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Reduction of E-cadherin by human defensin-5 in esophageal squamous cells

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ABSTRACT

Barrett's esophagus (BE) is metaplastic columnar epithelium converted from normal squamous epithelia in the distal esophagus that is thought to be a precancerous lesion of esophageal adenocarcinoma. BE is attributed to gastroesophageal reflux disease (GERD), and therefore gastric acid or bile acids are thought to be factors that cause epithelial cell damage and inflammation in the gastro-esophageal junction. The decrease of adherent junction molecules, E-cadherin has been reported to be associated with the progression of the Barrett's carcinoma, but the initiation of BE is not sufficiently understood. BE is characterized by the presence of goblet cells and occasionally Paneth cells are observed at the base of the crypts. The Paneth cells possess dense granules, in which human antimicrobial peptide human defensin-5 (HD-5) are stored and secreted out of the cells. This study determined the roles of HD-5 produced from metaplastic Paneth cells against adjacent to squamous cells in the gastro-esophageal junction. A human squamous cell line Het-1A, was incubated with the synthetic HD-5 peptide as a model of squamous cell in the gastro-esophageal junctions, and alterations of E-cadherin were investigated. Immunocytochemistry, flow-cytometry, and Western blotting showed that the expression of E-cadherin protein was decreased. And a partial recovery from the decrease was observed by treatment with a CD10/neprilysin inhibitor (thiorphan). In conclusion, E-cadherin expression in squamous cells was reduced by HD-5 using *in vitro* experiments. In gastro-esophageal junction, HD-5 produced from metaplastic Paneth cells may therefore accelerate the initiation of BE.

1. Introduction

Esophageal adenocarcinoma, which is a rapidly increasing cancers nationwide, is thought to arise from the metaplastic epithelium. Metaplasia is defined as Barrett's esophagus (BE) which is converted from squamous epithelial cells to columnar cells in the distal esophagus [1]. Numerous investigations for the resolution of the occurrence of BE have been conducted in Western countries. The main therapeutic strategy for early intervention of esophageal adenocarcinoma is to restore esophageal squamous epithelium from metaplasia [2,3]. Until now, the clinical and pathological evidence has suggested that BE is derived as a consequence of the chronic inflammation so to the exposure to refluxed gastric acids and bile acids. Chemoprevention with proton pump inhibitors, NSAIDs, or statins could lower the risk of BE [4,5].

Esophageal epithelium damaged by the acids has been shown to increase permeability because the structural barriers are injured [6]. E-cadherin is a component of adherent junctions and forms cell–cell interactions. E-cadherin intracellularly binds to β-catenin which is further connected to the actin cytoskeletons, keeping the cell integrity and eventually allowing tissue morphogenesis [7]. Moreover, abnormal cadherin function is linked to inflammatory disorders and cancers. The down regulation of E-cadherin in esophageal cancer has been reported in many papers and is considered to be a prognostic marker in the cancer patients [8]. Loss of E-cadherin expression is observed in esophageal adenocarcinoma and the stepwise reduction progresses in the sequence of metaplasia–dysplasia–adenocarcinoma progression. E-cadherin methylation is observed in 54.7% of BE in our previous experiment using capture micro dissection and methylation specific polymerase chain reaction (PCR) assay [9]. Therefore, E-cadherin is one of the

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most important molecules involved with the Barrett's carcinogenesis.

Pathological examinations in BE have indicated that Goblet cell metaplasia is frequently observed and aberrant Paneth cells are sometimes found in columnar epithelial cells [10]. Specialized columnar epithelium composed of goblet cells, occasionally possesses Paneth cells in the base of the crypt structure. A previous study noted that human defensin 5 (HD-5), a Paneth cell specific marker, is expressed in BE or gastric intestinal metaplasia similar to the expression in the small intestinal epithelia [11]. Defensins are antimicrobial peptides that were first discovered as antibacterial molecules, and their multifunctional properties have been unmasked. Defensins play many functional roles in mammalian cells including monocytes or some epithelial cells [12–14]. Therefore HD-5 secreted from metaplastic Paneth cells in the gastro-esophageal junction may affect the normal esophageal epithelial cells while they are replaced by the intestinal epithelial cells. The present study determined whether HD-5 affected the expression and production of E-cadherin in an esophageal epithelial cell line *in vitro*.

2. Materials and methods

2.1. Cell culture conditions

The human esophageal squamous cell line, Het1A, purchased from America Tissue Culture Collection (ATCC# CRL-2692) was maintained in bronchial epithelial cell basal medium (BEBM) supplemented by bronchial epithelial growth media kit (Lonza, Walkersville, MD). Cell culture dishes were coated with a mixture of 0.01 mg/ml fibronectin (Asahi Glass Co., Chiba, Japan), 0.03 mg/ml bovine collagen type I (BD bioscience, Bedford, MA) and

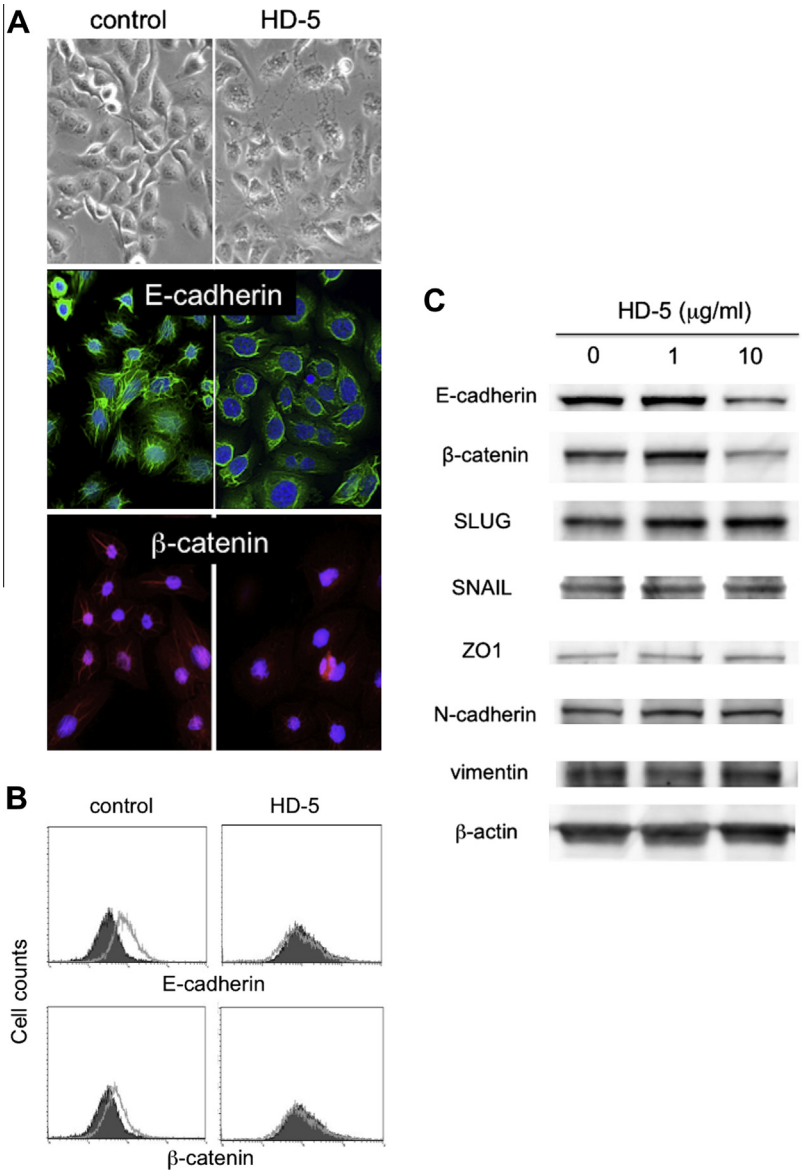

Fig. 1. E-cadherin reduction in Het-1A cells by exposure of HD-5. Right panels were Het-1A human squamous cells supplemented by HD-5 in the culture media, and left panels are controls. Magnification was 1000×. (A) Bright field photos demonstrate no significant change in cell morphology (Top panel). Immunocytochemistry of E-cadherin (Middle panel) and β-cetenins (Bottom panel) were compared between with or without HD-5. The expression of E-cadherin was reduced. (B) Immunocytochemical study was followed by FACS analysis. HD-5 markedly decreased E-cadherin protein, and β-catenin faintly. (C) The cells cultured with different concentration of HD-5 (0, 1, 10 μg/ml) and harvested cells were analyzed by Western blotting. E-cadherin and β-catenin was down-regulated, and ECAD-1 transcriptional factor; SLUG, SNAIL, tight junction molecule; ZO-1, and epithelial–mesenchymal transition activating factor; N-cadherin, vimentin were not different. β-actin was used as internal control.

0.01 mg/ml bovine serum albumin (Wako, Osaka Japan). Cells were cultured in 5% CO_2 at 37 °C and stimulated by reagents in semi confluent condition. Synthetic HD-5 was purchased from the Peptide Institute Inc., (Osaka, Japan). The protease inhibitor chloroquine (Sigma–Aldrich, St Louis, MO), Proteasome inhibitor, MG132 (Enzo life Science, Farmingdale, NY) and autophagy inhibitor, 3-Methyladenine (3-MA, R & D systems, Minneapolis, MN), CD10/neprilysin inhibitor, thiorphan (Enzo Life Sciences, Farmingdale, NY) were used for inhibition of protein degradation.

2.2. Protein extraction and Western blotting

The collected cells were washed with PBS, whole proteins were extracted with Mammalian Cell Extraction Kit (BioVision, Mountain View, CA), and the protein concentrations were measured with BioRad Protein Assay Kit (Bio-Rad, Hercules, CA). Adjusted proteins (20 μg) were boiled in Laemmli Sample Buffer (Bio-Rad, Hercules, CA) and separated by electrophoresis on 12.5% polyacrylamide Mini-PROTEIN TGX Gels (Bio-Rad, Hercules, CA), transferred onto nitrocellulose membrane (Bio-Rad, Hercules, CA). Sequentially, the membrane was blocked with 1% Bovine Serum Albumin (BSA, Sigma–Aldrich, St Louis, MO) in PBS with 0.1% Tween-20 (T-PBS) for 1 h, incubated with primary antibody over night at 4 °C, with secondary antibody for 60 min, and developed with the Super-Signal West Pico or Femto enhanced chemiluminescence system (Thermo Science, Rockford, IL). The luminescence was detected by LAS3000 (Fuji Photo Film Co., Kanagawa, Japan) and the intensity of the visualized bands was measured with Image-J. The primary and secondary antibodies are described in Supplementary Table 1.

2.3. Immunocytochemistry and FACS analysis

Cultured cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton-X (Nacalai Tesque Inc., Kyoto, Japan) for 15 min, blocked in 10% BSA for 1 h, incubated in primary antibodies and reacted with secondary antibody conjugated with Alexa 488 or 594 for 1 h. DAPI (Lonza, Walkersville, MD) were used for nuclear staining. The cells were mounted in anti-fade mounting medium (Vector Laboratories Inc., Burlingame, CA) and observed under the fluorescence microscopy (KEYENCE Corp., Osaka, Japan).

Trypsin-treated cells were collected, fixed in 70% cold ethanol, and the density of the cells was adjusted to 10^6 in 100 ml of MACS Buffer (Milteny Biotec, Auburn CA). Further reactions were described above with some modifications. All procedure was performed in MACS Buffer. The cells were resuspended to 2×10^6/

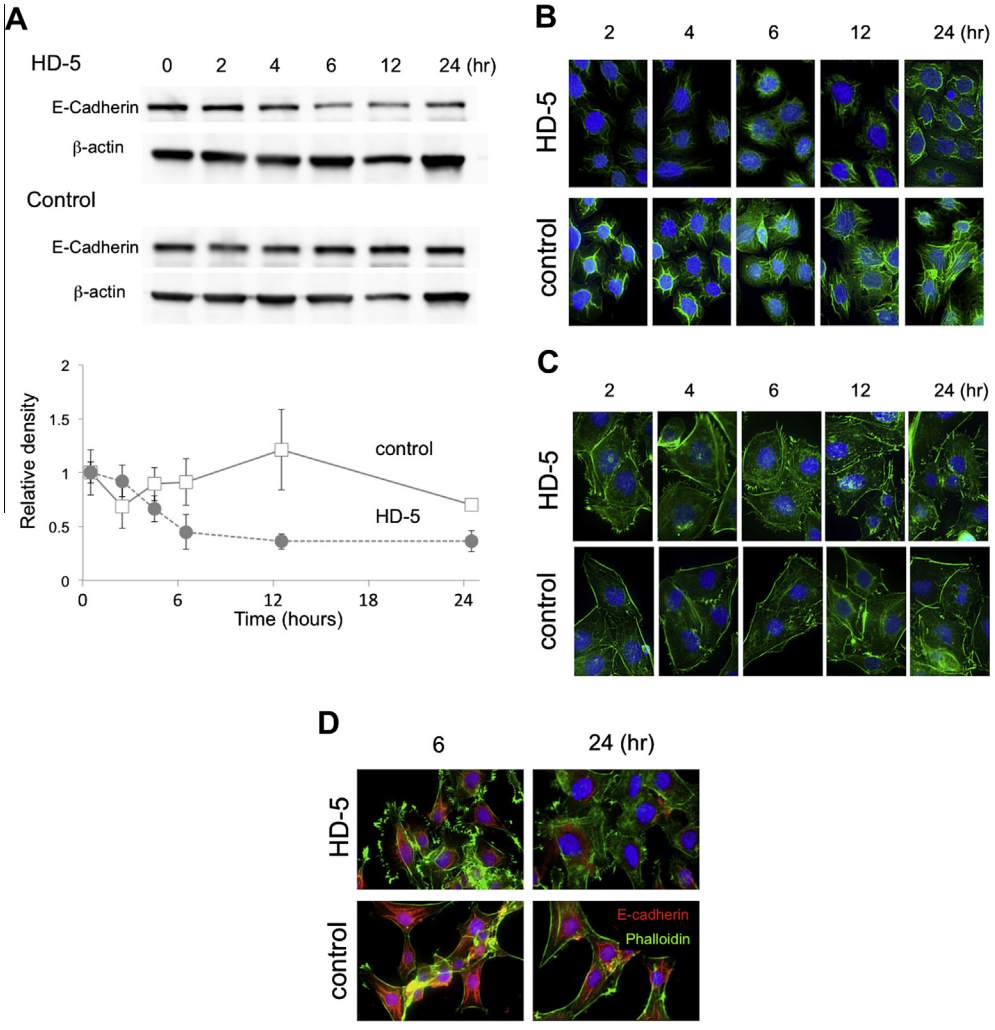

Fig. 2. Time course reduction of cytoskeleton molecules. (A) Western blotting showed that E-cadherin expression was reduced from earlier than 4 h after incubation with HD-5. (B,C) The immunocytochemical analysis demonstrated E-cadherin expression (B) was already reduced at 2 h, while F-actin stained with phalloidin (C) was not significantly reduced. The F-actin on the cell membrane was protruding after 6 h. (D) Double staining of E-cadherin and phalloidin remarkably showed the protruding actin filament on the cell surface and reduced cytoplasmic E-cadherin.

ml and analyzed with FACS Caliber (BD Bioscience, Fukushima, Japan).

2.4. Electron microscopic analysis

For conventional transmission electron microscopy, attached cell layers were fixed in half-strength Karnofsky fixative, followed by further fixation in 1% osmium tetroxide in distilled water. After en bloc staining with uranyl acetate, specimens were dehydrated in ethanol and embedded in Epson 812 (Taab, Berkshire, United Kingdom). Ultra thin sections were stained with uranyl acetate and lead citrate.

2.5. Statistical analysis

The statistical analysis was assessed by Mann–Whitney test for the analysis of the difference between two groups, and statistical significance was defined when $P < 0.05$.

3. Results

3.1. Adherent junctions in squamous epithelial cell lines were altered

Het-1A, a normal human esophageal squamous cell line, was maintained on the fibronectin-coated plates in bronchial cell basal medium. The epithelial cells formed spindle shapes with attachment to the plate and adjoining cells with adherent junction (Fig. 1A, top panel). The cells were incubated with HD-5 to determine the alterations in cell morphology of squamous cells *in vivo*, in order to determine the effect of HD-5 produced from metaplastic Paneth cells. The morphology was not remarkably changed when the cells were incubated with 10 μg/ml HD-5. One of the most important molecules in the junction is E-cadherin, a core component facilitating cell–cell recognition and adhesion. Immunocytochemical observation demonstrated that the expression of E-cadherin was reduced and the cytoskeletal structure was deformed by HD-5 exposure (Fig. 1A). Similar staining patterns were observed in the immunostaining of β-catenin, which binds to E-cadherin to form a cadherin–catenin complex to support epithelial cell integrity and morphogenesis. The intensity of the fluorescence was confirmed with FACS analysis as well as immunocytochemistry (Fig. 1B).

Het-1A cells were treated with the different concentrations of HD-5 to assess the expression levels of E-cadherin-related molecules, junctional molecules, and differentiation markers, by Western blotting. 10 μg/ml of HD-5 reduced E-cadherin and β-catenin, though snail or slug, transcriptional repressors of E-cadherin, were not increased (Fig. 1C). Furthermore the mRNA levels of E-cadherin, β-catenin and slug were constant following exposure to extracellular HD-5 (Supplementary Fig. 1A). Recent studies revealed that these two molecules induce the epithelial–mesenchymal transition in some cancer cells, in which N-cadherin or vimentin are overexpressed and used as the mesenchymal markers. They were not changed in the Western blotting assay. The tight junction molecule ZO-1 was analyzed because cadherin plays the important roles in adherent junctions. The level of ZO-1 was not affected by the HD-5 either.

3.2. HD-5 altered the morphology of cytoskeletal molecules

The time course experiment with Western blotting showed a reduction of E-cadherin at 4 h or later and the expression did not recovered within 24 h (Fig. 2A). Since E-cadherin protein production was not affected, HD-5 was found to regulate E-cadherin downstream of the protein synthesis (Supplementary Fig. 1B). Immunocytochemistry demonstrated the quick response of intracellular E-cadherin skeletal structures (Fig. 2B). Actin filaments were also assessed to determine the association of the cytoskeletal molecules that are closely related to the adherent junctions. The F-actin staining pattern were assessed by phalloidin staining and demonstrated that the both of cell surface structure and intracellular cytoskeleton were sedimentary at the cell surface (Fig. 2C and D). An ultrastructural examination demonstrated cell–cell attachment was distorted in HD-5 treated cells. Cell membranes were separated and the surface of the cells was fluffy with many protrusions that had a club-like appearances (Fig. 3).

3.3. HD-5 regulated E-cadherin expression independent of the major degradation pathways

The degradation processes were the focus of later experiments, since E-cadherin protein is commonly ubiquitinated and subjected to the proteasomal degradation. The post-translational regulation is frequently observed in the recycling of adherent junction molecules, E-cadherin and catenin, that are critical for cell–cell attachment in epithelial cells. Protease inhibitors were applied to the cells to investigate whether the degradation of E-cadherin is regulated by the exposure to HD-5. A protease inhibitor, chloroquine and proteasome inhibitor, MG-132 were investigated. Autophagy

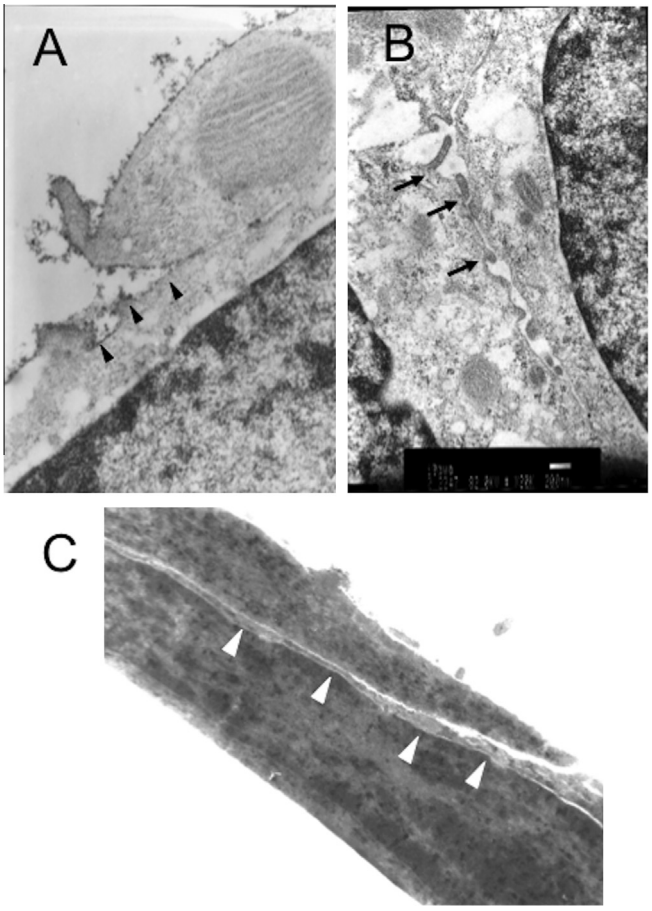

Fig. 3. The ultrastructure of the intracellular gap of the treated squamous esophageal cells. (A) Cultured Het-1A cells were treated with 10 μg/ml HD-5 and were observed by electron microscopy. The cell membrane was detached from adjacent cell membrane, and the microdense nodules are indicated by closed rectangles. The original magnification was 30,000×. (B) A high magnification view showing the club-like appearance (arrows) on the intracellular membrane. (C) Control Het-1A cells without HD-5 exposure demonstrated a smooth intracellular structure indicated by white rectangles. The original magnification was 25,000×.

is also an important recycling pathway, which is a bulky system of the degradation of intracellular components. The autophagy inhibitor, 3MA was also used in the cell culture experimental assay. None of these inhibitors recovered the reduction of E-cadherin expression by HD-5 (Fig. 4A–C). Cadherin, which is isolated from calcium dependent adhesion molecules, is stabilized against

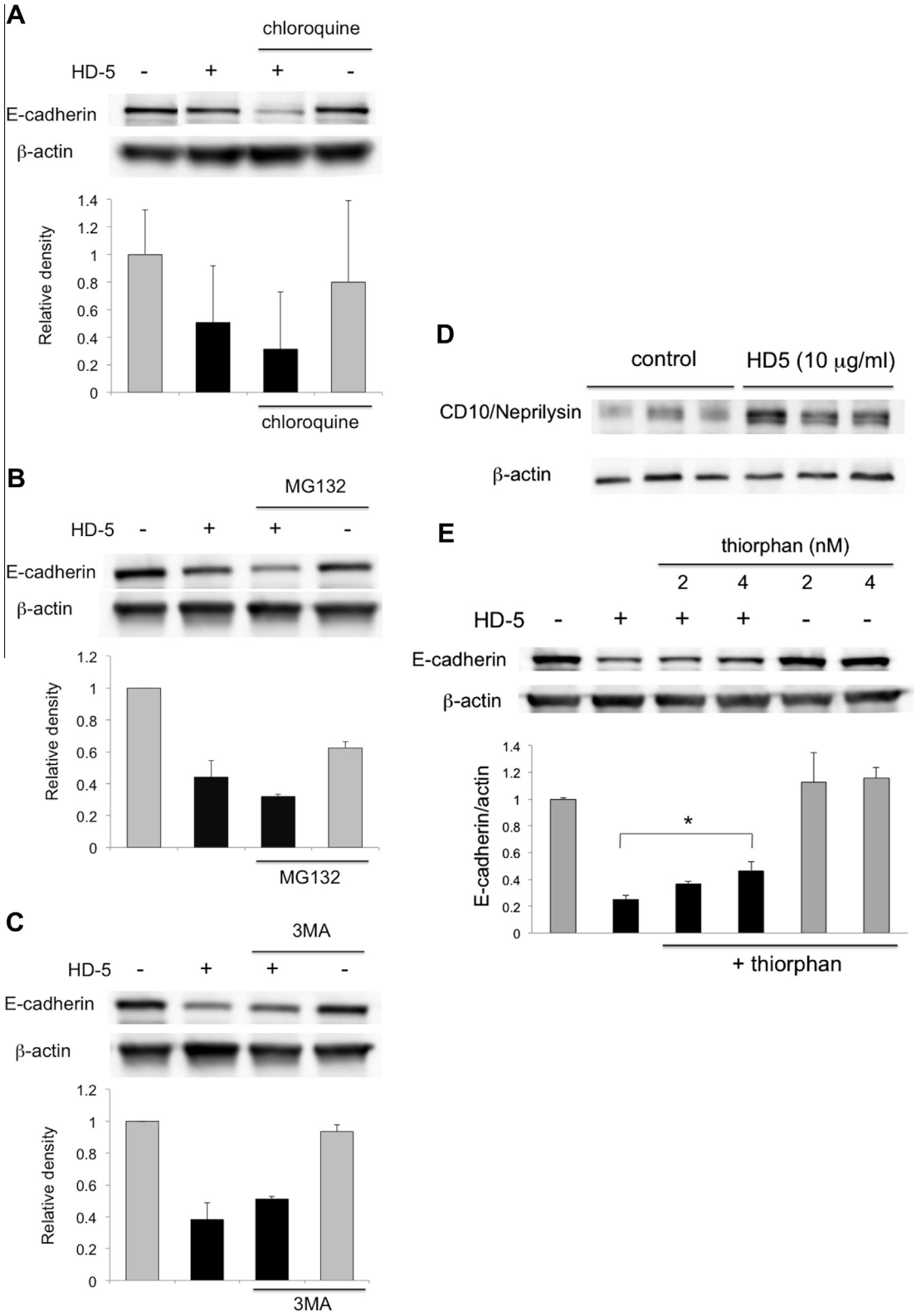

Fig. 4. Inhibition of E-cadherin degradations by the specific inhibitors. Protease inhibitor chloroquine (A), proteasome inhibitor MG-132 (B) or Autophagy inhibitor 3-MA (C) was added to the culture medium. None of the inhibitors rescued the E-cadherin reduction by HD-5. (D) A Western blotting analysis demonstrated the expression of the CD10/neprilysin protein in Het1A cells, and indicated that the level was up-regulated by HD-5 exposure. (E) Het1A cells were cultured with thiorphan, neprilysin specific inhibitor, and either treated with HD-5 or used as control. The E-cadherin degradation by HD-5 was partially blocked by thiorphan at the concentration of 4 nM. The E-cadherin expression was not returned to basal protein level by a higher concentration of the inhibitor. Error bars, mean ± SEM. $^{*}P < 0.05$.

proteinase cleavage when Ca^{2+} is present. Recovery from the reduction of E-cadherin was confirmed by the addition of calcium chloride (data not shown). An endopeptidase, neprilysin (EC 3.4.24.11) was expressed in Het1A cells and the protein expression was up-regulated by HD-5 exposure (Fig. 4D). Treatment with neprilysin inhibitor, thiorphan led to a partial recovery of the inhibition of E-cadherin protein expression induced by HD-5 (Fig. 4E).

4. Discussion

BE is characterized by the replacement of squamous epithelium in the esophagus with columnar epithelium, and is considered to be precursor to esophageal adenocarcinoma [15]. Therefore, it is important to investigate the clinicopathological alteration of the squamous-columnar epithelia. The current study investigated whether metaplastic Paneth cell-derived defensin altered the expression of the adherent junction molecule E-cadherin expression in *in vitro* analysis using immobilized normal squamous cell lines. Metaplastic Paneth cells are observed in the stomach under conditions of chronic gastritis, as well as in the esophagus of subjects with gastro-esophageal reflux disease (GERD) [11,16–18]. The cells are induced by *Helicobacter pylori* (*H. pylori*) infection in the stomach and the Paneth cell defensin reduces colonization of the bacteria [19]. The intestinal metaplasia in the stomach is, therefore, considered an adaptation response of the human being against external bacteria. Intestinal metaplasia in the esophagus might be an adaptation after injury or chronic inflammation of reflux esophagitis, as well as of gastritis in the stomach. One of the primary inducible factors of BE is bile acids, based on the rat reflux model the underwent gastrojejunostomy [20]. A mouse model that overexpressed interleukin-1β genes specifically in the esophagus has been developed very recently and it demonstrated that both bile acids and inflammation activate Barrett-like metaplasia [21]. The current observations indicated that epithelial injury in squamous cells at the gastro-esophageal junction is alternatively caused by the HD-5 that is produced by the metaplastic Paneth cells.

Enteric human defensins have been extensively studied as antimicrobial peptides, which eliminate microorganisms in the intestine, maintaining the homeostasis in the gut microenvironment. Members of the defensins contribute to not only innate immune mechanisms against microorganisms, but also to the acquired immunity in monocytic cells and/or epithelial cells [22,23]. In addition, β-defensins increase proliferation of epithelial cells and angiogenesis of vascular endothelial cells [24,25]. Human neutrophil defensins also increases respiratory epithelial cell proliferation and wound closure in the lung [26]. Neutrophil defensins mediate lung injury through loss of capillary-epithelial barrier functions [27]. The defensins disrupt capillary endothelial cells targeting low-density lipoprotein-related receptor and increase permeability in the lung of the transgenic mouse model. The current study predicted a novel role for defensins, wherein they disrupts E-cadherin protein during the regeneration of gastro-esophageal junction. Taken together, our finding indicate that defensin family proteins are potential mediators of many biological functions, including tissue repair, in addition to natural innate defense. The current limited results indicated the possibility that Barrett's metaplasia expansion to the distal esophagus was accelerated by the HD-5 produced from metaplastic Paneth cells. The pathological examination confirmed the correlation between defensin produced from metaplastic Paneth cells and existing squamous epithelial cells in the esophagus.

The expression of E-cadherin is frequently regulated mainly in two levels. One is the transcriptional level by Slug or Snail, and the other is degradation process in which ubiquitinated cadherin is transferred and degraded in the proteasomes [28]. Exposure of Het1A cells to HD-5 did not affect the expression of E-cadherin mRNA, and the protein expressions of Slug or Snail were stable. Expression level of E-cadherin was down-regulated in Western blotting and immunocytochemistry. Furthermore, the defensin disrupted β-catenin and actin filaments in direct or indirect manner. The experimental results suggested that CD10/neprilysin partially controlled the expression of E-cadherin by HD-5, though the detailed mechanisms were unclear. CD10/neprilysin is a widely expressed metalloendopeptidase, but there is no direct evidence that CD10/neprilysin cleaves E-cadherin [29,30]. Other regulatory pathways may also control the expression and functions of E-cadherin.

In conclusion, the current study found a novel functional role of human defensin. HD-5 produced by the Paneth cells reduced the expression of E-cadherin, and the reduction was post-translationally regulated in *in vitro* experiments using a squamous cell line. The metaplastic Paneth cell-derived defensin observed in gastro-esophageal junctions might alter the nature of esophageal squamous epithelium, and possibly accelerate the cascade of BE.

Contributors

Y.N. and K.M. started this project, designed the study, and performed experiments in molecular biology; H.T. analyzed data and wrote the paper; S.I. and A.I.Y. performed electro microscopy; K.A. and N.U. analyzed pathology samples and the data; S.K., M.T., T.G. performed cell culture and protein analysis, Y.I. and T.I. performed cell analysis; M.F. and Y.K. designed this study and wrote the paper.

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Appendix A. Supplementary data

Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.026.

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