. The interassay precision was determined in duplicates for three (high, medium, low) levels of IL-32 samples, measured on 10 subsequent independent assays. Analytical recovery was measured using a negative control medium (RPMI-1640 containing 5% FBS) spiked with a predetermined concentration of recombinant IL-32α. These spiked media were serially diluted and then assayed in duplicate.

Establishment of Mammalian Cells Stably Expressing IL-32 and Identification of Intact IL-32 Using mAb KU32-52

In order to confirm whether KU32-52 could detect intact IL-32 in mammalian cells, K562 cells were used to transfect IL-32. cDNA of human IL-32α or IL-32β was cloned into mammalian expression vector pcDNA3.1$^+$ and then 1 μg each of plasmid DNA was transfected using the Amaxa Nucleofector Technology (Amaxa, Köln, Germany). One day after transfection, the transfectants were subjected to selection for three weeks in the presence of 1 mg/ml of G-418. The stable transfectants were maintained in RPMI-1640 culture medium containing 10% FBS and 400 μg/ml of G-418. Subsequently, constitutive expression of IL-32α or β was confirmed by Western blotting. Briefly, proteins were resolved by 12% SDS-PAGE and transferred onto a polyvinylidenedifluoride (PVDF) membrane (Millipore Co., Billerica, MA, U.S.A.). The membrane was blocked with 5% skimmed milk and then incubated with mAb KU32-52 at RT for 1 h. After washing with PBS containing 0.05% Tween-20 (PBST) solution, the membrane was reacted with peroxidase-conjugated anti-mouse IgG at RT for 1 h and treated with enhanced chemiluminescence (ECL) solution (Amersham Biosciences, Uppsala, Sweden), followed by exposure to X-ray film.

In order to detect the IL-32 secreted from the stable IL-32α or β expressing K562 transfectants, the stable transfectants were treated with 50 nM phorbol 12-myristate 13-acetate (PMA) for 3 days, and IL-32 levels were evaluated by the sandwich ELISA system described above.

Study Population

Blood samples and tissues were obtained from the Chungnam National University College of Medicine with informed consent from all subjects. In this study, sera from 16 patients with gastric carcinoma and from 10 healthy donors were used. The samples were allowed to clot and then stored at −70°C until assay. According to the TNM classification and histopathologic grading system, there were 4 cases of gastric carcinoma patients with stage IA, 6 cases of stage II, 3 cases of stage IIIA, and 3 cases of stage IV. We acquired the approval of the University Ethics Committee.

Statistical Analysis

Differences between gastric carcinoma patients and normal donor group were tested using Student's *t*-test. Differences were considered significant at $p<0.05$.

RESULTS

Isotyping Analysis of Monoclonal Antibodies and Imunohistochemical Staining of IL-32 Expressed in Gastric Tumor Tissues

The immunoglobulin isotyping analysis revealed that the subclasses of mAbs KU32-09 and KU32-52 were all IgG1. Western blot analysis revealed that KU32-52 was reactive to the recombinants IL-32α, IL-32β, IL-32γ, and IL-32δ (data not shown). KU32-09 could not detect IL-32 by Western blotting (data not shown), whereas this KU32-09 was shown to react on immunohistochemistry (Fig. 1). IL-32 expression in human gastric tumor tissues was analyzed by immunohistochemical staining with mAb KU32-52. As shown in Fig. 1, IL-32 was not detected in tissues stained with a negative isotype control mouse IgG1 antibody. However, the IL-32 staining by using mAb KU32-09 showed strong cytoplasmic and nuclear expressions in 29% (12/29) of stomach cancer patients. The IL-32 staining by using mAb KU32-09 also showed strong cytoplasmic and nuclear expressions in the lung and cervical cancer tissues

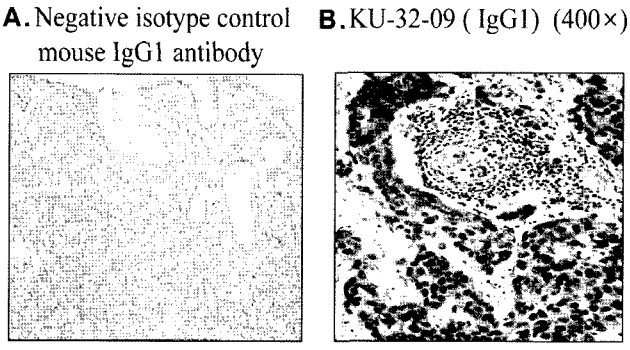

Fig. 1. Representative immunohistochemical staining of IL-32 in gastric cancers. The tissue samples of stomach from 29 individual patients were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin.

Sections from the paraffin blocks were examined for IL-32 expression using mAb raised against IL-32. These data represent one of 29 cases of the gastric cancer tissues examined. **A**. Negative isotype control with mouse IgG1 antibody shows negative staining (original magnification, ×200). **B**. IL-32 is strongly positive in the nuclei and cytoplasms of gastric cancer cells (original magnification, ×400).

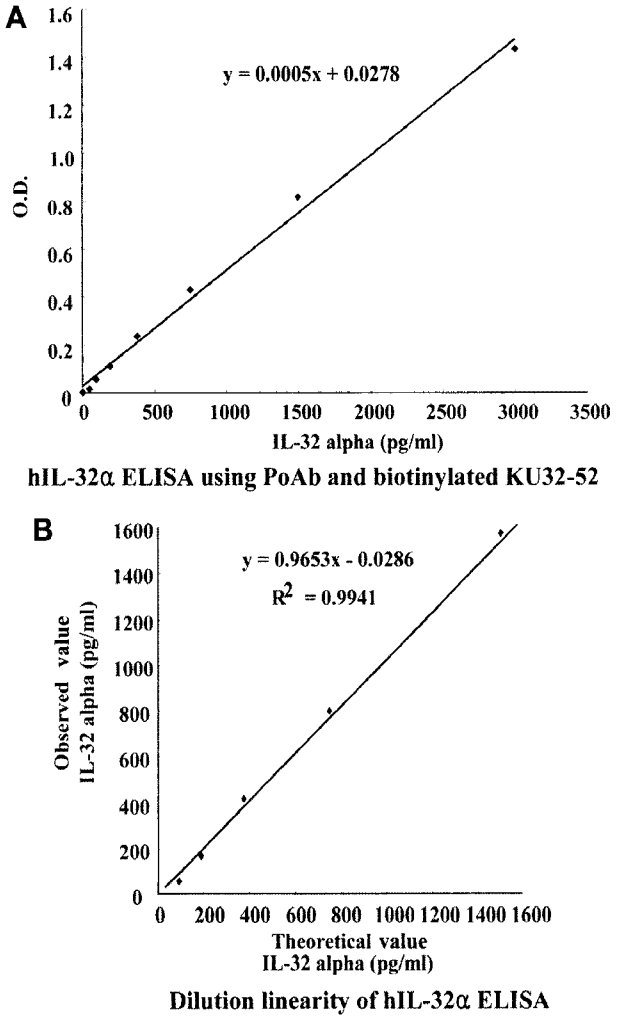

Fig. 2. ELISA for IL-32 using coating with polyclonal Ab and capturing biotinylated mAb KU32-52.
A. ELISA for hIL-32α using poAb and biotinylated mAb KU32-52 as coating and capturing antibody, respectively. Recombinant IL-32α was added, followed by biotinylated KU32-52, into microtiter plate wells precoated with goat anti-IL-32 antibody, and the bound biotinylated KU32-52 was probed with streptavidin conjugated to HRP. Standard calibration curve was generated by the established ELISA, where the recombinant hIL-32α was used as the standard. **B.** Dilution linearity of IL-32 ELISA. IL-32α was serially diluted and the IL-32 level detected by using the established ELISA as described in (**A**).

(data not shown). These results suggested that this mAb KU32-09 can not bind to the antigenic determinant of denatured IL-32, but can detect the native IL-32 expressed in tissues.

Evaluation of IL-32 ELISA

Serially diluted IL-32α, β proteins or other cytokines were added into wells precoated with goat anti-IL-32 antibody, followed by capturing of IL-32 by biotinylated-KU32-52 for 1 h of incubation. A streptavidin peroxidase was further incubated after washing. These sandwich ELISAs were highly specific, had a minimal detection limit of 80 pg/ml (mean±3SD of zero calibrator), and measured up to 3,000 pg/ml

Table 1. Evaluation of ELISA for hIL-32 using polyclonal anti-hIL-32 antibody and biotinylated mAb KU32-52 as coating and capturing antibody, respectively.

A. Run-to-run precision

	Control	N	X	SD	%CV	Recovery (%)
IL-32α (pg/ml)	Low (250 pg/ml)	10	255.8	59.1	23.09	102.3
	Medium (1,000 pg/ml)	10	1,166.9	105.8	9.07	116.7
	High (2,500 pg/ml)	10	2,449.5	306.7	12.5	98.0

B. Within-run precision

	Control	N	X	SD	%CV	Recovery (%)
IL-32α (pg/ml)	Low (250 pg/ml)	10	265.4	49.1	18.5	106.2
	Medium (1,000 pg/ml)	10	892.3	35.1	3.9	89.2
	High (2,500 pg/ml)	10	2,048.5	94.7	4.6	81.9

C. Dilution linearity

Dilution factor	Expected (pg/ml)	Observed (pg/ml)	Recovery (%)
1	3,000	2,847.2	94.9
2	1,500	1,748.0	116.5
4	750	905.5	120.7
8	375	406.3	108.4

D. Cross-reaction of monoclonal antibody for hIL-32α and the related cytokines

Cytokines	Cross-reaction (%)
hIL-32α	100
hIL-32β	100
hIL-1α	<0.1
hIL-1β	<0.1
hIL-2	<0.1
hIL-6	<0.1
hIL-8	<0.1
hIL-10	<0.1
hIL-18	<0.1
hTNF-α	<0.1

(Fig. 2A). An ELISA, using coating with goat anti-IL-32 antibody and capturing biotinylated mAb KU32-52, had no cross-reaction with other cytokines such as hIL-1α, hIL-1β, hIL-2, hIL-6, hIL-8, hIL-10, hIL-18, and hTNF-α. Intra-assay coefficients of variation were 18.5% to 4.6% (n=10), and interassay coefficients were 23% to 9% (n=10) (Table 1). The accuracy for the determination of IL-32 in the medium was evaluated after the medium was fortified with known amounts of IL-32 as indicated (Fig. 2B).

Identification of Intact IL-32 in Mammalian Cells Using KU32-52

Next, we investigated whether KU32-52 could detect intact IL-32 in mammalian cells. Thus, K562 cells were transfected

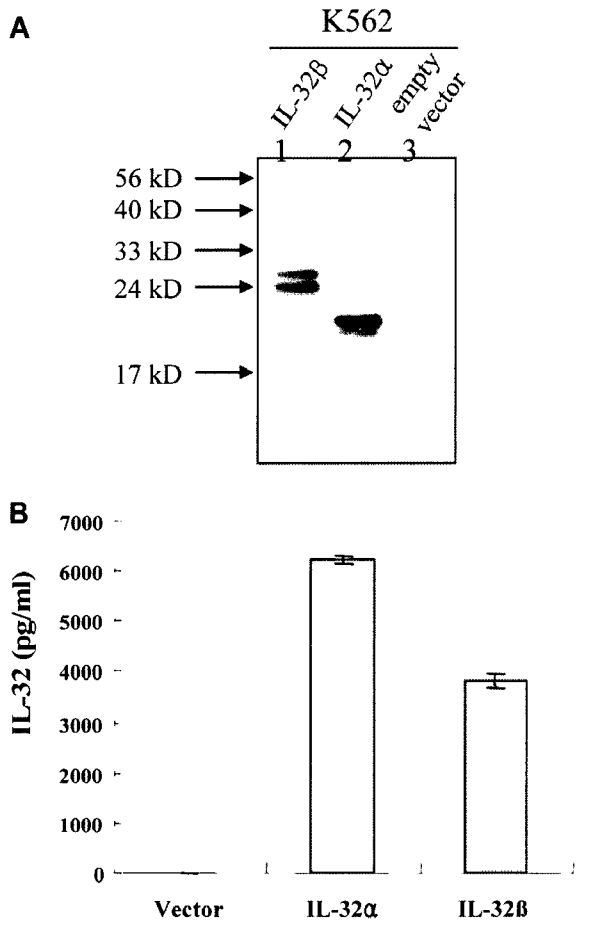

Fig. 3. Detection of expressed or secreted IL-32 from the stable K-562 transfectants expressing IL-32α or IL-32β.
A. Detection of expressed IL-32 in the transfectants expressing IL-32α or IL-32β. The cell lysates of the stable transfectants were resolved on SDS polyacrylamide gel and Western blotted. IL-32 protein was probed by using mAb KU32-52. **B.** Evaluation of secreted IL-32 from the supernatants of PMA-treated stable transfectants K562/IL-32α or K562/IL-32β. During the study on the effects of IL-32 on K562 differentiation induced by PMA treatment, we could detect IL-32α or IL-32β in culture supernatants.

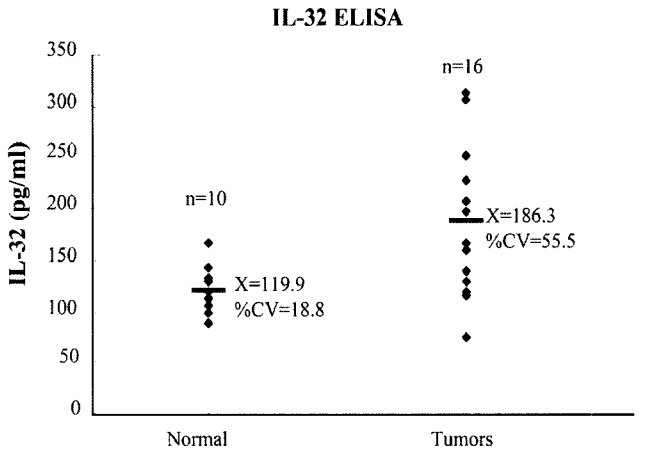

Fig. 4. IL-32 protein levels in the plasma of stomach cancer patients and healthy individuals.
Plasma samples obtained from 16 gastric carcinoma patients and from 10 normal healthy donors were evaluated for reactivity with IL-32 antibody using an ELISA. The p value between patients and normal individuals was slightly over 0.05 (p=0.068).

with IL-32 cDNA because IL-32 was not detected in these cell lines by Western blot using anti-IL-32 antibodies. After transfection of cDNA of human IL-32α or IL-32β plasmid DNA into K562 cells followed by exposure to G418 for selection for three weeks, constitutive expression of IL-32α or IL-32β was confirmed by Western blotting using KU32-52 (Fig. 3A). We detected the secreted IL-32α (20 kDa) or IL-32β (25 kDa) during the investigation of the effects of IL-32 on PMA-induced differentiation of erythroleukemia K562 cell. There are glycosylation sites in IL-32 [12], showing two bands each of IL-32α or IL-32β after post-translation in cells (Fig. 3A). An ELISA, using coating with polyclonal antibody and capturing biotinylated mAb KU32-52, showed that IL-32α and IL-32β were secreted from the K562/IL-32α and K562/IL-32β cells, respectively, when treated with PMA. This ELISA system could identify both IL-32α and IL-32β from PMA-activated IL-32α-or IL-32β-expressing K-562 cells (Fig. 3B). As this is an ectopic expression, the IL-32 proteins might have been leaked from dead or damaged cells.

Serum IL-32 Protein Levels in Gastric Cancer Patients

To investigate the potential of IL-32 as a serological marker for gastric cancers, we analyzed sera obtained from 16 gastric cancer patients and 10 healthy donors using a sandwich ELISA (Fig. 4). The average IL-32 level in the sera of the gastric cancer patients was 189 pg/ml with a range of 115–305 pg/ml. This value was higher than that of healthy blood donors (p=0.068). The average IL-32 level in the sera of 10 healthy control men was 109 pg/ml, which is near the detection limit (80 pg/ml), with a range of 87–167 pg/ml. These results support that the amounts of IL-32 secreted from the cells were small, compared with those in the cytosol [12], thus indicating that the IL-32 level was slightly increased compared with the healthy control men.

Discussion

The initial NK4 cDNA contained a signal peptide sequence without a transmembrane domain and was never expressed as a recombinant protein [7]. Therefore, it has been assumed that the protein coding for NK4 is a secreted protein. However, it still remains controversial whether IL-32 acts on cells from outside or inside, because the newly identified isoforms of IL-32 lack the putative signal peptide [9]. In this study, monoclonal antibodies reactive to IL-32 isoforms were prepared and characterized for studying the expression and secretion patterns of a new cytokine IL-32 in gastric

cancer. One mAb, KU32-09, was shown to react strongly on immunohistochemistry in the tissues of gastric cancer (Fig. 1) as well as in the tissues of rheumatoid arthritis [11]. In addition, marked expression of IL-32 was detected in colon mucosa using this KU32-09 [19]. These results suggest that KU32-09 can be effectively used in the functional study of IL-32 in the pathogenesis of cancers and inflammatory diseases. In order to detect IL-32 levels secreted from the cell lines and in the sera of gastric cancer patients, we established and evaluated a sandwich ELISA, based on the combination of antibody pairs. The combination of coating with polyclonal antibody and capturing with biotinylated mAb KU32-52 was proven to optimize the sandwich ELISA system. The bound biotinylated KU32-52 was probed with a streptavidin-HRP, indicating that this sandwich ELISA can sensitively and specifically detect 100 pg/ml of IL-32α. KU32-52 can detect both IL-32α and IL-32β in a sandwich ELISA, but not other cytokines. By applying this sandwich ELISA, we could detect IL-32α and IL-32β in the culture supernatant of PMA-treated K562/IL-32α or K562/IL-32β transfectants although this did not mean that these cell lines secrete IL-32α and IL-32β. The IL-32 level in sera was lower than 6 ng/ml (Fig. 3B) and we could not detect secreted IL-32 protein by Western blotting using mAb KU32-52 (data not shown), whereas intracellular IL-32 protein was detected (Fig. 3A), indicating that the amounts of IL-32 secreted from the cells were small, compared with those in the cytosol [12]. These results suggest that the new cytokine IL-32 usually exerts a role inside the cells, and a small amount of IL-32 is secreted from the cells.

In these studies, we have optimized and evaluated IL-32 ELISA: IL-32 mAbs can be used in the detection of expressed IL-32 in certain cells and in tissues of patients with chronic inflammatory diseases or some cancers. IL-32 ELISA systems can effectively detect secreted IL-32 in certain cells and patient's tissues or sera.

Acknowledgments

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