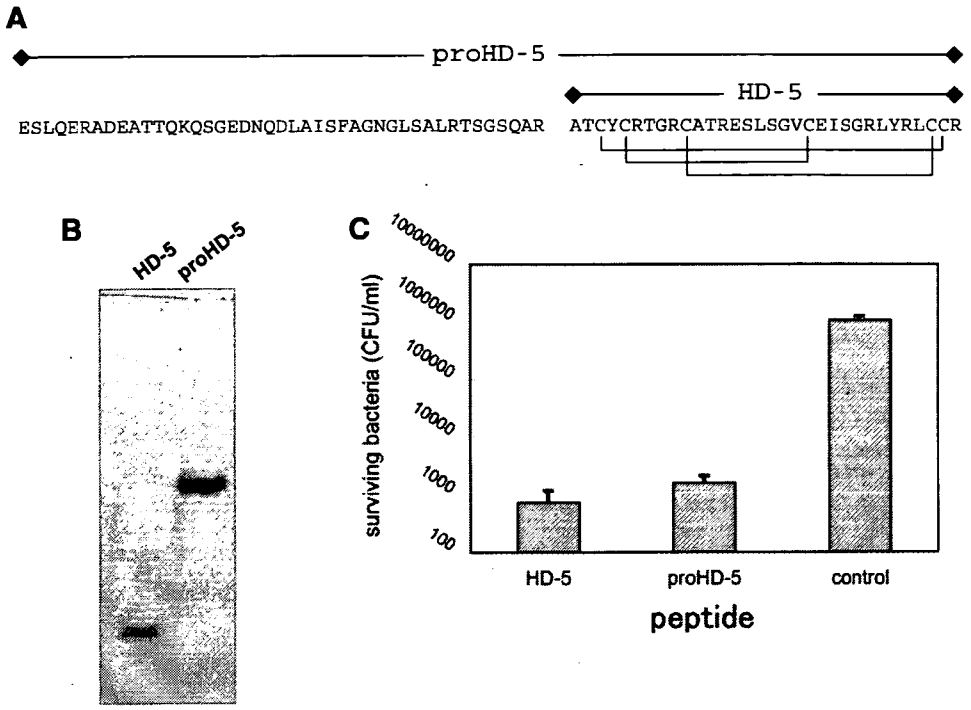

Fig. 1. Recombinant peptides of human enteric defensins. (A) Amino acid sequences of proHD-5 and HD-5. (B) Two micrograms of purified defensins were resolved on a 12.5% acrylamide AU–gel and stained with Coomassie blue. HD-5 moved faster than proHD-5 because mobility depends on size and charge of the peptide. (C) The bactericidal activity of each defensin was determined against *Salmonella typhimurium* PhoP-null. Each peptide (10 μg/ml in final conc.) was incubated with 10^6 CFU/ml of bacterial cells for 1 h and surviving bacteria were then counted. HD-5 and proHD-5 showed equivalent bactericidal activities.

form migrated significantly slower than the folded HD-5 and proHD-5, respectively (Fig. 2A and C). Since the reduced peptide is sensitive to trypsin treatment, the band was diminished in the gel. The folded proHD-5 was cleaved in order to form the mature HD-5 peptide which was resistant to the cleavage (Fig. 2C), confirming previous report [10]. The reduced peptides, which lost the bactericidal activities, demonstrated that the disulfide bonds confer not only the ability of stabilization of the molecule but also the physiological activity (Fig. 2B and D). The mass of the folded and the denatured proHD-5, confirmed with MALDI-TOF MS, was 8100.3 and 8105.9, respectively. The reduced peptide was 6 amino mass units longer than the folded peptide.

Defensin expressions in normal ileum

HD-5 from CD mucosa was sensitive to trypsin digestion

The expression of the peptides was determined by AU–PAGE Western blotting (Fig. 3). In order to detect the mature peptide from the crude extract, we applied trypsin cleavage. Mature HD-5 was not found in any of the materials without trypsin treatment, but it was detected as a fast migrating band in all of the samples from the normal small intestine after enzymatic digestion. In contrast, the mature HD-5 was diminished in 3 cases of the 6 CD patients (cases 3, 4, and 8), suggesting that the HD-5 that was derived from half of the CD patients was sensitive to trypsin and that the conformation of the mature active HD-5 was changed as the reduced recombinant defensins were digested by the trypsin.

Purification of denatured proHD-5 peptide from CD intestine

Small intestinal tissue was acid-extracted and 5 mg crude sample was developed by reversed phase-HPLC. The chromatograms showed no significant difference between the control and the CD patient (Fig. 4A). The defensin-rich fractions were determined by a bactericidal assay (Fig. 4B). The fraction demonstrating the strongest activity was then applied to the N-terminal protein sequence and ESLQERADEA..., which is identical to pro-peptide of HD-5, was obtained. A MALDI-TOF MS analysis showed 8102.0 amino mass units from the normal tissue (Fig. 4C, upper panel), identifying the pro-HD-5 including three disulphide bonds in the molecule. An HPLC fraction from CD case 3 was able to apply a mass spectrometry study and the 8107.6 amino mass units were observed (Fig. 4C, lower panel). The 6 amino mass units' increase, in comparison to the proHD-5 purified from the normal control, was identical to the reduced proHD-5. Therefore, the proHD-5 found in the CD intestine was expected to form an unfolded struc-

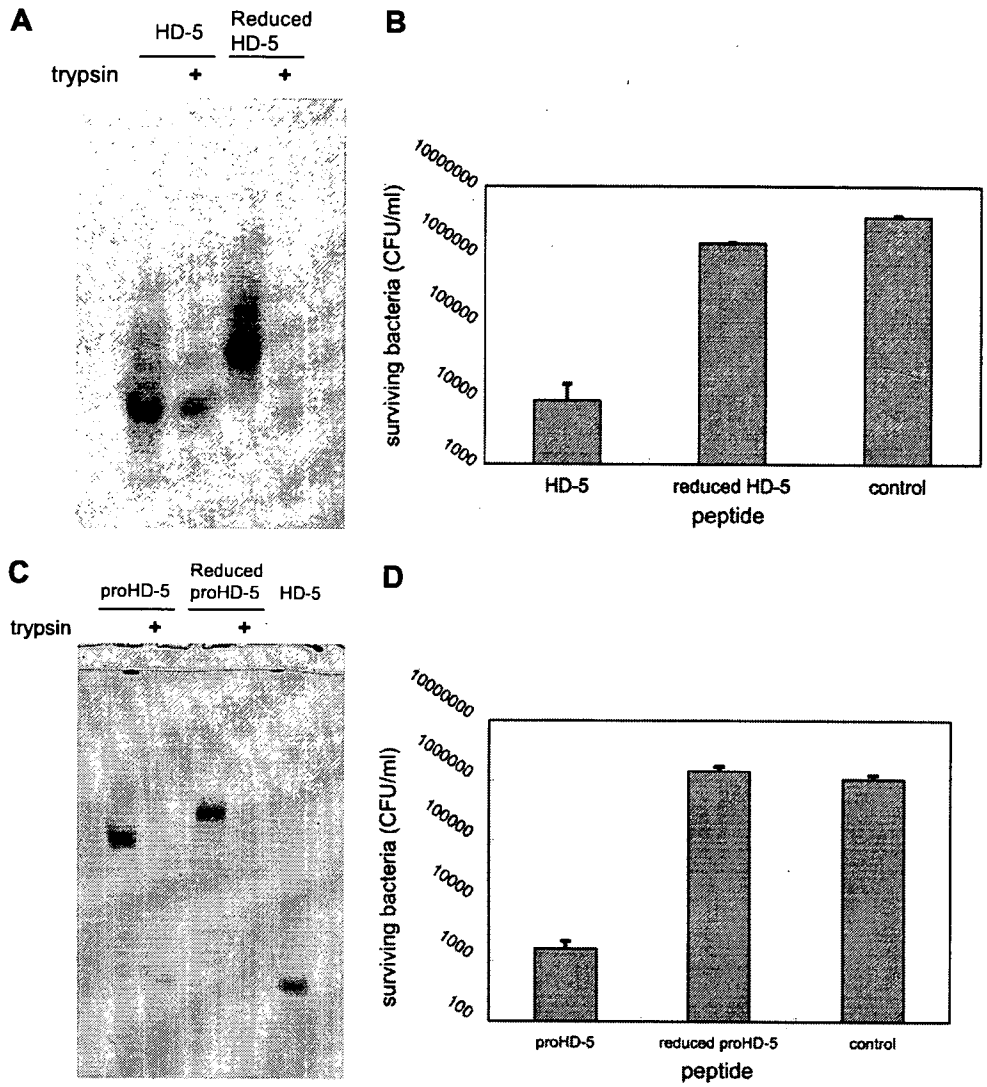

Fig. 2. Characterization of recombinant reduced HD-5 and proHD-5. (A,C) The recombinant HD-5 and proHD-5 peptides were reduced by DTT and purified by HPLC. Both forms were incubated with or without 0.5 μg of the trypsin overnight and loaded on AU–PAGE followed by Coomassie blue staining. The reduced forms moved slower than the folded form and were sensitive to the trypsin treatment. (B,D) Almost all of the bactericidal activity of HD-5 and proHD-5 (10 μg/ml in final conc.) were lost after the peptide was reduced by DTT.

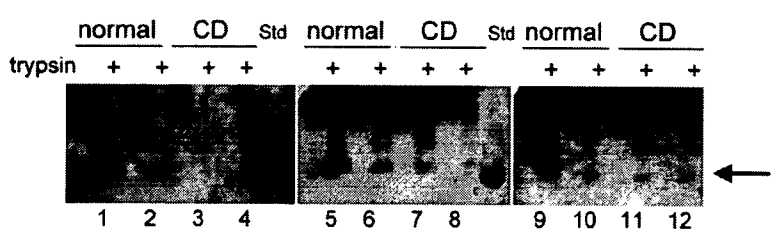

Fig. 3. Trypsin-sensitive HD-5 expression in CD small intestine. The samples (500 μg each lane) were loaded with AU–PAGE and transferred to the membrane for immunodetection. The mature HD-5 was detected as the fast migrating band (arrow). The trypsin sensitive HD-5 were observed in 3 out of 6 CD cases (cases 3, 4, and 8) and the expression of HD-5 was reduced in 3 other cases (cases 7, 11, and 12).

ture with no disulphide bridges. The reduced HD-5 was found in this study, demonstrating that the HD-5 peptide may be regulated by a post-translational modification in nature.

Discussion

The epithelial cells are the first barrier against luminal bacteria and respond to many bacterial antigens. The

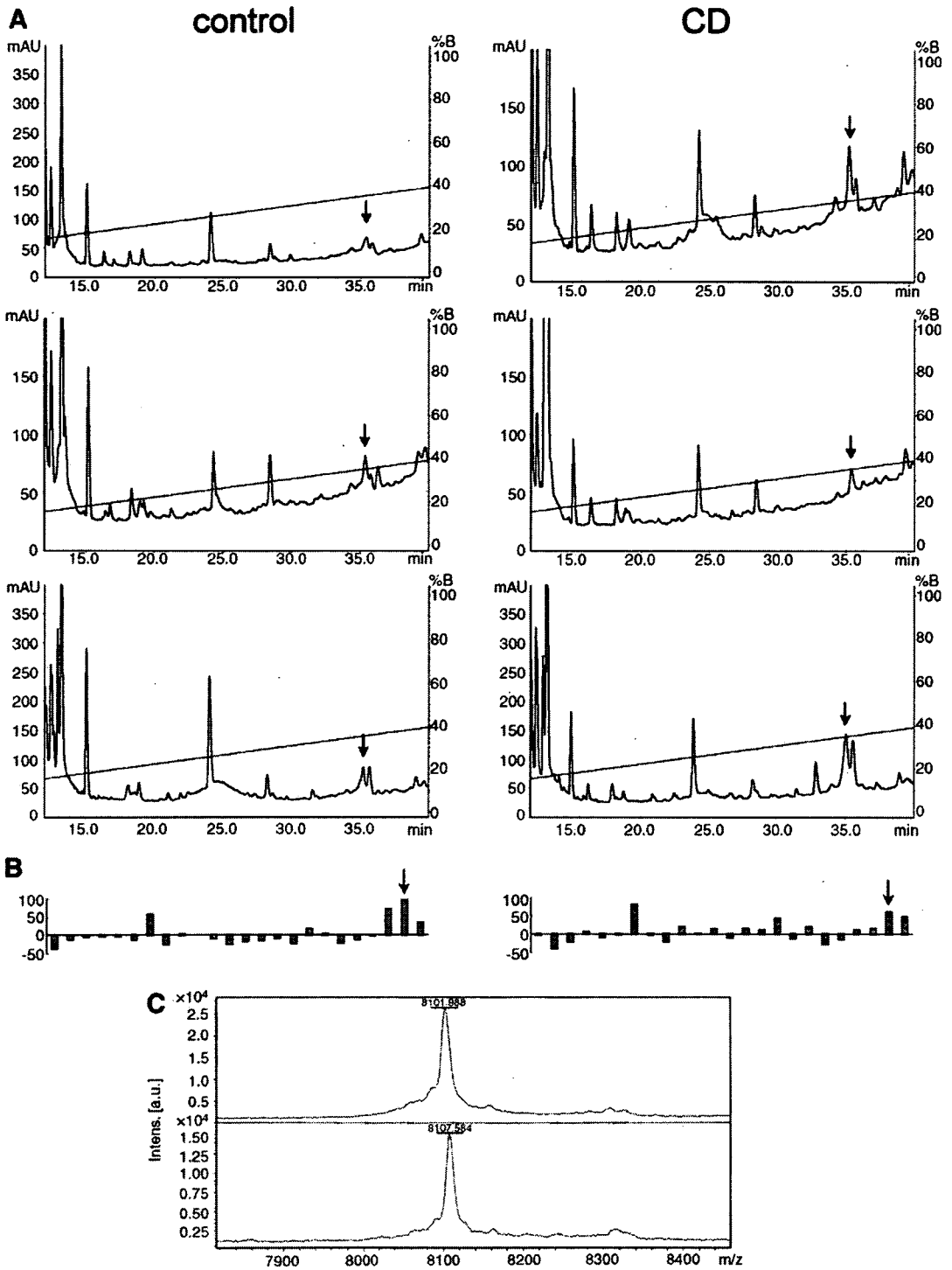

Fig. 4. Purification of native and reduced proHD-5 from the small intestine. (A) The intestinal crude extract was applied to the C18 column. The chromatogram patterns were compared between the control (left panel) and the CD patients (right panel). No significant differences were observed between these two groups. The arrows denoted the fractions containing proHD-5, determined with protein sequence. (B) Each fraction was applied to the bactericidal assay described in Materials and methods. The fractions of proHD-5 showed strong activity in both groups (arrow). Each bar presented the means of three experiments. (C) MALDI-TOF MS demonstrated that six amino mass units' increase in the CD patient (lower panel, case 3), presenting the disulphide bonds were reduced.

small intestinal Paneth cells, which are specialized columnar cells characterized by dense granules, are one of the major contributors to the innate immunity [2,21,22]. Two enteric defensins, HD-5 and HD-6, have been found in the Paneth cells [7,8]. The localization of HD-5 is determined by immunohistochemistry and its bactericidal

activity has already been demonstrated [9]. The Paneth cells are located at the bottom of the small intestinal crypt, and the granules are remarkably stained strongly in the serum.

One of the major roles of the Paneth cells is to act as a host defense against microbes secreting granules constituting an antimicrobial peptide mixture, determined with isolated crypts from the mouse small intestine [2]. The isolated crypts from the matrilysin knockout mouse lacks mature defensins and exhibits decreased antimicrobial activity. The *in vivo* study of the knockout mouse demonstrates the functional roles of defensins against orally challenged bacteria and the activation process [6]. Proteolytic activation is required for mouse intestinal defensins and this activation is completed in the Paneth cell granules before secretion. Mouse defensins are stored as mature forms in the intestinal tissue but human defensins are stored as pro-peptides and cleaved by a digestive enzyme, trypsin after the Paneth cell granules are secreted into the crypt rumen [10]. The bactericidal activity of the crude extract has been determined in recent papers [23]. In our study, proHD-5 was purified as the most bioactive molecule from low molecular proteins, though the mature HD-5 was not purified. The bactericidal activities of both the recombinant HD-5 and proHD-5 were also determined against *Salmonella* strains. It was concluded that HD-5 is stored as pro-peptides and already possesses bactericidal activity even without cleavage by trypsin, which is a cleavage enzyme of human enteric defensin after the Paneth cell granules are secreted into the luminal surface.

This activation process of the human enteric defensin is partially different from the mouse defensins that have been well studied in previous papers. The series of recombinant peptide studies has already demonstrated the relationship between the enzymatic activation and the characteristics of the disulfide bonds. Some of the experiments have focused on the roles of the disulfide bonds in the mouse defensins to demonstrate stability against enzymatic cleavage [11]. The disulfide bridges of human defensin contributed to stabilize the structure of the molecule and to keep the activity, as well as the mouse defensins. The human defensin was resistant to enzymatic digestion so that the trypsin treatment followed by AU–PAGE enabled us to detect the defensin as fast migrating bands. We coincidentally found that the HD-5 peptide diminished after the trypsin treatment in 3 cases out of 6 CD patients. We hypothesized that the process of disulfide bridge formation may be disturbed in the CD Paneth cells. The chromatograms of the CD intestine did not differ from the normal control. The peak of the proHD-5 was also conserved in all cases. The mass spectrum demonstrated the abnormal folding of the native proHD-5 that was purified from a CD patient. The protective roles of the disulfide bonds from the enzymatic degradation were also demonstrated using the recombinant peptides. We preliminarily found that the crypts that were isolated from the CD patients lacked bactericidal activity (data not shown). Taken together, the innate immune deficiency involved in CD may due to the abnormal activation process.

The intracellular synthesis of the disulfide bonds of the defensins remains unclear. For generating synthetic peptides with disulfide bonds, oxidative folding with reduced and oxidized glutathione has been successful [24], and the bacterial production of recombinant peptides has also been applied. The detailed mechanism of the modification, as well as mammalian production, will require further study. It is well known that protein folding is formed in the endoplasmic reticulum and that disulfide bonds are facilitated by the chaperones [25]. Therefore, stress induced reduction–oxidation unbalance in CD patients may cause a defensin reduction in the Paneth cells in the small intestine.

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ARTICLE

Immunolocalization of a Novel Collectin CL-K1 in Murine Tissues

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SUMMARY We have recently identified a novel collectin, CL-K1, that may play a role in innate immunity as a member of the collectin family. In this study using mice, we investigated the tissue distribution of CL-K1 for better understanding of its pathophysiological relevance. Real-time PCR analyses demonstrated that CL-K1 mRNA was expressed in all tissues tested. Immunohistochemical analyses demonstrated that CL-K1 was expressed in proximal tubules of kidney, in mucosa of the gastrointestinal tract, and in bronchial glands of bronchioles similar to the localization of SP-A and SP-D in these pulmonary structures. Immunohistochemistry also showed that CL-K1 was highly expressed in hepatocytes around the central veins in liver, which suggests that murine CL-K1 may be mainly produced in the liver and secreted into the blood stream as is human CL-K1. CL-K1 was especially detected in vascular smooth muscle in several types of tissues. In addition, it was also expressed in intestinal Paneth cells, in mesangial cells of kidney, in pancreatic islet D cells, and in neurons of the brain. It is of interest that this profile of CL-K1 expression is unique among the collectins. Together these histological findings may be useful for understanding the biological function of this novel collectin. (J Histochem Cytochem 56:243–252, 2008)

KEY WORDS
CL-K1
Colec11
collectin
mannin-binding lectin
mouse
somatostatin
Paneth cells

COLLECTINS ARE A FAMILY of proteins that contain two characteristic structures, a collagen-like region and a carbohydrate recognition domain (CRD) (Drickamer 1988). There are three classical collectins in humans: mannan-binding lectin (MBL) (Kawasaki et al. 1983; Sastry et al. 1991; Laursen et al. 1998; Laursen and Nielsen 2000) and surfactant proteins A and D (SP-A and SP-D) (Benson et al. 1985; Haagsman et al. 1987; Andersen et al. 1992). MBL, a plasma collectin synthesized in the liver (Sastry et al. 1991; Andersen et al. 1992; Hansen et al. 2002), can kill bacteria through activation of the complement pathway or by opsonization via collectin receptors (Kawasaki et al. 1989; Schweinle et al. 1989). SP-A and SP-D are mainly produced by alveolar type II cells and Clara cells in the lung (White et al. 1985; Lu et al. 1992; Madsen et al. 2000,2003; Paananen et al. 2001) and can mediate opsonization of bacteria and neutralization of viral growth. In addition, SP-A and SP-D associate directly with macrophages and stimulate phagocytosis or oxidant-dependent microbial clearance (Sano and Kuroki 2005). Thus, collectins play an important role in innate immunity.

Recently, cDNAs encoding three novel collectins, collectin liver 1 (CL-L1) (Ohtani et al. 1999), collectin placenta 1 (CL-P1) (Nakamura et al. 2001; Ohtani et al. 2001), and collectin kidney 1 (CL-K1) (Keshi et al. 2006) were isolated and characterized by our group as well as by other investigators. CL-L1 is mainly expressed in liver as a cytoplasmic protein. CL-P1 is a membrane-type collectin expressed in vascular endothelial cells, which binds to oxidized low-density lipoprotein (OxLDL) as a scavenger receptor. We have very recently demonstrated a novel human and murine collectin, CL-K1 (Keshi et al. 2006). According to the Mouse Genome Informatics database (http://www.informatics.jax.org/), CL-K1 was first cloned and deposited as RIKEN cDNA 1010001H16 in 2001. It was later assigned the name Colec11 (collectin sub-

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family member 11) in 2003. The Colec11 gene name is that used in the major databases including the Genome Expression Omnibus (http://www.ncbi.nlm.nih.gov/projects/geo/) that has 675 entries for expression data of this gene as determined by cDNA microarray. CL-K1 harbors a 25-amino acid signal sequence and is a secreted type of collectin present in human plasma. CL-K1 can also bind to microbial lipopolysaccharide (LPS) and lipoteichoic acid (LTA), suggesting that it may play an important role in innate immunity. However, little is known about the tissue distribution of CL-K1. In the present study we generated specific antibody against this collectin for use in immunohistochemistry (IHC) and determined the tissue distribution of CL-K1 in mice.

Materials and Methods

Animals and Tissues

Nine-week-old male C57Bl/6Ncrj mice (Charles River; Tokyo, Japan) were housed at 22C under 12 hr light/dark cycle (lights on at 7 AM) conditions and were allowed access to food and water ad libitum. For histology and IHC, mice were anesthetized with 2.5% avertin and perfused through the left ventricle with 20 ml of ice-cold PBS and then with 4% paraformaldehyde in PBS at 4C for 20 min. Various tissues were then collected, and specimens were dehydrated and embedded in paraffin. For double staining of CD31 and CL-K1, various mouse tissues were fixed by IHV Zinc Fixative (BD Biosciences Pharmingen; San Diego, CA) at room temperature for 24 hr and embedded in paraffin. Five-μm-thick sections were stained for IHC with Mayer's hematoxylin. All experiments were carried out in accordance with the rules and guidelines of the Animal Experiment Committee of Asahikawa Medical College.

RNA Isolation and First-strand cDNA Synthesis

Total RNA was isolated from small pieces of mouse tissue (80–100 μg) using Trizol reagent (Invitrogen; Carlsbad, CA). RNA was reverse-transcribed using RETROscript (Ambion; Austin, TX). One μg of total RNA was mixed with 2 μl of random decamers and nuclease-free water in a total volume of 12 μl and heated at 80C for 3 min. The mixture was then chilled on ice and incubated with 2 μl of 10X RT buffer, 4 μl dNTP mix, 1 μl RNase inhibitor, and 1 μl reverse transcriptase at 44C for 60 min. Reaction mixtures were further incubated for 10 min at 92C. cDNA was stored at −30C until used for real-time PCR.

Analysis of mRNA Expression by Real-time PCR

Real-time PCR was performed with the Real Time PCR system (7500; Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. A Taqman probe primer set for CL-K1 (Mm01289834-m1) was purchased from Applied Biosystems.

Antibodies

Recombinant human CL-K1 including the neck and CRD domains (amino acids 107–271) together with six histidines was expressed in *Escherichia coli* GI724 using pPLH3 expression vector as described previously (Keshi et al. 2006). CL-K1-CRD-his protein was extracted and purified with Ni-NTA Agarose (Qiagen; Valencia, CA) according to the manufacturer's instructions. The N-terminal amino acid sequence of the purified recombinant protein was confirmed to be CL-K1-CRD-his. The purified recombinant protein was further characterized as CL-K1-CRD-his by SDS-PAGE and immunoblotting. New Zealand White rabbits were injected three times at 2-week intervals with 200 μg of the above fusion protein in incomplete Freund's adjuvant. After immunization, whole sera from rabbits were applied to HiTrap Protein G HP (Amersham Biosciences; Piscataway, NJ), and anti-CL-K1 rabbit IgG fractions were eluted with 0.1 M glycine–HCl buffer (pH 2.5). Furthermore, the anti-CL-K1 IgG was affinity purified using a CL-K1-CRD-his-conjugated antigen column, HiTrap NHS-activated HP (Amersham Biosciences), as described previously (Takeuchi et al. 1997). The IgG fraction, which passed through the CL-K1 antigen column, was used as the control IgG. Extent of purification was determined by ELISA as described.

ELISA

Microtiter plates were coated overnight at 4C with 10 μg/ml of various collectins, namely, CL-L1-CRD-his, CL-P1-CRD-his, CL-K1-CRD-his, mouse CL-K1-CRD-his, and MBL-CRD-his, in the coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 0.05% NaN_3, pH 9.6). Plates were washed with TBS (Tris-buffered saline containing 20 mM Tris–HCl and 140 mM NaCl, pH 7.4)/TC (0.05% Tween 20 and 5 mM $CaCl_2$) and incubated at 37C for 1 hr with various preparations of anti-CL-K1 antibodies containing the IgG fraction of the anti-CL-K1 serum, the affinity-purified anti-CL-K1 IgG, or the control IgG fraction. After washing, they were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Chemicon International; Temecula, CA) followed by color development using a TMB Peroxidase Substrate System (Kierkegaard and Perry Laboratories; Gaithersburg, MD). The reaction was stopped with 1 M phosphoric acid, and absorbance was measured at 450 nm.

Immunocytochemistry

CHO-K1 cells (ATCC; Rockville, MD) were stably transfected with human CL-K1 expression vectors as de-

scribed previously (Keshi et al. 2006). Transfected cells (CHO/CL-K1) were plated in 14-mm wells of 35-mm plastic culture dishes (Matsunami Glass Industries; Tokyo, Japan) and cultured in Ham's F-12 medium containing 5% FBS. CHO/CL-K1 cells were fixed with 4% paraformaldehyde in PBS at 4C, permeabilized, and blocked in BlockAce (Dainippon Seiyaku; Osaka, Japan) for 1 hr at room temperature. Cells were then incubated with affinity-purified CL-K1 IgG or control IgG (1 μg/ml) overnight at 4C followed by treatment with anti-rabbit IgG-conjugated Alexa 488 and TO-PRO-3 (Molecular Probes; Eugene, OR). Fluorescent images were observed with a confocal laser-scanning microscope (CLSM, FV1000; Olympus Optical, Tokyo, Japan). All immunofluorescence images show fluorescence overlaid on phase contrast images.

IHC and Immunofluorescence Analyses

IHC staining was carried out with the avidin–biotin complex method and, for immunofluorescence, the indirect fluorescence staining method was used. Five-μm-thick tissue sections were cut and placed onto slides, and almost all sets of slides were processed together in the following steps. Slides were deparaffinized through a series of xylene and ethanol baths. Sections were blocked in BlockAce (Dainippon Seiyaku) for 1 hr at room temperature and then incubated in affinity-purified anti-CL-K1 IgG or control IgG (5 μg/ml) overnight at 4C. Each section was incubated with biotinylated guinea pig anti-rabbit IgG for 1 hr followed by incubation with avidin–biotin–alkaline phosphatase complex for 1 hr. Finally, the sections were treated with Alkaline Phosphatase Substrate Kit II (Vector Laboratories; Burlingame, CA). Endogenous alkaline phosphatase activity was blocked with Levamisol solution (Vector Laboratories). Sections were briefly counterstained with hematoxylin. In the case of immunofluorescence staining, secondary antibodies were incubated with Alexa Fluor 488 anti-rabbit IgG and TO-PRO-3 (Molecular Probes) for 1 hr. For double staining with somatostatin, glucagon, or insulin (Santa Cruz Biotechnology; Santa Cruz, CA) of stomach and pancreas, secondary antibody was used together with Alexa Fluor 594 anti-goat IgG. IHC and fluorescent images were examined with a CLSM. For double staining with mouse CD31 (BD Biosciences Pharmingen), secondary antibody was used together with Alexa Flour 594 anti-rat IgG.

Results

CL-K1 mRNA Expression in Murine Tissues

To investigate the tissue distribution of CL-K1 mRNA, real-time PCR analyses were performed with RNAs purified from a number of mouse tissues. We used specific primer pairs and a Taqman probe to detect the CL-K1 neck–CRD fragment. Data in Figure 1 show

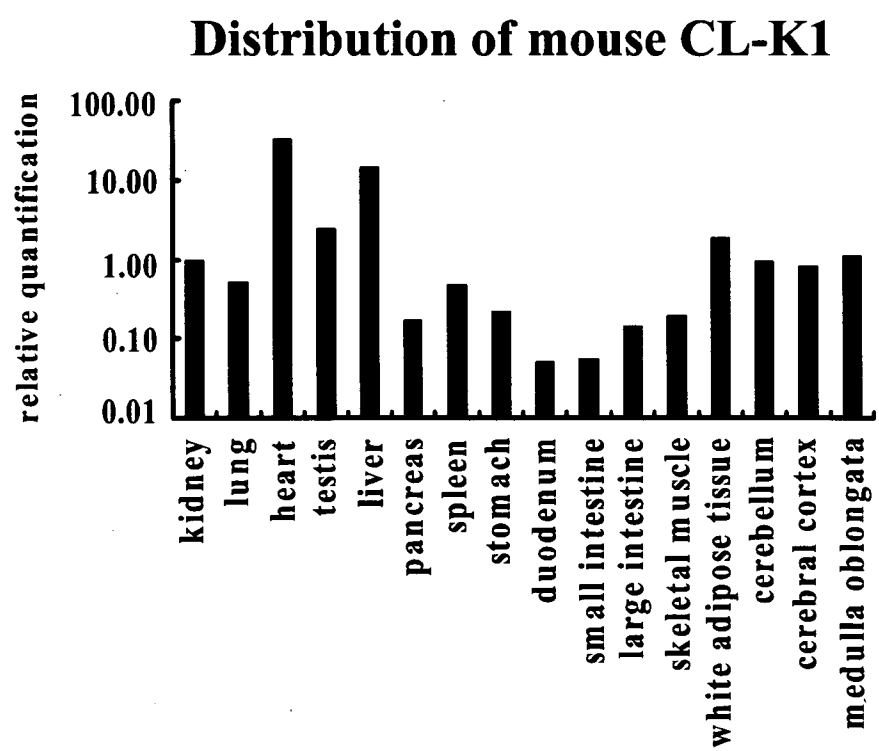

Figure 1 Estimation of the amount of CL-K1 mRNA in different tissues. Relative mRNA levels were measured by TaqMan RT-PCR. Data were normalized based on the value of 18S rRNA.

CL-K1 mRNA per 18S RNA and were further normalized to kidney defined as 1.0. Data of CL-K1 mRNA expression were normalized to kidney because we identified CL-K1 from kidney. Using real-time PCR analysis, CL-K1 mRNA was detectable in almost all organs tested (Figure 1). CL-K1 mRNA was found at the highest level in heart, and a relatively high expression of CL-K1 mRNA was detected in liver, testis, white adipose tissue, brain, and kidney.

Characterization of the CL-K1 Affinity Antibody

We first generated a specific antibody against CL-K1 for use in IHC. This antibody was raised against the CL-K1 neck–CRD region, which is highly conserved in humans, mice, and rats (Keshi et al. 2006). To increase antibody titer, we used a CL-K1 affinity column after IgG purification. As shown in Figures 2A and 2B, an ELISA for measuring titers of several antibodies against CL-K1 recombinant proteins revealed that CL-K1 antibodies were strongly reactive with the mouse CL-K1 protein. Because the affinity-purified antibody had an ~10 times higher titer than the unpurified preparation, we used the former in the following experiments. Figure 2B indicates that the CL-K1 IgG reacted specifically with CL-K1 rather than with CL-L1, CL-P1, or MBL. Figure 2C shows that the affinity-purified antibody was capable of detecting mouse CL-K1 as well as human CL-K1. CL-K1 overexpressed CHO cells (CHO/CL-K1), or empty vector transfected CHO cells (mock) were stained with the affinity-purified CL-K1 or

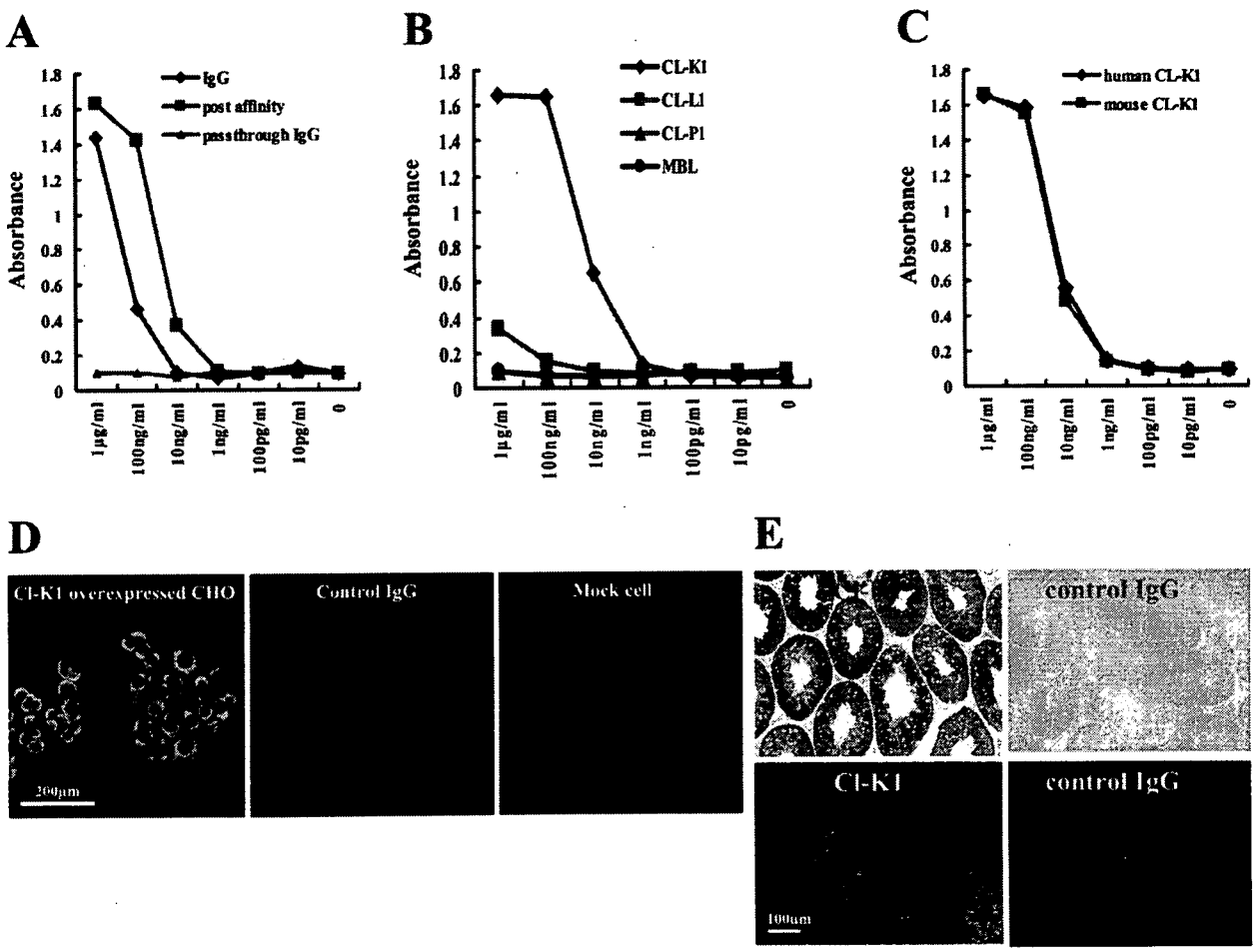

Figure 2 Specificity of our CL-K1 polyclonal antibody was analyzed by ELISA, immunocytochemistry, and immunohistochemistry (IHC). The anti-CL-K1 IgG fraction (IgG) was purified from rabbit serum. After IgG purification, the affinity antibody (postaffinity) was purified on an antigen column, and the pass-through IgG was used as the control IgG (pass-through IgG). **(A)** Results of ELISA analysis using anti-CL-K1 IgG, postaffinity antibody, or pass-through IgG. ELISA analyses of anti-CL-K1 antibodies against human CL-K1. **(B)** Results of ELISA analyses of anti-CL-K1 affinity antibody reactivity with other collectins, namely, CL-L1, CL-P1, and MBL. **(C)** Cross-reactivity between human and murine CL-K1 recombinant protein. **(D)** Immunofluorescence in CHO cells overexpressing CL-K1 (left and middle panels) as well as in empty vector expressed CHO cells (mock cells) (right panel). **(E)** IHC staining and immunofluorescence staining with affinity antibody or control IgG in murine testis.

control antibody, respectively. As shown in Figure 2D (left and middle panels), the CL-K1 affinity-purified antibody could detect human CL-K1 protein in the cytoplasm of CHO/CL-K1 cells, whereas the control IgG could not. In addition, the CL-K1 antibody failed to react with anything in CHO/mock cells, as shown in the right panel of Figure 2D, clearly demonstrating the high specificity of the affinity-purified antibody.

CL-K1 Expression in Murine Tissues

IHC and immunofluorescent analyses were performed in several murine tissues to investigate expression of the CL-K1 protein. Figure 2E shows that CL-K1 antibody could react with the testis, but the pass-through IgG used as a control could not detect any antigen in the testis, suggesting a specificity of the CL-K1 antibody. Using the CL-K1 affinity-purified IgG, IHC and immunofluorescent analyses were performed with tissues of murine kidney, lung, heart, testis, liver, pancreas, digestive organs (including the esophagus, stomach, small intestine, and large intestine), and brain. Figures 3A and 3B show results of immunofluorescence analysis of renal cortex demonstrating that CL-K1 was expressed in mesangial cells, podocyte, or microvascular endothelial cells of glomerulus (Figure 3A, red arrow) and in the brush border of proximal tubules (Figure 3A, yellow arrow). To further characterize the CL-K1 immunoreactive cells in the renal cortex, immunofluorescent analysis using both CL-K1 and antibody against CD31, a marker for endothelial cells, was performed. As demonstrated in Figure 3C, the merge image showed that endothelial cells do not express CL-K1, supporting the fact that CL-K1 may be expressed in the mesangial cells. Figure 3B shows that CL-K1 was also expressed in the vascular portion of the kidney. As shown in Figures 4A and 4B, CL-K1 was observed in the vascular portion of the heart and small intestine as well as in those of kidney. Furthermore, the double-immunofluorescence analyses presented in Figures 4C and 4D indicated that CL-K1 was expressed specifically in smooth muscle cells but not in endothelial cells. This indicates that vascular smooth muscle cells in all tissues are made up of primary cells expressing CL-K1.

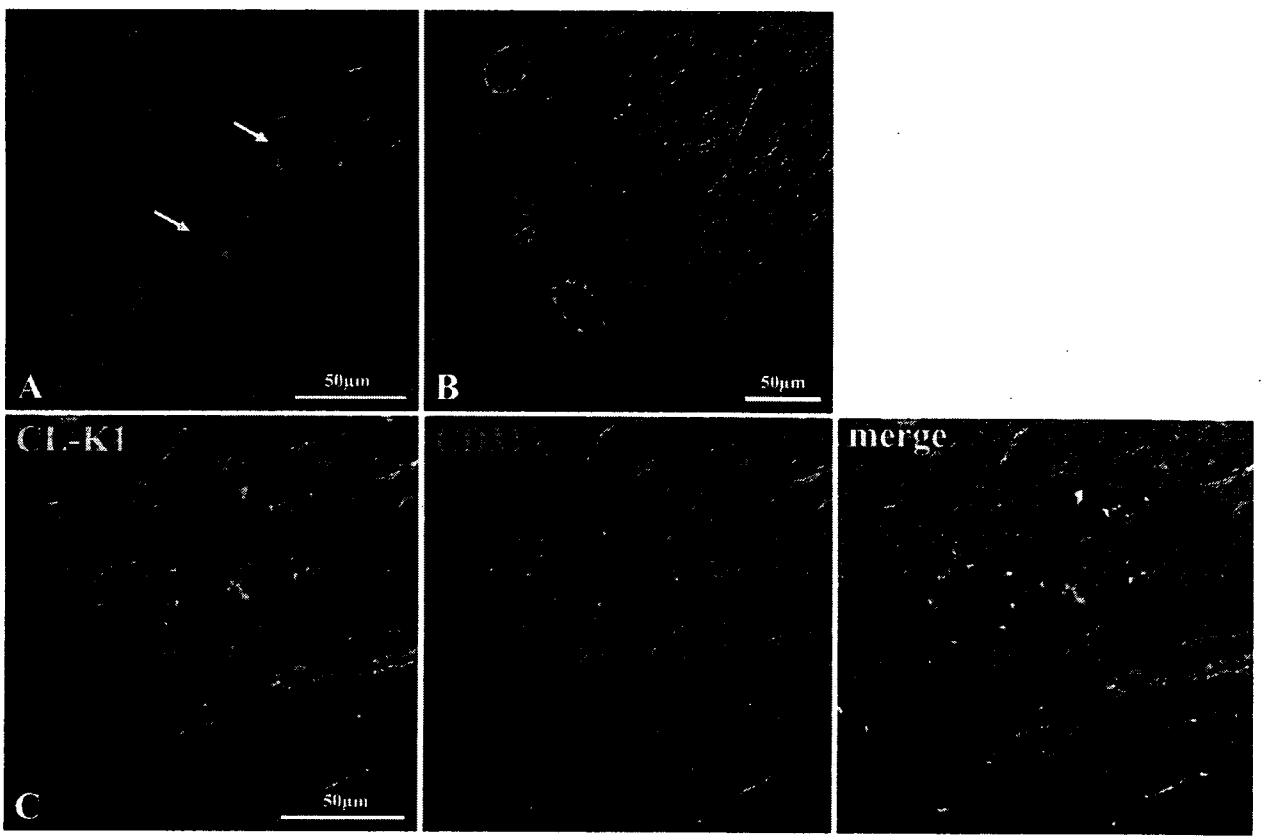

Figure 3 IHC of murine renal cortex (**A**) and vascular smooth muscle cells in kidney (**B**). CL-K1 protein was expressed in mesangial cells in glomerulus (red arrow in **A**) and in brush border of proximal tubules (yellow arrow in **A**). Double immunofluorescence staining (**C**) demonstrates that CL-K1 was not colocalized in microvascular endothelial cell.

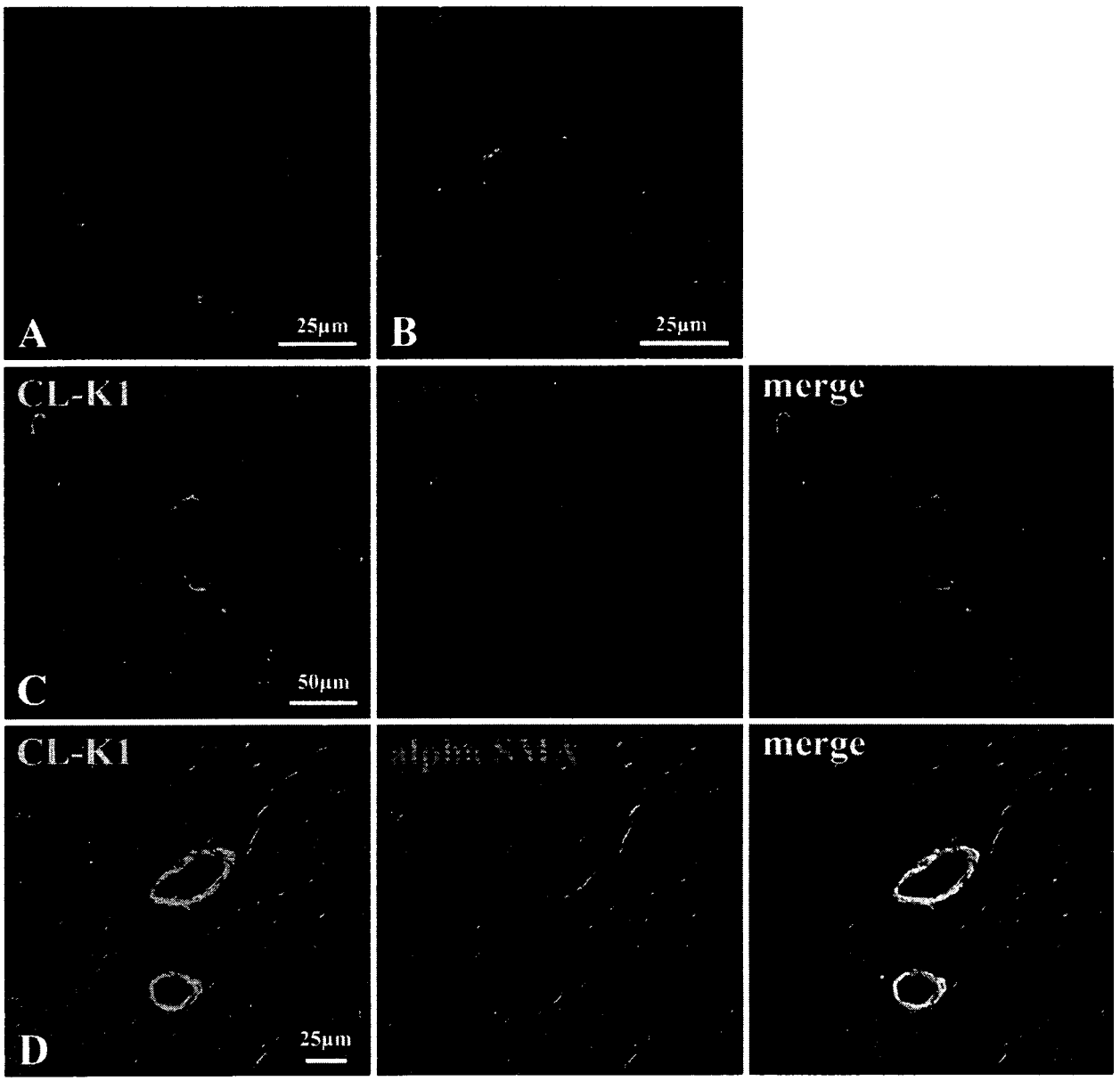

Figure 4 IHC of vascular cells in heart (**A**) and small intestine (**B**). CL-K1 expression was detected in vascular portion in heart (**A**) and small intestine (**B**). Double immunofluorescence staining (**C,D**) demonstrates that CL-K1 was colocalized in vascular smooth muscle cells but not in endothelial cells.

IHC localization of CL-K1 in lung, heart, testis, and brain is shown in Figure 5. CL-K1 expression was strong in bronchial glands of bronchium (Figures 5A–5C). CL-K1 was also expressed in bronchial glands of bronchioles (Figures 5A and 5B, red arrow) and respiratory bronchioles (Figure 5C, black arrow). Figure 5D indicates that CL-K1 was expressed in whole myocardium as well as in the vascular portion of this tissue, but not in endocardium. Figure 5E shows that CL-K1 was expressed in the cytoplasm of spermatocytes. In brain, CL-K1 was abundantly and ubiquitously expressed in neurons of the central nervous system (data not shown). Figure 5F indicates that representative neurons were stained in the medulla oblongata. IHC localization of CL-K1 in liver and pancreas is shown in Figure 6. CL-K1 was expressed in hepatocytes, especially around the central veins (Figure 6A, black arrow). Figures 6B and 6C show that CL-K1 was expressed in pancreatic acinar cells and islet cells. In the case of the islets, CL-K1 was especially expressed in

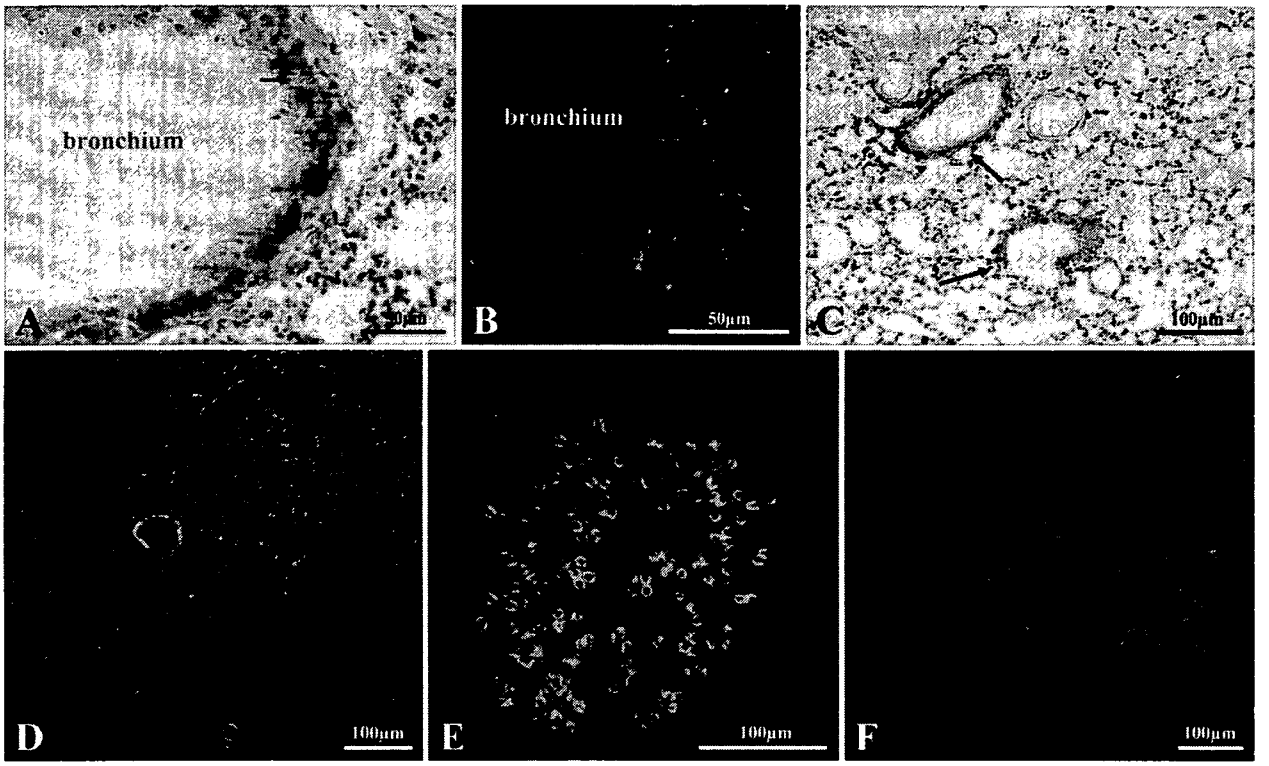

Figure 5 IHC localization of CL-K1 in murine lung, heart, testis, and brain. CL-K1 expression was especially strong in bronchial glands of bronchium (red arrow in **A,B**). In peripheral lung (**C**), CL-K1 was also expressed in respiratory bronchioles (black arrow). In heart and testis, CL-K1 was expressed in lamina elastica of coronary artery in myocardium (**D**) and in cytoplasm of spermatocytes (**E**). (**F**) Representative neurons stained with CL-K1 antibody in the reticular formation of the medulla oblongata.

the marginal cells. Double-immunofluorescence analyses presented in Figure 6D indicate that CL-K1 was expressed specifically in D cells that produce somatostatin but not in α- and β-cells, which produce glucagon and insulin, respectively. Figure 7 shows IHC localization of CL-K1 in murine digestive tract. CL-K1 was expressed in epithelial cells of all mucosa of the digestive tract including the esophagus (Figure 7A), stomach (Figures 7B and 7E), small intestine (Figure 7C), and large intestine (Figure 7D). CL-K1 was strongly stained on the surface of esophageal mucosa. In stomach, CL-K1 was expressed in whole mucosa of gastric glands. Double-immunofluorescence analyses revealed that CL-K1 in stomach was also specifically localized in D cells containing somatostatin. In small intestinal mucosa, CL-K1 was expressed in Paneth cells as well as in intestinal crypt (Figure 7C, yellow arrow). In the large intestine, CL-K1 was expressed in epithelial mucosa (Figure 7D).

Discussion

Collectins interact with glycoconjugated and lipid moieties present on the surface of microorganisms and allergens, as well as with receptors on host cells. Through these interactions, they play a crucial role in innate immunity. However, a single type of collectin cannot meet the requirements for all functions of innate immunity, and several collectins are required for host defense (van de Wetering et al. 2004). In our previous report we demonstrated that CL-K1 could bind to bacterial LPS and LTA. Thus, this novel collectin may be involved in host defense against microorganisms. With regard to the tissue distribution of human CL-K1, we have shown by RT-PCR that CL-K1 mRNA is expressed in most human tissues (Keshi et al. 2006). The present study using mice was carried out to determine the precise tissue distribution of CL-K1 protein expression to reach a better understanding of the biological functions of this novel collectin. For this purpose, we generated a new affinity-purified anti-CL-K1 antibody. This polyclonal antibody raised against the CL-K1 neck–CRD domain recognized full-length CL-K1 overexpressed in CHO cells. We have previously demonstrated by RT-PCR that CL-K1 mRNA expression is ubiquitous in human tissues (Keshi et al. 2006). In this study, we quantitatively evaluated the tissue expression of CL-K1 mRNA in mice using real-time PCR. Real-time PCR study demonstrated that

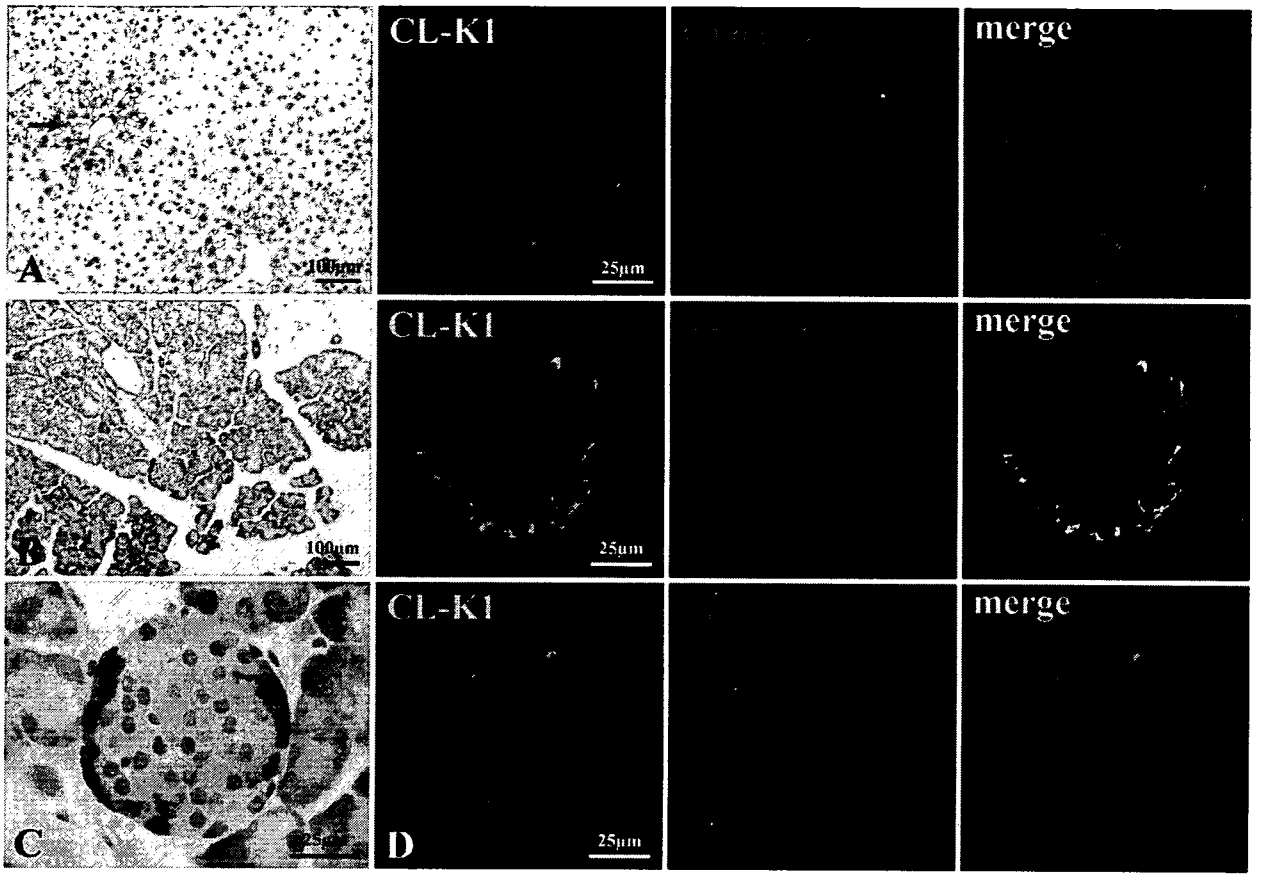

Figure 6 IHC localization of CL-K1 in liver and pancreas. In liver (**A**), CL-K1 was expressed in hepatocytes. A relatively high expression of CL-K1 was seen in hepatocytes around the central vein (black arrow). In pancreas (**B**), CL-K1 was expressed not only in acinar cells but also in islet cells (**C**). Double immunofluorescence staining (**D**) demonstrates that CL-K1 was colocalized in somatostatin-containing D cells but not in glucagon-containing α-cells or insulin-containing β-cells.

CL-K1 mRNA was distributed in all organs. Among the murine tissues expressing CL-K1 mRNA (see Figure 1), a relatively high level of expression was observed in heart, liver, testis, kidney, and white adipose tissue. Results of immunostaining of these tissues clearly demonstrated that heart, liver, testis, and kidney express CL-K1 protein, in strong agreement with the observations of mRNA expression by real-time PCR. The major finding in the present study was that CL-K1 was expressed in proximal tubules in kidney, bronchial glands of bronchioles, and mucosa of gastrointestinal tract. CL-K1 is a secreted type of collectin and would be expected to be secreted into lumen of these various tissues. This expression pattern is similar to those of SP-A and SP-D in the bronchial glands of bronchioles (Madsen et al. 2000,2003). Sites of CL-K1 expression in kidney, lung, and gastrointestinal tract coincide with areas subject to microbial growth, suggesting that CL-K1 has an important role in defense against microorganisms invading the urinary tract, respiratory tract, and lumen of the digestive tract. In kidney, CL-K1 was identified in mesangial cells of glomeruli, in addition to the proximal tubules. We have reported in our recent publication that CL-K1 is made in the liver and may secrete into the blood stream (Keshi et al. 2006). In addition, molecular mass of CL-K1 is ~37 kDa. One may speculate that collectin could be passively deposited in the mesangium. It is therefore speculated that CL-K1 immunoreactivity found in the mesangial cells may be passively deposited from systemic circulation, but at this time we are unable to rule out the possibility. However, the possibility may be low because native CL-K1 exists as an oligomer structure in the blood and its molecular mass is >100 kDa, as described in our recent publication (Keshi et al. 2006). These evidences indicate that CL-K1 immunoreactive products in the mesangial cells could not be passively deposited. Further studies such as in situ hybridization are needed to clarify whether CL-K1 is indeed produced by mesangial cells or other cells stained with the

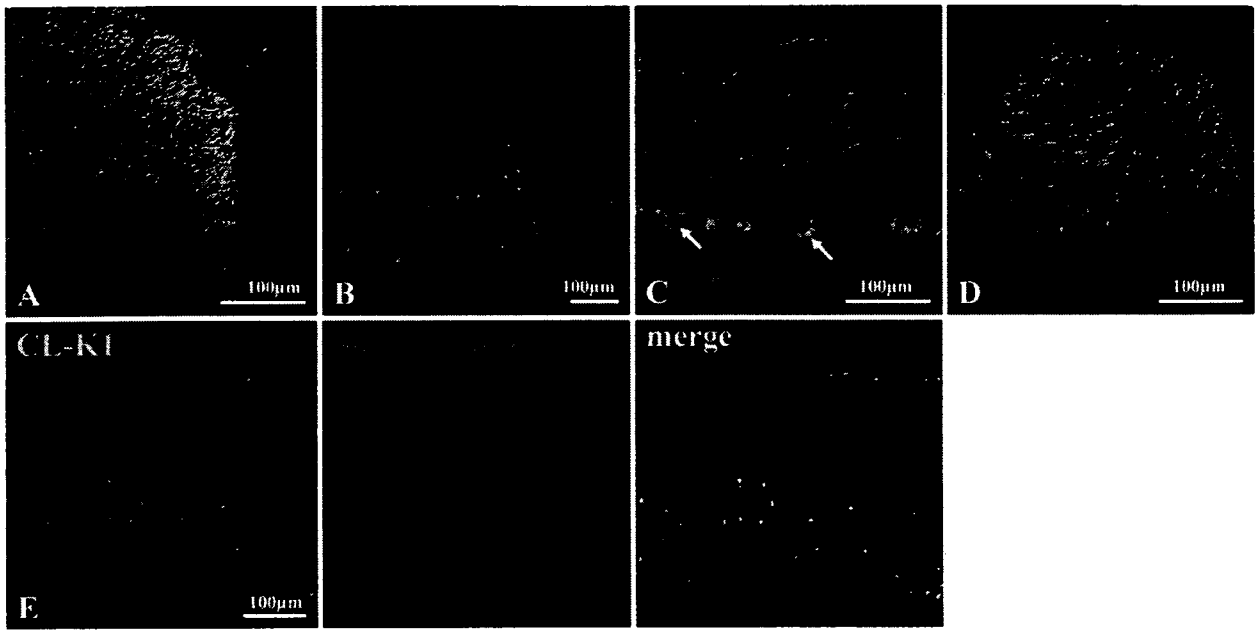

Figure 7 IHC localization of CL-K1 in gastrointestinal tract. In esophagus (**A**), stomach (**B**), small intestine (**C**), and large intestine (**D**), CL-K1 was expressed in epithelium. In stomach, CL-K1 was colocalized with somatostatin in somatostatin-containing cells (**E**). In small intestine, CL-K1 was expressed in Paneth cells (yellow arrow in **C**). In large intestine, CL-K1 was expressed in epithelial mucosa (**D**).

CL-K1 antibody. Recent studies on IgA glomerulonephritis have demonstrated that IgA2 harboring polysaccharide chains tend to be agglutinated with each other so that deposits of IgA2 accumulate in mesangial cells and activate the lectin pathway in glomeruli (Hisano et al. 2001,2005; Oortwijn et al. 2006). These experiments indicate that IgA2 with sugar chains are important in agglutination and adhesion in glomeruli. However, characterization of the ligands involved has not been carried out. Our findings suggest that CL-K1 may be involved in the triggering of glomerulonephritis because it would act as a ligand against polysaccharides with IgA. This concept will be further explored in a future study. On the other hand, results of the real-time PCR and IHC clearly demonstrated that CL-K1 mRNA was highly expressed in liver, and that CL-K1 protein expression was homogeneously localized in hepatocytes where it was especially high around the central veins. We have already shown that CL-K1 protein is secreted into human blood (Keshi et al. 2006). These results suggest that murine CL-K1 is mainly produced in hepatocytes in the liver and secreted into the blood stream, as is human CL-K1. In pancreas, CL-K1 was expressed in acinar cells and islet cells. According to immunostaining results, it is of interest that CL-K1 was strongly associated with somatostatin in the islets, but not with insulin or glucagon. Moreover, in gastric mucosa, cells producing CL-K1 corresponded to those producing somatostatin. Somatostatin is a peptide hormone known to regulate the endocrine system, affect neurotransmission, and inhibit release of a variety of secondary hormones. Recently, several reports have implicated somatostatin in innate immunity (Seboek et al. 2004; Zavros et al. 2004). These results also suggest that somatostatin may have a special relationship with CL-K1 in host defense mechanisms. In small intestine, CL-K1 was highly expressed in Paneth cells that contain epithelial granulocytes in the basement area of crypts. Defensins are secreted from Paneth cells and contribute to mucosal barrier function through their potent antimicrobial activities (Ouellette and Lualdi 1990; Ouellette et al. 1992a,b; Ayabe et al. 2000). The fact that CL-K1 was localized in Paneth cells indicates that this molecule would be advantageous in host defense because it would likely be secreted into the lumen together with defensins with which they would play a cooperative role as antimicrobial molecules. In the central nervous system, CL-K1 was mainly expressed in neurons of the brain. Because CL-K1 expression was localized in the cytoplasm and not in dendritic portion of the cell, it would not contribute to any specific neuronal network formation. The relatively high expression of CL-K1 mRNA observed in the central nervous system was in agreement with IHC observations. In lung, gastrointestinal tract, and testis, CL-K1 was expressed in the region exposed to outer environment, indicating that CL-K1 plays an important role in innate immunity systems as other collectins. On the other hand, CL-K1 expressed in

heart, liver, and brain may play unexpected roles because the sites of CL-K1 expression are unlikely involved in host defense. We do not know the physiological relevance of CL-K1 expressed in heart, liver, and neurons in brain. Further studies are needed to clarify what kinds of biological actions CL-K1 possesses, in addition to its expected action as a collectin.

In conclusion, we determined the tissue distribution of CL-K1 protein in mice. These findings may be useful for understanding the biological significance of this novel collectin in future studies.

Acknowledgments

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The Journal of Histochemistry & Cytochemistry

Hypoxia Inducible Factor-1–Independent Pathways in Tumor Angiogenesis

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Abstract Among the factors that can stimulate angiogenesis, vascular endothelial growth factor has emerged as one of the most important, and inhibition of vascular endothelial growth factor has recently shown efficacy in the treatment of advanced colorectal cancer. Hypoxia develops within solid tumors and is one of the most potent stimuli of vascular endothelial growth factor expression. This effect is mediated primarily by hypoxia inducible factor-1 (HIF-1), often considered a master regulator of angiogenesis in hypoxia. Consequently, inhibition of HIF-1 has been proposed as a strategy to block tumor angiogenesis therapeutically. However, accumulating evidence indicates that HIF-independent pathways can also control angiogenesis. This review highlights some of the key signaling pathways independent of HIF-1 that can stimulate angiogenesis in hypoxia. Understanding the full spectrum of molecular pathways that control tumor angiogenesis is critical for the optimal design of targeted therapies.

Background

Angiogenesis is a hallmark feature of human malignancies. The induction of vascular endothelial growth factor (VEGF) is an essential component of tumor angiogenesis, and this is mediated by multiple interacting genetic and environmental signals (1). Oncogenic mutations that are critical for the tumorigenic process in general can stimulate VEGF in particular, and hypoxia dramatically enhances this up-regulation. Hypoxia inducible factor-1 (HIF-1) is a primary regulator of VEGF during hypoxic conditions.

HIF-1 is a heterodimeric basic helix-loop-helix transcription factor composed of two subunits, HIF-1α and HIF-1β (ARNT), and HIF-1α is the key regulatory component (2). In the presence of oxygen, HIF-1α is hydroxylated on conserved prolyl residues within the oxygen-dependent degradation domain by prolyl hydroxylases and binds to von Hippel-Lindau protein, which in turn targets it for degradation through the ubiquitin-proteasome pathway (3). However, in hypoxic conditions, prolyl hydroxylase is inactive, resulting in stabilization of HIF-1α. HIF-1 transcriptional activity is also enhanced in hypoxia by "factor-inhibiting HIF-1," an oxygen-sensitive enzyme that hydroxylates asparagine residues at the C-terminal transactivation domain of HIF-1α to displace p300/CBP coactivator proteins (4). The HIF-1 complex recognizes a consensus hypoxia response element in the promoter of a broad range of target genes, including VEGF, platelet-derived growth factor, and transforming growth factor-α, that mediate hypoxic responses including angiogenesis. Early xenograft studies of embryonic stem cells from *HIF-1α*$^{-/-}$ mice showed that VEGF levels and markers of vascularization were significantly reduced (5), indicating a key role for HIF-1 in angiogenesis.

Angiogenesis Is Preserved in HIF-1–Deficient Tumor Xenografts

To better delineate the role of HIF-1 in human tumors, *in vivo* xenograft studies using cells, in which HIF-1 was targeted genetically, have been done. Knockdown of HIF-1α through small interfering RNA in a DLD-1 colon cancer cell xenograft reduced tumor growth but surprisingly did not block tumor angiogenesis (6). The microvessel density of HIF-1–deficient xenografts was equivalent to control xenografts expressing HIF-1 (26.1 ± 6.3 per field versus 28.7 ± 8.6 per field, respectively). In addition, microvessel perfusion, as visualized by intravascular lectin, was not altered in HIF-1–deficient tumors. Surprisingly, the induction of VEGF was not abrogated in HIF-1–deficient xenograft tissue or in HIF-1–deficent cells *in vitro*. VEGF levels were reduced ~50%, indicating that substantial amounts of VEGF were still produced. Independent studies of HIF-1α$^{-/-}$ ES cells confirmed that angiogenesis, as measured by microvessel density, was preserved when HIF-1α was knocked-out. There was an ~50% decrease in VEGF mRNA levels, but no significant changes in VEGF protein levels were observed (7).

The persistent expression of VEGF can potentially explain the preservation of the angiogenic phenotype, but an additional angiogenic factor, interleukin 8 (IL-8), was found to be induced specifically in these HIF-1–deficient tumors (6). When neutralizing antibodies to IL-8 were given to mice bearing HIF-1–deficient tumors, there were reductions in microvessel density

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(from 27.5 ± 3.2 per field to 14.7 ± 3.5 per field) and diameter (from 22.4 to 5.9 μm), indicating the functional significance of IL-8 in the regulation of angiogenesis. A lectin perfusion study verified that vascular integrity was severely compromised in HIF-1 – deficient xenografts when IL-8 was simultaneously blocked, and vessels were markedly narrowed and fragmented. These findings suggested that HIF-1 was not the only factor regulating angiogenesis in hypoxia. Furthermore, the angiogenic response seemed to be highly adaptable, as targeting a single angiogenic factor resulted in the induction of an independent factor. Thus, combinations of antiangiogenic agents that target different factors may be necessary to offset such compensatory responses and maximize therapeutic outcomes.

The Ras Oncogene and HIF-1 – Independent Regulation of VEGF

The specific molecular pathways that underlie the HIF-1 – independent regulation of VEGF have begun to be elucidated, and the RAS oncogene seems to play a pivotal role (Fig. 1). This was first shown in *H-RAS* transformed embryonic fibroblasts from *HIF-1α*$^{-/-}$ mice (8). Surprisingly, angiogenesis in these "tumors" was well preserved, implying that oncogenic *H-RAS* can compensate for the loss of HIF to maintain angiogenesis *in vivo*. In the absence of HIF-1α, the hypoxic induction of hypoxia-responsive genes, such as glucose tranporter-1 and phosphoglycerate kinase was abolished, but the induction of VEGF was still observed. Mouse hepatoma cells deficient in ARNT, the binding partner of HIF-1α, display a persistent hypoxic induction of VEGF mRNA, again indicating that pathways independent of HIF-1 may regulate VEGF in epithelially derived cancer cells (9). Subsequent studies have shown the specific role of the *K-RAS* isoform in human cancer cells using small interfering RNA against HIF-1α and site-directed mutagenesis of HIF-1 binding sites in the human VEGF promoter (10).

Several mechanisms for RAS-mediated regulation of VEGF in hypoxia have been proposed. Oncogenic *K-RAS* in combination

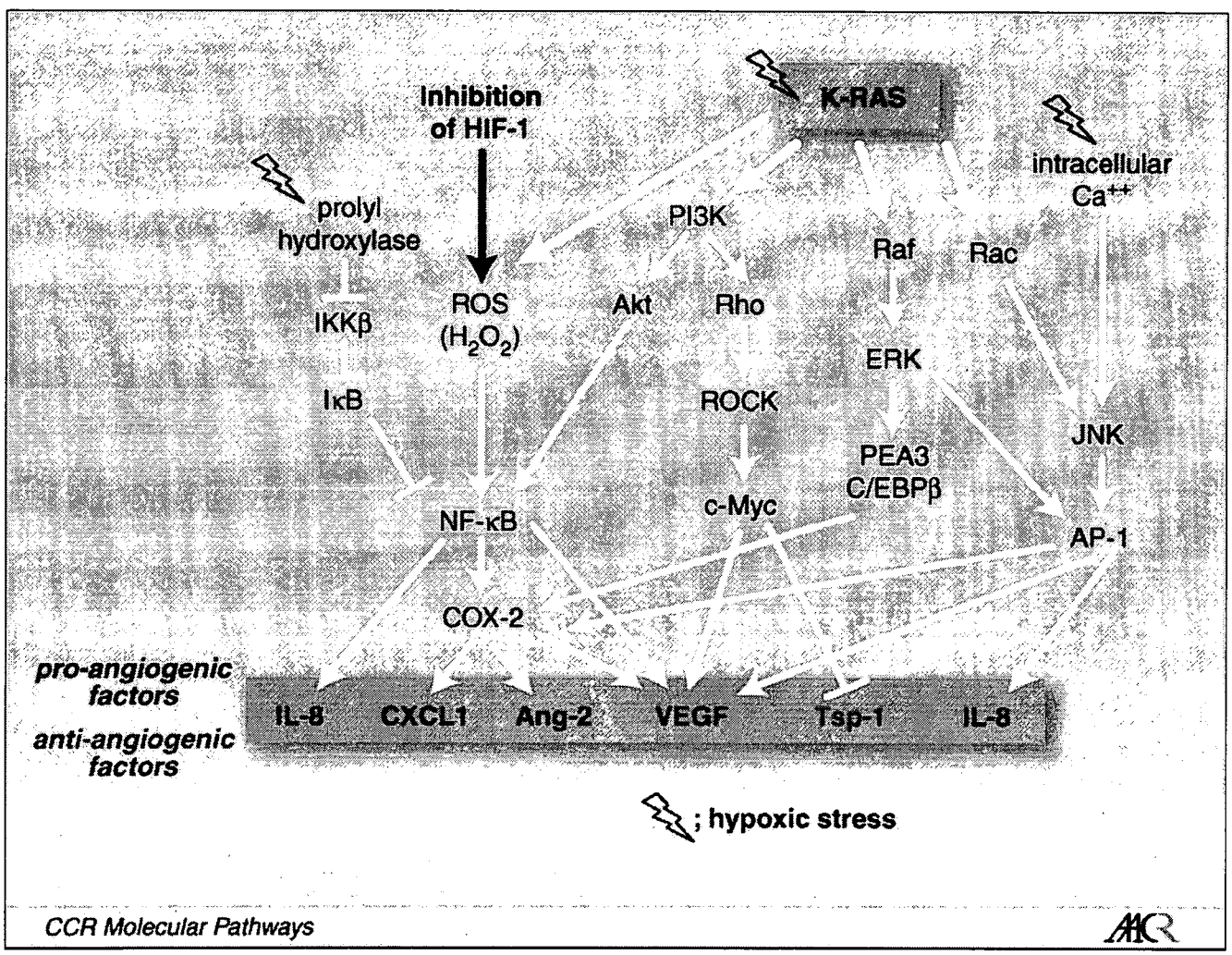

Fig. 1. Signal transduction pathways that can stimulate angiogenesis independently of HIF-1. Oncogenic Ras plays a central role in these HIF-1 – independent pathways; multiple effector pathways, including PI3K, Raf, and Rac, can be induced by Ras, which in turn stimulate transcription factors through protein kinases to up-regulate proangiogenic factors and down-regulate antiangiogenic factors. It should be noted that many of these factors can be synergistically regulated by hypoxia and Ras. In addition, NF-kB is induced by hypoxia specifically through accumulation of hydrogen peroxide when HIF-1 is blocked, and this compensatory pathway plays an important role to maintain angiogenesis in the absence of HIF-1 by up-regulating IL-8. Ang-2, angiopoietin-2; CXCL-1, CXCl chemokine ligand-1; ERK, extracellular signal-regulated kinase; H_2O_2, hydrogen peroxide; IkB, inhibitor of NF-kB; IKKb, IkB kinase; JNK, c-Jun kinase; ROCK, Rho kinase; Tsp-1, thrombospondin-1.

with hypoxia can synergistically up-regulate VEGF (10). Hypoxia can activate multiple RAS effector pathways, including extracellular signal-regulated kinase, c-Jun-NH_2 kinase, p38, Akt, and Rho. Among these pathways, extracellular signal-regulated kinase and Akt, but not c-Jun-NH_2 kinase and p38, were activated by hypoxia in colon cancer cells (11). Akt is a major down-stream target of phosphoinositide-3 kinase (PI3K), and inhibition of PI3K either by a dominant negative construct or specific inhibitors, such as LY-294002, strongly down-regulated the hypoxic induction of VEGF. Despite the crucial role of Akt in the regulation of VEGF under normoxic conditions (12, 13), inhibition of Akt did not attenuate the hypoxic induction. Instead, an alternative target of PI3K, the Rho/Rho kinase pathway, mediated the hypoxic induction of VEGF (11). The combination of hypoxia and oncogenic *K-RAS* synergistically increased levels of GTP-bound Rho via PI3K. Induction of Rho by hypoxia has also recently been shown in endothelial cells (14), indicating a universal role of Rho signaling in hypoxic stress. The synergistic up-regulation of the VEGF promoter by hypoxia and *K-RAS* was attenuated by the inhibition of either PI3K or Rho kinase, and this was observed in HIF-1 deficient colon cancer cells.

VEGF promoter reporter assays and electrophoretic mobility shift assays identified a *cis*-regulatory element that was responsive to signaling by PI3K/Rho/Rho kinase. This pathway converged on the c-Myc oncogene (11). Hypoxia induced phosphorylation of c-Myc through PI3K/Rho/Rho kinase, and this was required for the induction of VEGF in the absence of HIF-1. A role for c-Myc in angiogenesis has been previously illustrated by the widespread vascular abnormalities seen in Myc-deficient mouse embryos (15) and by a pancreatic β-cell carcinogenesis model in which the angiogenic switch is regulated by Myc through induction of IL-1β (16). Hypoxia and c-Myc are linked to the regulation of VEGF through the activation of a novel *cis*-regulatory element in the VEGF gene promoter (11, 17). As an aside, signaling through this PI3K/RHO/Rho kinase/MYC pathway can simultaneously down-regulate thrombospondin-1, an endogenous antiangiogenic factor (18).

Another important transcription factor that can mediate hypoxic responses is nuclear factor-κB (NF-κB). The mechanism of NF-κB activation by hypoxia is not straightforward. Changes in the redox potential in hypoxic cells due to the generation of reactive oxygen species (ROS) in mitochondria can result in NF-κB activation (19). In addition, activation of NF-κB during hypoxic conditions can be mediated by decreased prolyl hydroxylation and subsequent degradation of IκB kinase-β (20). Finally, NF-κB can also be activated by oncogenic RAS, primarily through PI3K-Akt (21).

Previous studies have clearly shown that NF-κB can regulate VEGF transcription (22–24). Curiously, analyses of the VEGF promoter have not identified consensus and functional κB sites (25, 26), and NF-κB may regulate VEGF indirectly through other transcription factors. An essential role of activator protein-1 in NF-κB–dependent regulation of VEGF has been described (26), and there are at least two activator protein-1 sites involved in the hypoxic induction of VEGF transcription that do not depend upon HIF-1 (27). However, there is a complex link between NF-κB and HIF-1, and other studies have shown NF-κB–dependent induction of HIF-1. For example, NF-κB can induce HIF-1 when cells are stimulated by IL-1β (28), lipopolysaccharide (28), or ROS (29). Thus, NF-κB seems to induce VEGF through both HIF-1–dependent and HIF-1–independent mechanisms. As discussed later, a more critical role for NF-κB may be its induction of the angiogenic factor IL-8, and this process is HIF-1 independent (6).

Activated RAS can also control VEGF protein activity. RAS can stimulate the expression of several proteases, including matrix metalloproteases (matrix metalloproteinase-2 and matrix metalloproteinase-9) and urokinase-type plasminogen activator. As a consequence, the cellular release and activation of VEGF protein is enhanced, thereby increasing its extracellular levels (30). The hypoxic regulation of matrix metalloproteinase, urokinase-type plasminogen activator, and VEGF expression may also be independently mediated by the nonreceptor tyrosine kinases Syk and Lck (31).

HIF-1–Independent Activation of Angiogenic Factors other than VEGF

Although VEGF is one of the primary angiogenic factors induced in tumors, there are additional factors that play important roles, many of which do not depend upon the activity of HIF-1. For example, oncogenic *H-RAS* can induce IL-8, a potent angiogenic factor, through PI3K/Akt/NF-κB and Raf/extracellular signal-regulated kinase/activator protein-1 (32). In human ovarian cancer cells, hypoxia induces IL-8 through activation of NF-κB that is controlled by RAS-effector pathways including PI3K/Akt and p38 (33).

In colon cancer cells deficient in HIF-1, a strong induction of IL-8 was observed. This induction of IL-8 was mediated by the enhanced production of ROS under hypoxia and subsequent activation of NF-κB. Hypoxic conditions can lead to the increased production of ROS, and scavenging of H_2O_2 is often achieved by the increased production of pyruvate that occurs when cells shift from oxidative to glycolytic metabolism (34). Importantly, this shift depends upon HIF-1α (35, 36). The induction of NF-κB was blocked by ROS inhibitors. Exogenous administration of H_2O_2 stimulated the induction of IL-8, which was blocked by the NF-κB inhibitor BAY 11-7082. Furthermore, the *K-RAS* oncogene, which is commonly mutated in colon cancer, plays a critical role in this pathway. Knockdown of oncogenic *K-RAS* strongly attenuated the hypoxic induction of NF-κB reporter activity and IL-8 mRNA in HIF-1–deficient cells. In Caco2 cells that carry a wild-type *K-RAS* gene, expression of mutant *K-RAS* enhanced the induction of NF-κB by ROS. As an aside, NF-κB can also contribute to the induction of placental growth factor in RAS-transformed embryonic fibroblasts in concert with metal responsive transcription factor-1 in hypoxia (37).

Cyclooxygenase-2 (COX-2) is another key mediator of angiogenesis, and it can be induced both by RAS and hypoxia. Although HIF-1 can play an important role in the induction of COX-2 in hypoxic conditions (38), hypoxia can also up-regulate COX-2 through HIF-1–independent pathways including NF-κB (39). In addition, RAS acts through Rac/c-Jun-NH_2 kinase to phosphorylate c-Jun (activator protein-1) and Raf/extracellular signal-regulated kinase to activate CCAAT/enhancer binding protein β (CAAT/enhancer binding protein β) and/or Ets transcription factor PEA3, all of which are key regulators of COX-2 expression (40, 41). RAS effectors also play a role in stabilizing COX-2 mRNA (42). COX-2 mediates most of its

proangiogenic effects through the induction of prostaglandin E2. Although prostaglandin E2 can signal through HIF-1 (43), it can also activate a variety of other pathways including mitogen-activated protein kinase and PI3K/Akt that can potentially induce VEGF (44, 45). Furthermore, prostaglandin E2 can induce other angiogenic molecules including CXCL1, a proangiogenic chemokine (46), as well as the vascular remodeling protein angiopoietin-2. This induction of angiopoietin-2 is stimulated by hypoxia and does not depend upon HIF-1 (47). Thus, there are multiple pathways that are both HIF-dependent and HIF-independent that regulate the angiogenesis mediated by COX-2 and prostaglandin E2.

Clinical-Translational Advances

Although disrupting the function of a transcription factor is a challenge, several potential approaches to inhibit HIF-1 for therapeutic purposes have been identified (48). Echinomycin and polyamides seem to block binding of HIF-1 to its DNA hypoxia response element (49, 50), and chetomin can block the interaction of HIF-1 with CBP/p300 (51). These reagents have shown promising antitumor effects in preclinical studies. Other compounds that have been tested *in vitro* including heat shock protein 90 inhibitor 17-allyl-aminogeldanamycin and endogenous metabolite of estrogen, 2ME2, seem to function by targeting signaling pathways that activate HIF (52, 53). Phase II trials of 17-allyl-aminogeldanamycin in a wide variety of solid and hematologic malignancies are under way, and phase I trials of the closely related compound 17-dimethylaminoethylamino-17-demethoxygeldanamycin have also been initiated. Some of these phase II trials will correlate the RAS mutation status with clinical outcome. Because these compounds have multiple functions in addition to the inhibition of HIF-1, it may be a challenge to assess the relative contribution of HIF-1 inhibition to the observed therapeutic effects. Nevertheless, it will be critical to determine which HIF-1–independent mechanisms may be responsible for adaptive angiogenic responses that emerge.

In addition to the preclinical data previously discussed, several independent observations support the concept that acquired resistance to single-agent antiangiogenic therapy can develop. In a mouse model of pancreatic islet tumorigenesis, inhibition of VEGF receptors 1 and 2 with monoclonal antibodies initially blocked angiogenesis and tumor growth. However, tumor regrowth accompanied by revascularization was observed at 4 weeks. This resistance to anti-VEGF receptor therapy was characterized by the compensatory up-regulation of VEGF-A, the fibroblast growth factors (FGF) FGF1, FGF2, FGF7, and FGF8, ephrin-A1, and angiopoietin-2. The functional significance of these FGFs was verified when the addition of a neutralizing FGF trap then resulted in regression of tumor growth and angiogenesis (54). Similarly, in a clinical trial of 10 rectal cancer patients treated with bevacizumab alone, significant decreases in blood flow and microvessel density were observed after a 2-week period. Not surprisingly, there was a strong induction of plasma VEGF levels after the introduction of neutralizing VEGF antibody. However, plasma levels of placental growth factor, another angiogenic factor that binds to VEGF receptor 1, increased nearly 3-fold after treatment (55). These observations underscore the importance of compensatory angiogenic responses. Ultimately, the most successful antiangiogenic approaches may require combinations of agents that simultaneously target these adaptive pathways. In particular, inhibition of HIF-1–dependent, as well as HIF-1–independent pathways, may prove to be a compelling strategy.

Concluding Remarks

Targeting tumor angiogenesis is an effective component of the treatment strategy for cancer patients. The regulation of angiogenesis is a complex interplay between tumor genotype and the environment. Hypoxia is a universal feature of solid tumors, and one nearly ubiquitous factor that seems to mediate the hypoxic regulation of angiogenesis is HIF-1. Although HIF-1 is clearly an important therapeutic target, there are multiple pathways other than HIF-1 that can respond to hypoxia. Furthermore, *in vivo* studies have introduced the concept that alternative angiogenic pathways can be induced when a single factor, such as HIF-1, is blocked. Collectively, these observations underscore the complexity and diversity of the tumor angiogenic response. Delineation of the full spectrum of angiogenic mechanisms that are HIF-dependent, as well as HIF-independent, is therefore a prerequisite to the design of optimal combinations of antiangiogenic agents.

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