THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 18, pp. 15034–15043, April 27, 2012

Pulmonary Surfactant Protein A Protects Lung Epithelium from Cytotoxicity of Human β-Defensin 3*

Received for publication, September 26, 2011, and in revised form, March 7, 2012 Published, JBC Papers in Press, March 14, 2012, DOI 10.1074/jbc.M111.308056

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Background: The mechanisms by which hosts protect their own cells from cytotoxicity of defensins have been poorly understood.
Results: The cytotoxicity of human β-defensin 3 was significantly decreased by SP-A both *in vitro* and *in vivo*.
Conclusion: SP-A protects lung epithelium from tissue injury caused by excess amount of human β-defensin 3 secreted during inflammation.
Significance: Our results will promote therapeutic use of antimicrobial peptides.

Defensins are important molecules in the innate immune system that eliminate infectious microbes. They also exhibit cytotoxicity against host cells in higher concentrations. The mechanisms by which hosts protect their own cells from cytotoxicity of defensins have been poorly understood. We found that the cytotoxicity of human β-defensin 3 (hBD3) against lung epithelial cells was dose-dependently attenuated by pulmonary surfactant protein A (SP-A), a collectin implicated in host defense and regulation of inflammatory responses in the lung. The direct interaction between SP-A and hBD3 may be an important factor in decreasing this cytotoxicity because preincubation of epithelial cells with SP-A did not affect the cytotoxicity. Consistent with *in vitro* analysis, intratracheal administration of hBD3 to SP-A$^{-/-}$ mice resulted in more severe tissue damage compared with that in WT mice. These data indicate that SP-A protects lung epithelium from tissue injury caused by hBD3. Furthermore, we found that the functional region of SP-A lies within Tyr^{161}-Lys^{201}. Synthetic peptide corresponding to this region, tentatively called SP-A Y161-G200, also inhibited cytotoxicity of hBD3 in a dose-dependent manner. The SP-A Y161-G200 is a candidate as a therapeutic reagent that prevents tissue injury during inflammation.

Pulmonary surfactant contains two collectins, surfactant proteins A and D (SP-A and SP-D),[3] that belong to the C-type lectin superfamily. Pulmonary collectins are mosaic proteins, consisting of an amino-terminal domain, a collagen-like domain, a coiled-coil neck domain, and a carbohydrate recognition domain (CRD). These collectins have been shown to be implicated in host defense and regulation of inflammatory responses in the lung (1, 2).

Antimicrobial peptides (AMPs) are well conserved host defense molecules that have been found in both protostomes and deuterostomes. AMPs bind to pathogens and increase the permeability of their membranes (3, 4). It is thought that AMPs distinguish pathogens from host cells through recognition of the different surface structures of the cells. The cationic AMPs bind to negatively charged bacterial surfaces but not to phosphatidylcholine-rich host cell surfaces (5). In addition to microbicidal activities, these peptides play important roles in innate immune responses and wound healing. For example, they act as chemoattractant peptides or secretagogues to mediate and amplify the immune responses (6, 7). Furthermore, AMPs promote wound healing by modulating cell proliferation and migration (8). Although there is ample evidence of the important roles of AMPs in host defense, several reports have also pointed out the cytotoxic activity of AMPs against host cells in higher concentrations (9–11). Understanding the mechanisms by which the host prevents tissue injury caused by AMPs will result in greater recognition of the therapeutic use of AMPs.

Defensins are cationic AMPs displaying broad spectrum microbicidal activity against both bacteria and fungi. Human defensins are composed of two subclasses, α-defensins and β-defensins (hBDs), characterized by their distinct arrangement of three intramolecular disulfide bonds (3, 4). Six α-defensins have been identified specifically in neutrophils or Paneth cells. In contrast to restricted expression of α-defensins, hBDs have been isolated from various tissues including epithelial cells, neutrophils, phagocytes, and leukocytes and are also found in blood plasma and urine (3, 4). The expression of hBD1 is constitutive in cultured epithelial cells, whereas the expressions of hBD2 and hBD3 are increased by bacterial constituents and cytokines (12–15). Although little is known about the effects of defensins in disease development, the concentrations of defensins are also elevated in plasma or bronchoalveolar

* This work was supported by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.
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[3] The abbreviations used are: SP-A, surfactant protein A; AMP, antimicrobial peptide; BALF, bronchoalveolar lavage fluid; CRD, carbohydrate recognition domain; CRF, collagenase-resistant fragment; hBD, human β-defensin; LDH, lactate dehydrogenase; SP-D, surfactant protein D.

lavage fluid (BALF) of patients with pulmonary diseases (13–16).

Recent studies have indicated that human α-defensins 1 and 2 interact with SP-D and modulate the antiviral activity of SP-D (17, 18). Complex functional interactions have been demonstrated, *i.e.* they cooperate in some instances but compete in others. The viral strain and sequence of incubation of a virus with α-defensins or SP-D are responsible for different effects. Although functional interactions between defensins and collectins have been reported, the effects of collectins on cytotoxicity of defensins have not been studied. For the present study, we focused on the effects of pulmonary collectins on the cytotoxic activity of defensins against lung epithelial cells. Here, we report the protective role of SP-A against cytotoxicity of hBD3.

EXPERIMENTAL PROCEDURES

Synthetic Peptides—Human α-defensin 1, hBD1, hBD2, and hBD3 were from Peptide Institute Inc. (Osaka, Japan). SP-A Y161-G200 was synthesized by Greiner Bio-One Co., Ltd. (Tokyo, Japan).

Assay of Cytotoxic Activity—A549 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). THP-1 cells and H441 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FCS. A549 cells and H441 cells (1×10^4 cells/well) were seeded into a 96-well plate and allowed to adhere overnight. THP-1 cells (1×10^4 cells/well) were treated with 100 ng/ml phorbol 12-myristate 13-acetate (Sigma) for 48 h to induce differentiation toward macrophage-like cells and then used for the subsequent assay. After being washed with the medium, cells were incubated with defensins in the medium for an indicated time. We determined cytotoxic activity of defensins by measuring lactate dehydrogenase (LDH) leakage and membrane permeability. Activity of LDH in the supernatant was examined using the LDH cytotoxicity detection kit (Takara Bio Inc., Shiga, Japan). The lysate of non-treated cells, lysed with medium containing 2% Triton X-100, was used as high control to assess the total activity of LDH contained in the cells. The data were expressed as the percentages of absorbance at 492 nm for each sample to that for the high control. Remaining cells were stained with 25 μg/ml ethidium bromide and 5 μg/ml acridine orange to examine the permeability of cell membrane (19). All cells were stained with acridine orange, whereas only the cells with damaged membrane were stained with ethidium bromide. The cells were observed under fluorescence microscopy, and cytotoxic activity was determined as the percentage of cells stained with ethidium bromide in total cells counted.

Collectins—Recombinant human SP-A and SP-D were expressed in CHO-K1 cells using the glutamine synthetase gene amplification system and purified using a mannose-Sepharose 6B column (19). Chimeric protein with SP-A and SP-D, consisting of Glu^{1}-Pro^{80} of SP-A and Asp^{203}-Phe^{355} of SP-D, was prepared as described previously (20).

Preparation of Pulmonary Surfactant and Surfactant Lipids—BALF was collected from Sprague-Dawley rats and centrifuged at 700 × *g* for 3 min to remove cells. The supernatant was further centrifuged at 84,000 × *g* for 1 h. The pellet was homogenized in 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 1.64 M sodium bromide, and again centrifuged at 60,000 × *g* for 4 h. The pulmonary surfactant that formed a white membrane on top of the tube was collected and washed twice by centrifugation at 100,000 × *g* for 1 h with 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl. The content of proteins was determined using the BCA protein assay kit (Thermo Scientific). Surfactant lipids were extracted from purified surfactant by the method of Bligh and Dyer (21). The organic phase was dried completely under N_2 gas and resuspended with distilled water.

Surface Plasmon Resonance Analysis—SP-A or SP-D was immobilized on a sensor chip CM5 of the BIAcore 3000 system (BIAcore, Uppsala), according to the manufacturer's specifications. For the running buffer, 5 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 2 mM $CaCl_2$ were used. hBD3 was injected at a flow rate of 30 μl/min. Sensorgrams of the interactions obtained by using various concentrations of hBD3 (200–1200 nM) were analyzed by the BIAevaluation program. Where indicated, 5 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl and 2 mM EDTA or 5 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl and 2 mM $CaCl_2$ was used as a running buffer.

Assay of Antimicrobial Activity—Escherichia coli JM109 and *Staphylococcus aureus* were grown in LB and 3% tryptic soy broth, respectively. Bacteria in the exponential phase were collected by centrifugation and washed with 10 mM sodium phosphate, pH 7.4. The washed bacteria (1×10^3 cfu) were incubated in the same buffer containing hBD3 for 1 h at 37 °C. After incubation, suspensions were spread onto agar plates. The antimicrobial activity was assessed by counting the colony number.

Murine Model—Animal care and experiments were conducted according to the regulations of the Sapporo Medical University Animal Care and Use Committee. SP-A knock-out mice were purchased from The Jackson Laboratory (Bar Harbor, ME) (22). The original SP-A knock-out mice contained 129P2/OlaHsd and NIH Swiss Black background. Because we maintained the mice as homozygotes, we could not choose an appropriate strain for the WT control. Consequently, mice from the commercially available inbred strain C57BL/6 were used as the WT control. C57BL/6 mice were purchased from Sankyo Labo Service Corporation Inc. (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions and used when they were between 7 and 8 weeks of age.

Leakage of intracellular molecules is mostly believed to occur at the acute phase of tissue injury. In contrast, visible histological changes can be observed at the late phase. We therefore performed measurement of LDH activities and observed histological sections at different time points. Mice were anesthetized and instilled with hBD3 (1.5 mg of peptide/kg in 50 μl of saline) intratracheally after tracheostomy. Six hours after instillation, the mice were killed, and their lungs were lavaged with 1 ml of normal saline. The volume of recovered BALF was measured, and the number of leukocytes was counted after gentian violet staining. The activity of LDH in BALF was determined using a LDH cytotoxicity detection kit. To assess hBD3-induced histological changes of the lung, mice were killed 72 h after inhalation of hBD3. The lungs were fixed with 15% buffered formalin

phosphate and embedded in paraffin. Lung sections were stained with hematoxylin-eosin and examined under light microscopy.

Preparation of Collagenase-resistant Fragment (CRF)—SP-A was treated with collagenase III from *Clostridium histolyticum* (Advance Biofactures Corporation) at 37 °C for 22 h. After incubation, CRF was isolated by gel filtration using Superose 6 10/300 GL (GE Healthcare). The amino-terminal sequence of the purified CRF determined by the protein sequencer (Applied Biosystems) was Gly-Pro-Pro and Gly-Leu-Pro-Ala, indicating that the amino-terminal region and the collagenous domain of SP-A (Glu^{1}-Arg^{74} or Pro^{77}) are removed and the CRF starts at amino acid residue Gly^{75} or Gly^{78} of SP-A.

Digestion of CRF by Lysyl Endopeptidase—CRF was reduced in 0.25 M Tris-HCl, pH 8.5, 5 mM EDTA, 6 M guanidine hydrochloride and 1 mM DTT for 1 h at 37 °C under N_2 gas. 3 mM iodoacetic acid was then added and incubated further for 1 h at room temperature to block the reduced Cys residues. The modified CRF was dialyzed against 50 mM Tris-HCl, pH 9.0. Enzymatic digestion was performed with lysyl endopeptidase (Wako Pure Chemicals, Tokyo, Japan) at an enzyme to a substrate ratio of 1 to 20 in a solution containing 2 M urea and 50 mM Tris-HCl, pH 9.0, for 24 h at 37 °C. Peptides obtained by the digestion were separated by HPLC using Cosmosil $5C_8$-AR-300 column (Nacalai Tesque Inc., Kyoto, Japan). Elution was performed with a linear gradient of acetonitrile from 12.5 to 80% in 0.05% trifluoroacetic acid. Fractions were lyophilized and resuspended in distilled water. Aliquots of the fractions were used to examine the ability to attenuate cytotoxicity of hBD3 against A549 cells. The remaining sample was subjected to amino-terminal sequence analysis.

RESULTS

hBD3 Exhibits Cytotoxic Activity against Lung Epithelial Cells—To investigate the effects of pulmonary collectins on cytotoxicity of defensins expressed in the lung, we used A549 cells for *in vitro* experiments. Considering the tissue expression pattern and abundance of α-defensin 1 in neutrophils, we used α-defensin 1, hBD1, hBD2, and hBD3 in the present study. First, we examined the cytotoxicity of defensins against A549 cells at various concentrations. Among the four defensins tested, only hBD3 caused LDH leakage from treated cells in a concentration-dependent manner (Fig. 1*A*). To confirm that the LDH leakage reflected the damaged plasma membrane, we stained treated cells with acridine orange and ethidium bromide. As shown in Fig. 1*B*, cells with permeabilized membranes were stained red, whereas cells with intact membranes were stained green. Consistent with Fig. 1*A*, hBD3 but not other defensins increased membrane permeability (Fig. 1, *B* and *C*). The effects were dose-dependent, and >90% of the cells were permeabilized at 100 μg/ml. Similar results were obtained in experiments using H441 cells (Fig. 1, *D* and *E*) and THP-1 cells (Fig. 1, *F* and *G*). These data indicate that effects of hBD3 are not specific for certain cell types. Based on these observations, we focused on hBD3 in the following experiments.

SP-A Binds Directly to hBD3 and Attenuates Its Cytotoxicity—We next examined the effects of pulmonary collectins on cytotoxicity of hBD3. When cells were treated with hBD3 in the presence of SP-A, the LDH activity in the medium was decreased significantly in a dose-dependent manner. In contrast, SP-D did not affect the LDH leakage even at 50 μg/ml (Fig. 2*A*). These data indicate that the cytotoxicity of hBD3 is attenuated specifically by SP-A. The molar stoichiometry of hBD3: SP-A in the assay is shown in Table 1. The maximal inhibition was achieved at the ratio of 0.0017. The cytotoxicity of hBD3 was decreased to 40% of the control by SP-A at the concentration. The effects of pulmonary collectins on cytotoxicity were also confirmed in the experiments that assessed the membrane permeability (Fig. 2, *B* and *C*). Because SP-A is a constituent of pulmonary surfactant, it was determined that purified surfactant executes the same effect as purified SP-A. The addition of purified pulmonary surfactant significantly decreased the cytotoxicity of hBD3 (Fig. 2*D*). To clarify that the effects of surfactant are mediated by SP-A, we extracted surfactant lipids and tested their ability to decrease cytotoxicity of hBD3 (Fig. 2*E*). Although surfactant lipids slightly decreased cytotoxicity, they were less effective than whole surfactant. The addition of recombinant SP-A to surfactant lipids restored the anticytotoxic effects. Although some components in surfactant other than SP-A may exhibit anticytotoxic effects, these data clearly indicated that SP-A could express the significant protective effects in complex with pulmonary surfactant lipids. Next, we determined whether direct interaction between SP-A and the cells was necessary for attenuation of the cytotoxicity. Cells were preincubated with SP-A followed by treatment with hBD3. LDH activity in the medium of cells preincubated with SP-A was almost the same as that in those preincubated with the medium alone (Fig. 2*F*). These data indicate that direct interaction of SP-A with the cells does not cause the inhibitory activity for the cytotoxicity by hBD3 and suggest that direct binding of SP-A to hBD3 may be important for the effects of SP-A. Consequently, we determined the binding of SP-A to hBD3 by surface plasmon resonance analysis. The passage of hBD3 at various concentrations over SP-A immobilized on a sensor chip yielded a dissociation constant of $K_D = 1.73 \times 10^{-5}$ M (Fig. 3*A*). The binding of SP-A to a variety of ligands is Ca^{2+}-dependent. However, the addition of EDTA to a running buffer did not diminish the interaction obtained in the buffer containing $CaCl_2$ (Fig. 3*B*). To examine the electrostatic interaction between SP-A and hBD3, sensorgrams in running buffers containing different concentrations of NaCl were compared. The binding of hBD3 to SP-A was dramatically impaired in the buffer containing a high concentration of NaCl (Fig. 3*C*). These data indicate that SP-A interacts electrostatically with hBD3 and attenuates cytotoxicity against A549 cells. We also analyzed the binding of hBD3 to SP-D immobilized on a sensor chip. In contrast to expectations based on the results seen in Fig. 2, SP-D exhibited significant binding to hBD3 (Fig. 3*D*). These results suggest that the interaction between SP-A and hBD3 is important but not sufficient to inhibit cytotoxic activity.

SP-A Does Not Inhibit Antimicrobial Activity of hBD3—The antimicrobial activity of hBD3 is an important component in the host defense ability to eliminate infectious microbes. The

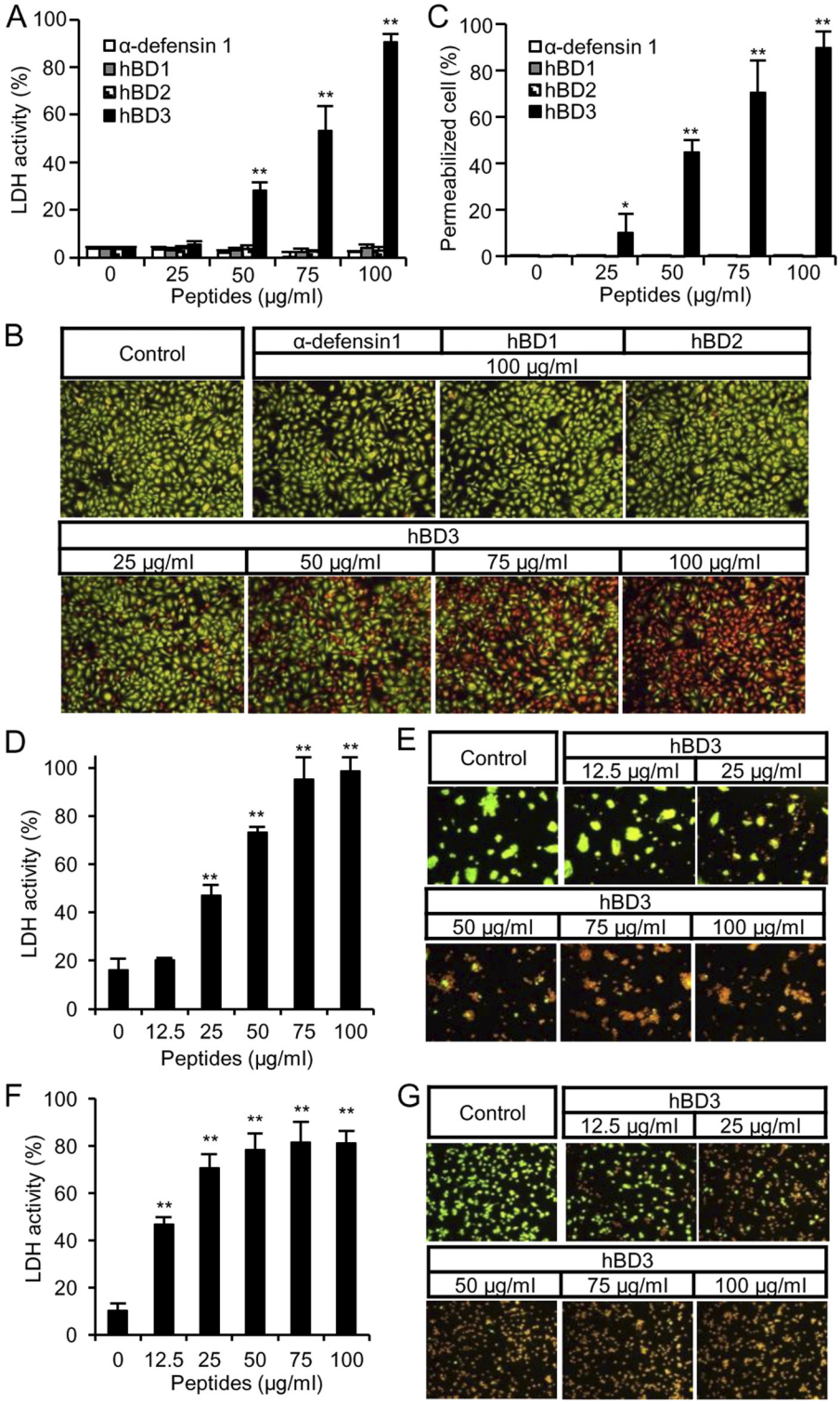

FIGURE 1. **hBD3 exhibits cytotoxic activity against lung epithelial cells.** A549 cells (*A–C*), H441 cells (*D* and *E*), and THP-1 cells (*F* and *G*) were incubated with various concentrations of defensins for 3 h. *A*, *D* and *F*, LDH activity leaked from cells into the medium was measured and is expressed as the percentage of absorbance at 492 nm to that for the high control. Remaining cells were stained with acridine orange and ethidium bromide. *B*, *E*, and *G*, representative specimens observed under fluorescence microscopy are indicated. *C*, percentages of the number of permeabilized cells to that of total cells are indicated. At least 300 cells were counted in each specimen. The data are mean ± S.D. (*error bars*) of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$ compared with the control which contained no peptide.

results described above prompted us to examine the effects of SP-A on the antimicrobial activity of hBD3. The antimicrobial activities of 50 μg/ml hBD3 against *S. aureus* and *E. coli* were not inhibited by the addition of 50 μg/ml SP-A (Fig. 4). The same results were obtained in experiments using a much lower concentration of hBD3 (Fig. 4, 5 μg/ml). These results suggest that SP-A decreases cytotoxic activity of hBD3 without affecting antimicrobial activity.

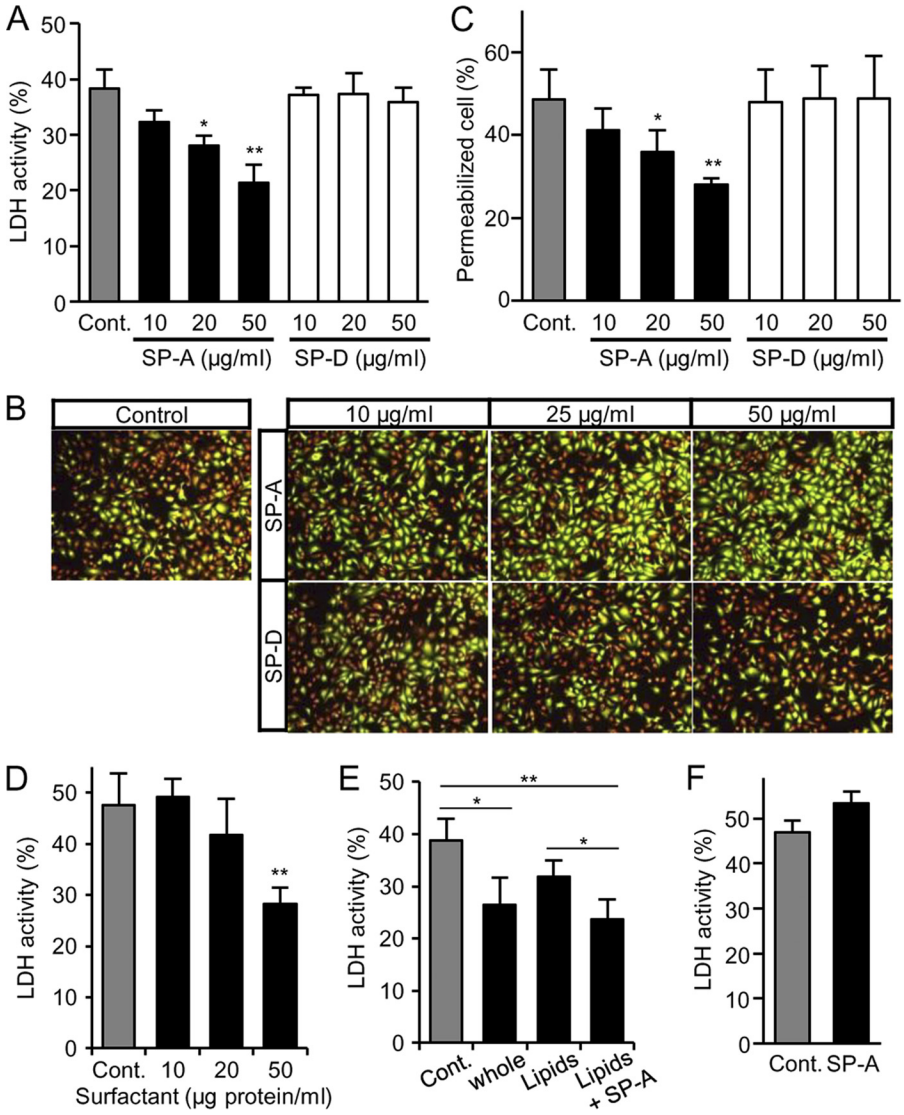

FIGURE 2. **SP-A but not SP-D attenuates cytotoxicity of hBD3.** A549 cells were incubated with 50 μg/ml hBD3 for 3 h in the presence of indicated concentrations of collectins. *A*, LDH activity leaked from cells into the medium was measured and is expressed as the percentage of absorbance at 492 nm to that for the high control. Remaining cells were stained with acridine orange and ethidium bromide. *B*, representative specimens observed under fluorescence microscopy are indicated. *C*, percentages of the number of permeabilized cells to that of total cells are indicated. At least 300 cells were counted in each specimen. *D*, A549 cells were incubated with 50 μg/ml hBD3 for 2 h in the presence of indicated concentrations of purified pulmonary surfactant. The concentration of surfactant was evaluated by the content of proteins. *E*, A549 cells were incubated with 50 μg/ml hBD3 for 2 h in the presence of whole surfactant, surfactant lipids, or mixture of surfactant lipids and 100 μg/ml recombinant SP-A. *F*, A549 cells were preincubated with 50 μg/ml SP-A for 1 h. Control cells were preincubated with medium alone for 1 h. After being washed with the fresh medium, cells were further incubated with 50 μg/ml hBD3 for 2 h. *D–F*, activity of LDH in the medium was measured. The data are mean ± S.D. (*error bars*) of three independent experiments. *A*, *C*, and *D*, *, $p < 0.05$ and **, $p < 0.01$ compared with the control which was treated with hBD3 alone. *E*, *, $p < 0.05$ compared with the indicated sample.

SP-A Protects Lung from Tissue Injury Caused by hBD3 in Vivo—In addition to the *in vitro* experiments, we examined the effects of SP-A on cytotoxicity of hBD3 *in vivo*. The WT and SP-A knock-out (SP-$A^{-/-}$) mice were instilled with hBD3 intratracheally. Six hours after instillation, LDH activities in BALF were measured (Fig. 5*A*). Deficiency of SP-A appeared to not affect integrity of lung epithelium because the LDH activities in BALF from WT and SP-$A^{-/-}$ mice instilled with saline were not significantly different. Instillation of hBD3 into WT mice significantly increased LDH activity in BALF compared with the saline control (1.48-fold increase). This indicates that hBD3 is toxic to lung epithelium in the physiological environment. The LDH activity in BALF of SP-$A^{-/-}$ mice instilled with hBD3 was more markedly increased compared with that of WT mice instilled with hBD3. The activity was increased to 2.48-fold of the saline control. These data indicate that lung epithelia of SP-$A^{-/-}$ mice were more susceptible to cytotoxicity of hBD3. It has been reported that an excess amount of defensins also results in overamplification of an inflammatory response followed by tissue injury. In these cases, a huge number of leukocytes are recruited to the alveolar space (23, 24). To examine whether SP-A is also involved in regulation of inflammatory responses induced by hBD3, we counted the number of leukocytes in BALF of mice instilled with hBD3 (Fig. 5*B*). Similar to the results of LDH activity measurement, the number of leukocytes was significantly increased in the BALF of SP-$A^{-/-}$ mice compared with that of WT mice. Histological examination revealed that hBD3 inhalation induced leukocyte infiltration,

interstitial edema, and alveolar septal thickening in murine lung tissue, whereas saline-treated murine lungs did not show any findings indicative of pulmonary inflammation or injury. These histological findings were more markedly seen in the SP-A$^{-/-}$ mice than in the WT mice (Fig. 5*C*). The lung damage observed in SP-A$^{-/-}$ mice instilled with hBD3 showed improvement with exogenously administered SP-A (Fig. 5*D*). These results suggest that SP-A regulates hBD3-induced inflammatory responses and prevents lung tissue from cytotoxicity of hBD3.

Tyr^{161}-Lyl^{201} Is Functional Region of SP-A—We tried to identify the functional region of SP-A using enzymatically digested SP-A and chimeric proteins. Schematic representations of recombinant proteins used in the experiments are shown in Fig. 6*A*. CRF, whose amino terminus and collagenase domains were removed by collagenase treatment, consists of the neck domain and CRD of SP-A. In chimeric protein, the neck domain and CRD of SP-A were replaced by those of SP-D. Purity of the proteins and efficiency of collagenase treatment were analyzed by SDS-PAGE. The proteins gave a single band at theoretical size in the gel (Fig. 6*B*). A549 cells were treated with hBD3 in the presence of CRF or chimeric protein, and cytotoxicity of hBD3 was assessed by measuring LDH activity in the medium and membrane permeability (Fig. 6, *C* and *D*). CRF decreased the LDH activity and membrane permeability to a level comparable with the sample incubated in the presence of SP-A. However, chimeric protein did not affect the cytotoxicity of hBD3. These data clearly indicate that the functional region of SP-A lies within the neck domain and/or CRD of SP-A.

For more detailed analysis, CRF was further digested by lysyl endopeptidase and separated by HPLC (Fig. 6*E*). The treatment of CRF by lysyl endopeptidase would produce four peptide fragments, Gly^{78}-Lys^{117}, Val^{118}-Lys^{159}, Tyr^{161}-Lys^{201} and Glu^{202}-Phe^{228}. The amino-terminal sequences determined by protein sequencer were Tyr-Asn-Thr for peak 1, Val-Phe-Ser and Glu-Gln-Xaa for peak 2, and Gly-Leu-Pro for peak 4. Peak 3 did not contain the peptide fragment. These data indicate that we recovered all of the four digested fragments. Next, the aliquots of these peaks were tested for their ability to attenuate the cytotoxicity of hBD3. Results indicated that peak 1 was the most efficient in decreasing the LDH leakage from A549 cells (Fig. 6*F*). These data suggest that the fragment Tyr^{161}-Lys^{201} is a functional region of SP-A.

TABLE 1

Molar stoichiometry of hBD3: SP-A in the cell protection studies

As shown in Fig. 2*A*, A549 cells were treated with 0.96 nmol of hBD3 in the presence of various concentrations of SP-A. The LDH activities in the medium were measured and expressed as the percentage of absorbance at 492 nm to that for control. The sample treated with hBD3 in the absence of SP-A was used as a control. The data are mean ± S.D. of three independent experiments. Because a monomeric SP-A seems to possess a functional region for anticytotoxic effect (see Figs. 6 and 7), we calculated the molar quantity of SP-A using molecular mass of monomeric protein (molecular mass = 36,000).

hBD3	SP-A	Molar ratio hBD3/SP-A	LDH activity
nmol	*nmol*		*% control*
0.96	0		100 (control)
0.96	27.72	0.0346	85.1 ± 6.6
0.96	55.44	0.0173	73.5 ± 3.4
0.96	138.6	0.0069	56.1 ± 3.9
0.96	277.2	0.0035	46.3 ± 2.7
0.96	554.4	0.0017	40.4 ± 1.5
0.96	831.6	0.0012	46.5 ± 7.3

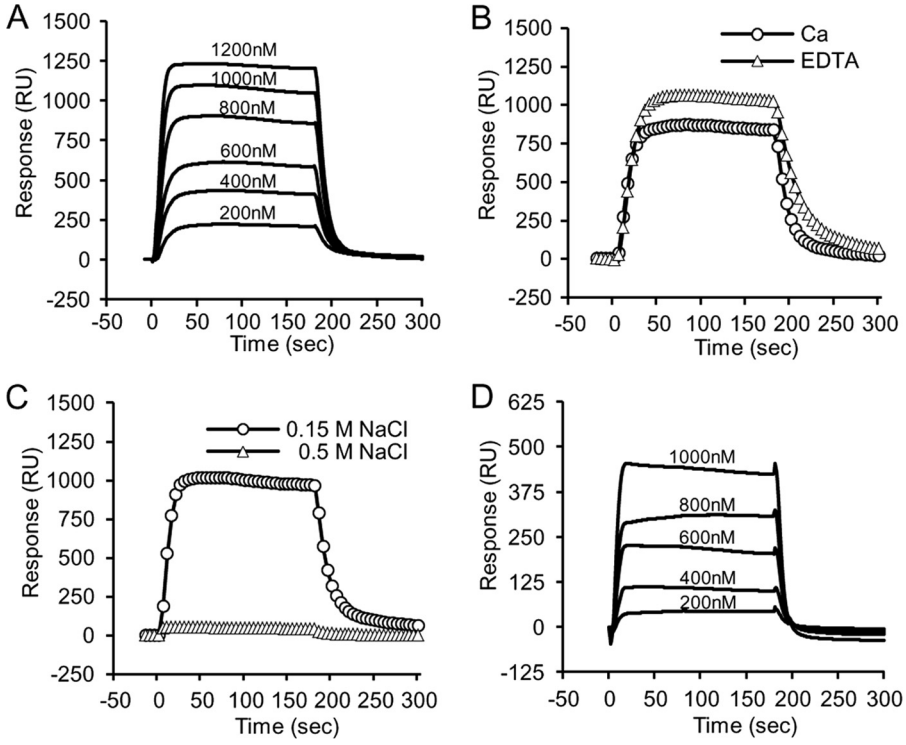

FIGURE 3. **SP-A electrostatically interacts with hBD3.** Binding of hBD3 to SP-A or SP-D was examined by surface plasmon resonance analysis. *A–C*, SP-A was immobilized on a sensor chip (620 resonance units (RU)). *A*, sensorgrams for binding of hBD3 to SP-A were overlaid at various concentrations of hBD3. *B*, sensorgrams for binding of hBD3 (600 nM) in a running buffer containing 2 mM $CaCl_2$ and that containing 2 mM EDTA were superposed. *C*, sensorgrams for binding of hBD3 (600 nM) in a running buffer containing 0.15 M or 0.5 M NaCl are superposed. *D*, sensorgrams for binding of hBD3 to SP-D immobilized on a sensor chip (1069 resonance units) are overlaid at various concentrations of hBD3.

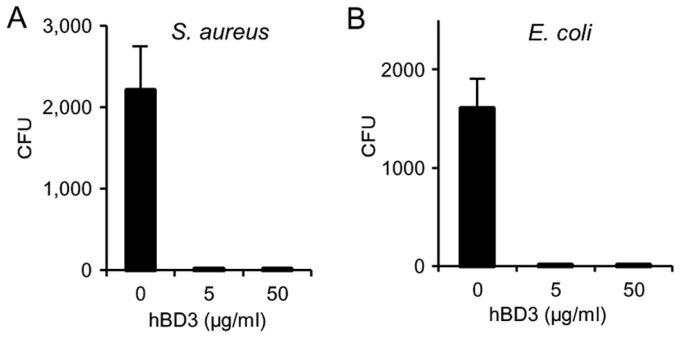

FIGURE 4. **SP-A does not affect the antimicrobial activity of hBD3.** *S. aureus* (*A*) and *E. coli* (*B*) were incubated with indicated concentrations of hBD3 for 1 h in the presence of 50 μg/ml SP-A. After incubation, suspensions were spread onto agar plates and cultured at 37 °C. The antimicrobial activity of hBD3 was assessed by counting colony numbers.

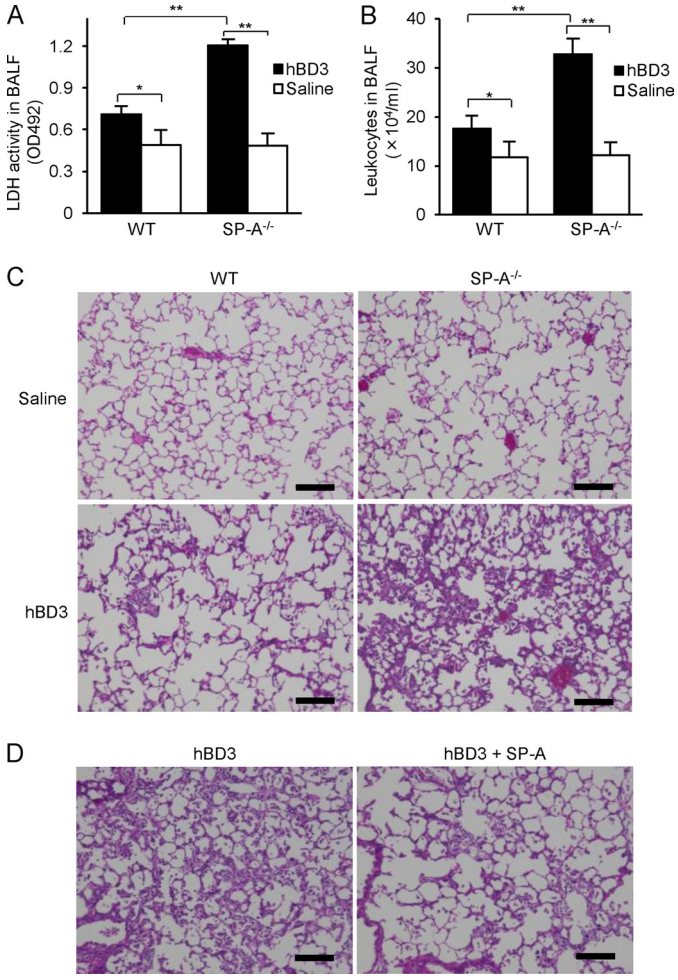

FIGURE 5. **SP-A protects the lung from tissue injury caused by hBD3 *in vivo*.** C57BL/6 (WT) and SP-A knock-out (SP-A$^{-/-}$) mice were instilled intratracheally with hBD3 or saline. Six hours after instillation, mice were sacrificed, and lungs were lavaged with saline. The LDH activity (*A*) and the number of leukocytes (*B*) in the BALF collected from each mouse were determined. The data are mean ± S.D. (*error bars*) of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$ compared with the indicated sample. *C*, for histological analysis, mice were sacrificed 72 h after inhalation of hBD3. The lungs were inflated with 15% buffered formalin phosphate, isolated, washed, and fixed. Paraffin-embedded sections from WT mice and SP-A$^{-/-}$ mice exposed to saline or hBD3 were stained with hematoxylin-eosin and observed under light microscopy. Representative specimens are indicated. *Scale bars*, 100 μm. *D*, SP-A$^{-/-}$ mice were instilled with hBD3 in the absence (*left*) or presence (*right*) of SP-A (1.5 mg/kg). After 72 h, the histological sections of the lungs from the treated mice were prepared as described in *C*. Representative specimens are indicated. *Scale bars*, 100 μm.

Synthetic Peptide of Tyr^{161}-Gly^{200} Attenuates Cytotoxicity of hBD3—To demonstrate the protective role of Tyr^{161}-Lys^{201} of SP-A and to reveal the potential of the peptide in therapeutic use, we synthesized the peptide and tested it in experiments using A549 cells. Concerning the electrostatic interaction between SP-A and hBD3, we deleted Lys residue at the carboxyl terminus and synthesized the peptide of Tyr^{161}-Gly^{200} to reduce positive charges in the peptide. The amino acid sequence of the peptide, tentatively named SP-A Y161-G200, is indicated in Fig. 7*A*. Purity of the synthetic peptide was checked by HPLC, and a single peak was detected at 214 nm (Fig. 7*B*). The retention time of SP-A Y161-G200 was slightly different from that of the Tyr^{161}-Lys^{201} fragment generated by enzymatic digestion of SP-A (Fig. 6*E*, peak 1). In the previous study, it was reported that the Asn^{187} residue of SP-A was attached by an *N*-linked sugar chain (25). In contrast, SP-A Y161-G200 was synthesized as a polypeptide without any modification. The difference in retention times may reflect the presence or absence of an *N*-linked sugar chain. When the biological activity was tested by *in vitro* assay, SP-A Y161-G200 dose-dependently attenuated the cytotoxicity of hBD3 against A549 cells (Fig. 7*C*). Much higher molar quantities of SP-A Y161-G200 were required to obtain the anticytotoxic effect comparable with SP-A itself (Figs. 2*A* and 7*C*). The weak anticytotoxic effect of synthetic peptide may have been due to poor solubility of the peptide. Indeed, the peak 1 revealed more effective anticytotoxic activity than SP-A itself (Fig. 6*F* and data not shown). Although more detailed study to optimize the peptide design is required, these data indicate that the synthetic peptide of SP-A Y161-G200 is enough to attenuate the cytotoxicity of hBD3.

DISCUSSION

Although the important roles of AMPs in host defense have been reported in many studies, molecular mechanisms underlying the negative regulation to prevent overamplification of inflammation and tissue injury by AMPs are poorly understood. Here, we have indicated one of the mechanisms to prevent the tissue injury by hBD3 in the lung.

In our assay system, only hBD3 exhibited cytotoxicity against lung epithelial cells (Fig. 1). The folding and arrangement of disulfide bonds of the synthetic hBD3 used in the present study were reported to be identical to natural peptide (26). Observed cytotoxicity of hBD3 was therefore not due to inappropriate quality of the peptide. This conclusion is in contrast with several other reports, which have demonstrated the cytotoxicity of α-defensin 1 and hBD2 against respiratory epithelial cells (27, 28). These different results must have been influenced by the presence of serum in the assay medium. Indeed, it has been reported that cytotoxicity of α-defensin 1 is completely abrogated by the addition of serum or BSA into the assay medium (9, 11). These data and results in Fig. 5*A* indicate that hBD3 is a potent cytotoxic peptide in physiological conditions. Interestingly, the inhibitory effect of SP-A was specific for cytotoxicity, and antimicrobial activity of hBD3 was not affected by SP-A (Fig. 4).

It is difficult to determine the exact concentrations of collectins *in vivo*. Because SP-A and SP-D are constituents of a pul-

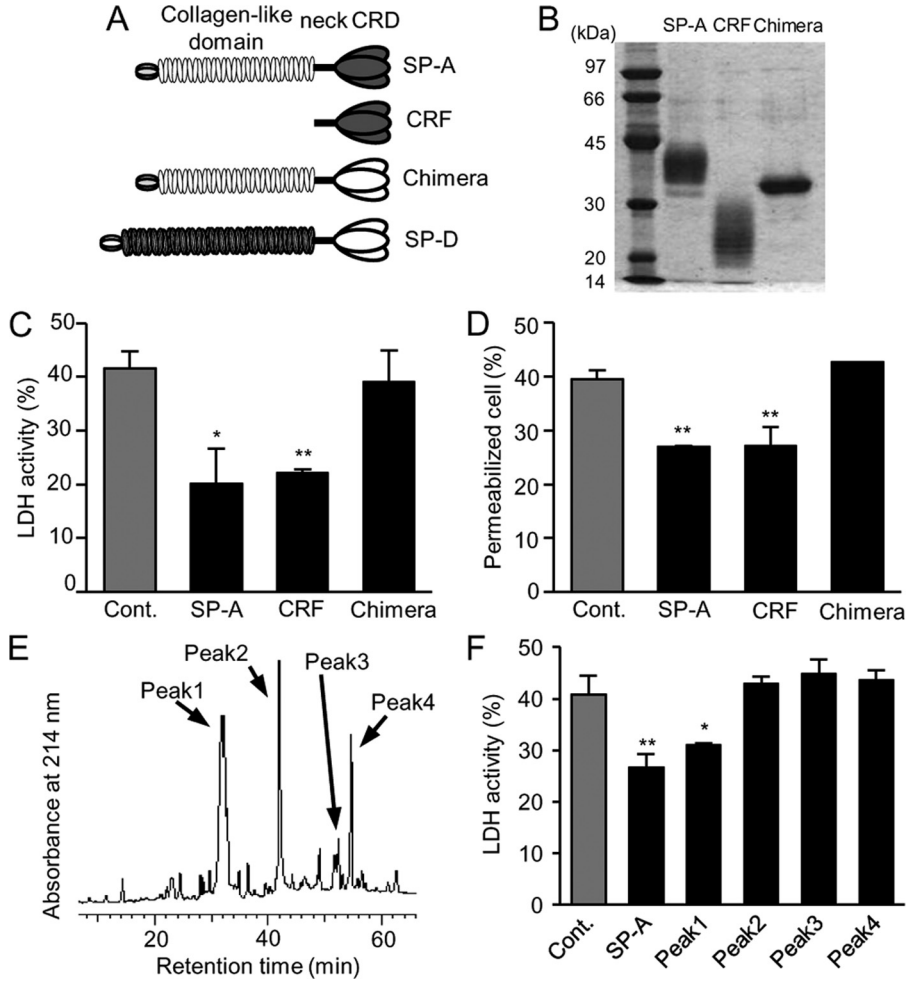

FIGURE 6. **Determination of the functional region in SP-A.** *A*, schematic representations of SP-A, CRF, and chimeric protein. *B*, recombinant proteins (4 μg each) subjected to 12% SDS-PAGE under reducing conditions and visualized with Coomassie Brilliant Blue staining. *C* and *D*, A549 cells incubated with 50 μg/ml hBD3 for 2 h in the presence of 50 μg/ml SP-A, CRF, or chimeric protein. The cytotoxicity of hBD3 was determined by measuring LDH activity leaked from cells into the medium (*C*) and cell membrane permeability (*D*). *E*, CRF digested by lysyl endopeptidase and subjected to HPLC. The elution was monitored by measuring the absorbance at 214 nm. *F*, testing of ability of each fraction to attenuate cytotoxicity of hBD3 against A549 cells. Cells were incubated with 50 μg/ml hBD3 for 2 h in the presence of separated peptides. After incubation, the activity of LDH in the medium was measured. SP-A (50 μg/ml) was used as a positive control. The data are mean ± S.D. (*error bars*) of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$ compared with the control which was treated with hBD3 alone.

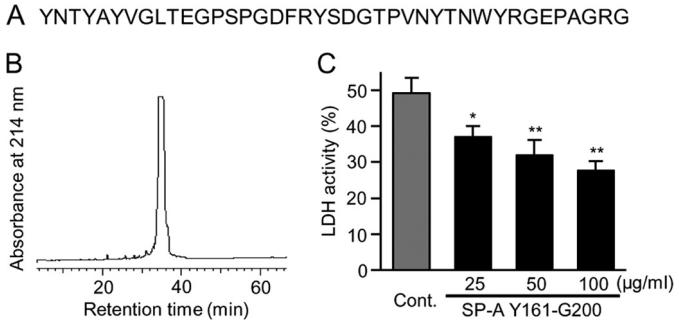

FIGURE 7. **Synthetic peptide corresponding to Tyr^{161}-Gly^{200} of SP-A attenuates cytotoxicity of hBD3.** *A*, amino acid sequence of SP-A Y161-G200. *B*, SP-A Y161-G200 subjected to HPLC using the Cosmosil $5C_8$-AR-300 column. Elution was performed with a linear gradient of acetonitrile from 12.5 to 80% in 0.05% trifluoroacetic acid and monitored by measuring the absorbance at 214 nm. *C*, A549 cells incubated with 50 μg/ml hBD3 for 2 h in the presence of indicated concentrations of SP-A Y161-G200. The cytotoxicity of hBD3 was determined by measuring LDH activity leaked from cells into the medium. The data are mean ± S.D. (*error bars*) of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$ compared with the control which was treated with hBD3 alone.

monary surfactant which exists in the epithelial lining fluid of the alveolus, it is not possible to determine the concentrations directly. However, we can estimate their concentrations according to previous studies, which calculate the concentrations based on the recovery of the proteins in the BALF. The calculated concentration of SP-A ranges from 180 to 1800 μg/ml (29, 30). Although they appear to vary in a diseased state, the concentrations of collectins used in our study are within the physiological ranges.

Hartshorn *et al.* reported that the neck domain and CRD of SP-D are sufficient for binding to defensins, and binding of SP-D is not calcium-dependent or inhibited by maltose, suggesting that binding of SP-D to defensins differs mechanistically from its binding to other microbial ligands (17). Similar results were obtained in binding analysis for SP-A and hBD3. The binding of SP-A to hBD3 was Ca^{2+}-independent, and SP-A appears to interact electrostatically with hBD3 (Fig. 3, *B* and *C*). These data suggest that SP-A interacts with hBD3 in a manner independent of its lectin activity. The interaction may be important for attenuation of cytotoxicity

because preincubation of cells with SP-A failed to attenuate the cytotoxicity of hBD3 (Fig. 2*F*). However, through surface plasmon resonance analysis, we found that hBD3 binds not only to SP-A but also to SP-D and BSA, despite the fact that neither exhibited an anticytotoxic effect (Figs. 2, *A* and *B*, 3*D*, and data not shown). These data suggest that electrostatic interaction is important but not sufficient to inhibit cytotoxic activity. Furthermore, the peak 1 in Fig. 6*E* did not inhibit the binding of hBD3 to SP-A immobilized on a sensor chip, indicating that SP-A more tightly interacts with hBD3 than the peptide contained in peak 1 (data not shown). Because peak 1 revealed a more efficient inhibition of the cytotoxicity of hBD3, these data suggest discrepancy between binding affinity and anticytotoxic effect. More complicated interactions may be involved in the protective role of SP-A. Several reports have indicated a correlation between hydrophobicity of AMPs and cytotoxicity (31–33). Consistent with the proposed hydrophobicity-cytotoxicity relationship, more hydrophobic analogous peptides of hBD3 exhibit greater cytotoxic activity (10, 34, 35). With respect to the structural aspect, the solution structure of hBD3 is characterized by an amphipathic dimeric structure, which is different from those of hBD1 and hBD2 (36). The identified functional region of SP-A is rich in aromatic and hydrophobic amino acid residues (Fig. 7*A*). This region corresponds to the lipid binding site of SP-A that forms a hydrophobic patch of aromatic rings and aliphatic side chains on the surface of CRD (37). It is possible that these aromatic and aliphatic amino acid residues are responsible for reducing cytotoxicity via hydrophobic interactions with the hydrophobic surface of hBD3.

The expression of hBD3 is increased by various stimuli such as inflammatory cytokines, bacteria, fungi, and viruses (14, 15, 38–41). In some cases, the expression was reported to be increased >10-fold from basal expression. These observations suggest that hBD3 causes tissue injury at the site of infection. Our data clearly indicate the ability of SP-A Y161-G200 to decrease cytotoxicity of hBD3 in a dose-dependent manner (Fig. 7*C*). We propose that SP-A Y161-G200 has potential for therapeutic use to prevent tissue injury during infections.

We propose here that hBD-3 is one of the molecules that can be cytotoxic during inflammation. Many kinds of host defense molecules are produced at the sites of infection. Among them, a variety of molecules, including reactive oxygen species, elastase, and cytokines, are reported to cause tissue injury. This makes it difficult to examine the cytotoxic effects of hBD-3 *in vivo*. Tissue injury might be induced by other cytotoxic molecules even if we simply delete hBD-3 alone. Nonetheless, further experiments using mouse β-defensin 14 (a mouse orthologue of hBD3) knock-out mice and SP-A knock-out mice will provide important information and evidence to verify proposed SP-A functions *in vivo*. To evaluate the effects of hBD3 and SP-A in lung injuries, the extent of tissue damage during infection in β-defensin 14 or SP-A knock-out mice should be compared with that in WT mice. These studies should be conducted in the future.

In conclusion, the cytotoxic activity of hBD3 was significantly decreased by SP-A both *in vitro* and *in vivo*. Our results suggest that SP-A protects lung epithelium from injury caused by excess amount of hBD3 secreted during inflammation. Furthermore, we suggest that the synthetic peptide corresponding to the functional region of SP-A is a candidate as a new therapeutic reagent derived from host molecule.

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