Biochemical and Biophysical Research Communications 432 (2013) 412–417

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Stabilin-2 acts as an engulfment receptor for the phosphatidylserine-dependent clearance of primary necrotic cells

Shilpa D'Souza [a,1], Seung-Yoon Park [b,1], In-San Kim [a,c,*]

[a] Department of Biochemistry and Cell Biology, Cell and Matrix Research Institute, School of Medicine, Kyungpook National University, Daegu 700-422, Republic of Korea
[b] Department of Biochemistry, School of Medicine, Dongguk University and Medical Institute of Dongguk University, Gyeongju 780-714, Republic of Korea
[c] Biomedical Research Institute, Korea Institute Science and Technology, Seoul, Republic of Korea

ARTICLE INFO

Article history:
Received 22 January 2013
Available online 15 February 2013

Keywords:
Phagocytosis
Necrotic cells
Phosphatidylserine
Stabilin-2

ABSTRACT

Phosphatidylserine (PS) exposed by necrotic cells serves as an engulfment signal for their effective clearance. However, the molecular mechanism responsible for the PS-dependent clearance of necrotic cells remains to be investigated. Here, we show that stabilin-2 acts as a receptor for necrotic cell clearance. Stabilin-2-mediated necrotic cell engulfment occurred in a PS-dependent manner. EGF-like domain repeat (a PS-recognition domain of stabilin-2) bound to necrotic cells and inhibited the stabilin-2-mediated engulfment. However, primary necrotic cells did not induce the anti-inflammatory effect in stabilin-2-expressing cells. These findings facilitate the elucidation of the molecular mechanisms responsible for the PS-dependent clearance of necrotic cells.

1. Introduction

Discrimination of dying cells (apoptotic and necrotic cells) from healthy viable cells and their clearance by the immune system are important during development and tissue remodeling, because it allows the effects of the noxious contents from dying cells, which can damage neighboring tissues, to be managed [1]. Apoptosis, a programmed mode of cell death, is directed by well-regulated inherent genes [2]. Early during apoptosis, the exposure of phosphatidylserine (PS) on cell surfaces serves as a well-characterized 'eat me' signal for apoptotic cell engulfment [3]. Phagocytes recognize PS on apoptotic cell surfaces either through membrane PS-receptors (Tim-4, BAI1, and Stabilin-1 & 2) or soluble bridging molecules (MFG-E8 and Gas6) [4]. On the other hand, necrosis is induced by external baleful stimuli like injury, or exposure to toxins, extreme temperatures, or chemicals that interrupt some vital function or disrupt physical integrity (primary necrosis). Necrosis can also occur in a programmed, non-accidental manner, when the plasma membranes of apoptotic cells become permeabilized (secondary necrosis) [5]. Although several soluble proteins, such as, mannose-binding lectin, ficolin-2 and -3, C-reactive protein, serum amyloid protein, and histidine-rich glycoprotein act as opsonins for the clearance of late apoptotic (secondary necrosis) and primary necrotic cells [6], the engulfment signals of necrotic cell clearance have not been fully characterized. Accumulating evidences indicate that the redeployment of PS, which distinguishes viable cells from dying cells, is an earmark displayed by both apoptotic and necrotic cells and serves as an 'eat-me' signal for phagocytes [7–10]. However, the molecular mechanism responsible for PS-dependent clearance of necrotic cells remains to be investigated.

Stabilin-2 (also known as HARE and FEEL-2) is a type 1 transmembrane receptor that consists of seven FAS1 domains, four EGF-like domain repeats, and an Link domain, and is expressed in sinusoidal endothelial cells of the spleen, liver, and lymph nodes as well as in a macrophages subpopulation [11,12]. Stabilin-2 is involved in the clearance of metabolic waste products, which include hyaluronic acid, heparin, advanced glycation end products, and acetylated LDL [13–16]. Our previous study showed that stabilin-2 directly binds to PS, and that this binding mediates the clearance of PS-exposed RBCs and apoptotic cells and directs the secretion of anti-inflammatory cytokine TGF-β [12]. The EGF-like domain repeats (EGFrp) in stabilin-2 have been previously shown to represent the PS-recognition domain [17,18]. Previously, we also found that stabilin-1 and -2 participate in PS-dependent erythrophagocytosis in mouse liver [19]. It has been shown that apoptotic and necrotic targets share an array of specialized macrophage receptor systems, including the thrombospondin–CD36–αvβ3 complex, CD14, and the complement component C1q, for their efficient disposal [10]. In *Caenorhabditis elegans*, it was found that a common set of engulfment genes is involved in clearance of both apoptotic and necrotic cell corpses [20]. These observations

* Corresponding author at: Department of Biochemistry and Cell Biology, Cell and Matrix Research Institute, School of Medicine, Kyungpook National University, Daegu 700-422, Republic of Korea. Fax: +82 53 422 1466.
E-mail address: iskim@knu.ac.kr (I.-S. Kim).
[1] These authors contributed equally to this study.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.01.133

suggest that stabilin-2 could participate in the clearance of necrotic cells. Thus, in the present study, we investigated the involvement of stabilin-2 in the clearance of primary necrotic cells at the molecular level and in their immunomodulatory response to stabilin-2.

2. Materials and methods

2.1. Reagents and antibodies

Normal mouse IgG, anti-phospho-Erk1/2, and HRP-conjugated anti-His antibodies were obtained from Santa Cruz Biotechnology. Anti-NF-κB p65, anti-phospho-JNK, and anti-phospho-p38 antibodies were purchased from Cell Signaling. Anti-Fas (clone CH11) and anti-Phosphatidylserine (clone 1H6) antibodies were purchased from Upstate. Mouse monoclonal anti-human stabilin-2 antibody (5G3) was generated as previously described [12]. All phospholipids were purchased from Avanti Polar Lipids.

2.2. Cell culture

Mouse fibroblast L cells stably transfected with stabilin-2 expression vector (L/Stab2 cells) or empty vector (L/Mock cells) were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen) and 400 μg/mL G418, as previously described [12]. Jurkat T cells (human T cell lymphoma) were maintained in RPMI 1640 medium containing 10% FBS. Necrosis and apoptosis was induced in Jurkat cells by subjecting them to hyperthermic conditions (55 °C, 30 min) or by incubating them with anti-Fas antibody (250 ng/mL, 5 h), respectively. PS exposure and loss of membrane integrity were determined by Annexin V-FITC labeling and PI staining, respectively, and flow cytometry.

2.3. Phagocytosis of necrotic cells

Necrotic Jurkat cells (1.5×10^6 cells/well) were added to L/Mock or L/Stab2 cells cultured on 6-well collagen-coated plates (IWAKI) at an initial density of 3×10^5 cells/well, and incubated at 37 °C for 1 h in DMEM media containing 10% FBS. Cells were then extensively washed with serum-free DMEM media, to remove any bound, non-engulfed necrotic Jurkat cells, and fixed in methanol and stained using a Diff Quick staining kit (IMEB Inc.). Uptake of necrotic cells was visualized under the light microscope. To verify and quantify necrotic cell engulfment, necrotic Jurkat cells were labeled with FITC for 30 min and then added to L/Stab2 cells. After incubation for 1 h at 37 °C, unbound necrotic cells were removed by extensive washing with serum-free DMEM media. Fluorescence derived from non-engulfed, bound cells was quenched by treating cells with 0.4% trypan blue for 5 min at room temperature and washing with serum-free DMEM media. Cells engulfing FITC-labeled necrotic Jurkat cells were then quantified under a confocal microscope. Phagocytic indices were determined by calculating the average number of necrotic cells ingested per phagocyte. At least 100 cells were scored per well, and the experiment was repeated at least three times.

In some experiments, L/Stab2 cells were pre-incubated with anti-stabilin-2 antibody (20 μg/mL), isotype-matched control IgG (20 μg/mL), or phosphatidylcholine (PC) and PS liposomes for 1 h at 37 °C before adding FITC-labeled necrotic Jurkat target cells. PC liposomes (100% PC) and PS liposomes (a 50:50 M ratio of PS:PC) were generated as previously described [21]. To blockade PS on the surfaces of necrotic Jurkat cells, cells were pre-incubated with anti-PS antibody (20 μg/mL) or isotype-matched control IgG (20 μg/mL) and then incubated with L/Stab2 cells for 1 h at 37 °C.

2.4. Binding assays

His-tagged recombinant proteins corresponding to the third EGF-like domain repeat (EGFrp) of stabilin-2 were generated as previously described [17]. The binding assay was as previously described [22]. In brief, necrotic Jurkat cells (1×10^6 cells/mL) were incubated with thioredoxin-EGFrp or thioredoxin protein in RPMI medium containing 0.2% BSA and 5 mM $CaCl_2$ for 1 h at 4 °C. Cells were then washed with PBS (pH 7.4) and lysed in ice-cold lysis buffer [10 mM Tris–Cl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.5% SDS, 0.02% sodium azide, 1 mM PMSF]. Equal amounts of protein were then separated by SDS–PAGE on 12% gel. Amounts of EGFrp proteins associated with necrotic Jurkat cells were determined by immunoblotting with HRP-conjugated anti-His antibody.

2.5. TGF-β assays

L/Stab2 cells were seeded on a 24-well collagen-coated plate at an initial density of 5×10^4 cells/well. The next day viable, apoptotic, or necrotic Jurkat cells were added to L/Stab2 cells at a density of 2.5×10^5 cells/well and incubated at 37 °C under serum-free conditions. Supernatants were collected 18 h later and evaluated for TGF-β production by ELISA (R&D system).

2.6. Analysis of NF-κB translocation

L/Stab2 cells were seeded on 6-well plates at an initial density of 3×10^5 cells/well. The next day cells were stimulated with apoptotic or necrotic cells in the presence or absence of LPS (5 μg/mL) under serum-free conditions. After incubation for 1 h, cells were extensively washed to remove non-engulfed cells and harvested. Cytosolic and nuclear fractions were then isolated as previously described [23] and immunoblotted using anti-NF-κB p65 antibody. In some experiments, L/Stab2 cells were pre-incubated with U0126 (an Erk1/2 inhibitor, Cell Signaling) and then stimulated with apoptotic or necrotic cells.

2.7. MAPK phosphorylation

L/Stab2 and L/Mock cells were seeded on 6-well collagen-coated plates at an initial density of 3×10^5 cells/well. The next day cells were serum staved for 6 h, stimulated with necrotic or apoptotic Jurkat cells (1×10^6 cells) by incubation for 15 min, extensively washed with serum-free medium, and harvested. The cells were then lysed in cold lysis buffer for 30 min on ice. Identical amounts of total cell lysates were immunoblotted using anti-phospho-Erk1/2, phosphor-JNK, phosphor-p38, or anti-actin antibody. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) kit (Amersham).

2.8. Statistical analysis

All results shown are the averages of three independent experiments. Statistical significances were determined using the Student's *t*-test. Statistical significance was accepted for P values <0.05.

3. Results and discussion

3.1. Stabilin-2 expressing cells engulfed primary necrotic corpses

It is known that stabilin-2 acts as a receptor for the engulfment of PS-exposed RBCs and apoptotic cells in a PS-dependent manner [12]. To explore the ability of stabilin-2-expressing cells to engulf

primary necrotic cells, Jurkat cells were rendered necrotic by heating at 55 °C for 30 min. PS exposure and loss of membrane integrity in heat-induced necrotic cells were cytofluorometrically evaluated by staining with FITC-annexin V and propidium iodide (PI), respectively. The plasma membranes of most of viable Jurkat cells were intact and cells were negative for Annexin V staining, whereas 89% of heat-killed necrotic cells were membrane-permeable and PS was observed on cell surfaces (Fig. 1A). When necrotic Jurkat cells were presented to L cells stably transfected with human stabilin-2 cDNA (L/Stab2) or empty vector (L/Mock), the necrotic cells were internalized by L/Stab2 cells, which exhibited nuclear dislocation due to necrotic cell engulfment (black arrowheads) (Fig. 1B). In contrast, L/Mock cells showed no internalization of dying cells (Fig. 1B). To verify the engulfment of primary necrotic cells by stabilin-2-expressing cells, FITC-labeled necrotic cells were incubated with L/Stab2 or L/Mock cells, and the engulfments of FITC-labeled cells were analyzed by removing fluorescence from non-engulfed, bound necrotic cells using the trypan blue quenching technique. L/Stab2 cells were found to engulf FITC-labeled necrotic cells efficiently, whereas L/Mock cells did not (Fig. 1C). To quantify phagocytic activities in both cells, phagocytic index was determined by counting the number of FITC-labeled engulfed targets per phagocyte. The phagocytic index of L/Stab2 cells for necrotic cell engulfment was 0.31, whereas phagocytic index of L/Mock cells was only 0.01 (Fig. 1D). Both L/Stab2 and L/Mock cells showed only basal levels of viable Jurkat cell engulfment (Fig. 1D). The role of stabilin-2 as a receptor, during the engulfment of primary necrotic Jurkat cells, was further investigated by blocking stabilin-2 on the surfaces of L/Stab2 cells with an antibody previously determined to block stabilin-2 function [12]. Treatment with anti-stabilin-2 antibody significantly reduced the uptake of necrotic cells by approximately 50%, whereas an isotype-matched control IgG had no effect (Fig. 2A and B). These results show that stabilin-2 plays an active role in efficient clearance of heat-killed primary necrotic corpses.

3.2. Stabilin-2 mediated the engulfment of primary necrotic cells in a PS-dependent manner

The exposure of PS, an important recognition marker of apoptotic cells, is also an important feature of primary necrotic cells and contributes to necrotic cell clearance [8–10]. To determine whether stabilin-2-mediated engulfment of necrotic cells is PS-dependent, phagocytosis assays were carried out in the presence of PS liposomes or PC liposomes (the negative control). Treatment with PS liposomes caused a dose-dependent reduction in the uptake of necrotic cells by L/Stab2 cells, whereas PC liposomes failed to inhibit necrotic cell engulfment even at a higher concentration (Fig. 3A). We further tested the involvement of PS in the engulfment of necrotic targets by blocking the PS exposed on necrotic cells, using an anti-PS antibody. Treatment of necrotic cells with anti-PS antibody significantly reduced their uptake by L/Stab2 cells, whereas isotype-matched control IgG had no effect (Fig. 3B). A previous study showed that the EGF-like domain repeats (EGFrp) in stabilin-2 are responsible for the PS recognition, and thus, directs cell corpse clearance [17,18]. To determine whether necrotic cells interact with the PS-binding domain of stabilin-2, a binding assay was performed between necrotic cells and recombinant EGFrp proteins. As is shown in Fig. 3C, thioredoxin-EGFrp proteins bound to necrotic cells in a dose-dependent manner, whereas thioredoxin proteins did not. To determine whether the EGFrp proteins inhibit the stabilin-2-mediated uptake of

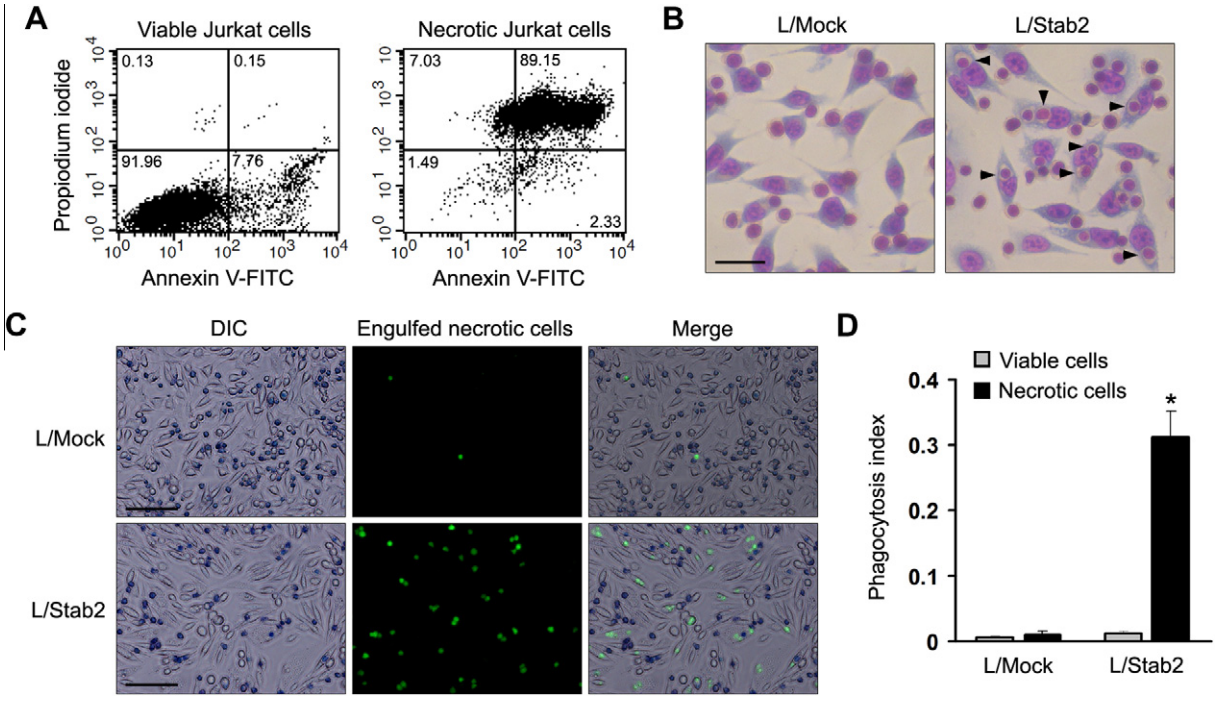

Fig. 1. Stabilin-2-expressing cells engulfed primary necrotic Jurkat cells (A) Flow cytometric analysis of membrane permeability and PS exposure in primary necrotic cells. Viable (untreated) and necrotic (heating at 55 °C) Jurkat cells were analyzed by flow cytometry for loss of membrane integrity and the accessibility of phosphatidylserine by staining with propidium Iodide (PI) and FITC-annexin V, respectively. (B) Necrotic Jurkat cells were added to L/Mock or L/Stab2 cells and then incubated for 1 h at 37 °C. After unbound necrotic cells were removed by washing, L/Mock or L/Stab2 cells were stained with a Diff Quick staining kit. Engulfed necrotic Jurkat cells (indicated by black arrowheads) were visualized under a light microscope. Scale bar, 25 μm. (C) Representative images of phagocytosis of FITC-labeled necrotic cells. L/Mock or L/Stab2 cells were incubated with FITC-labeled necrotic cells for 1 h at 37 °C. Necrotic cells engulfed in L/Mock or L/Stab2 cells are shown in green. Scale bar, 100 μm. (D) Microscopic quantification of necrotic cell phagocytosis in L/Mock and L/Stab2 cells. Phagocytic indices (number of ingested necrotic cells per phagocyte) were calculated. Results are means ± SD of three independent experiments. Students *t*-test: $^{*}P < 0.01$ versus L/Mock cells.

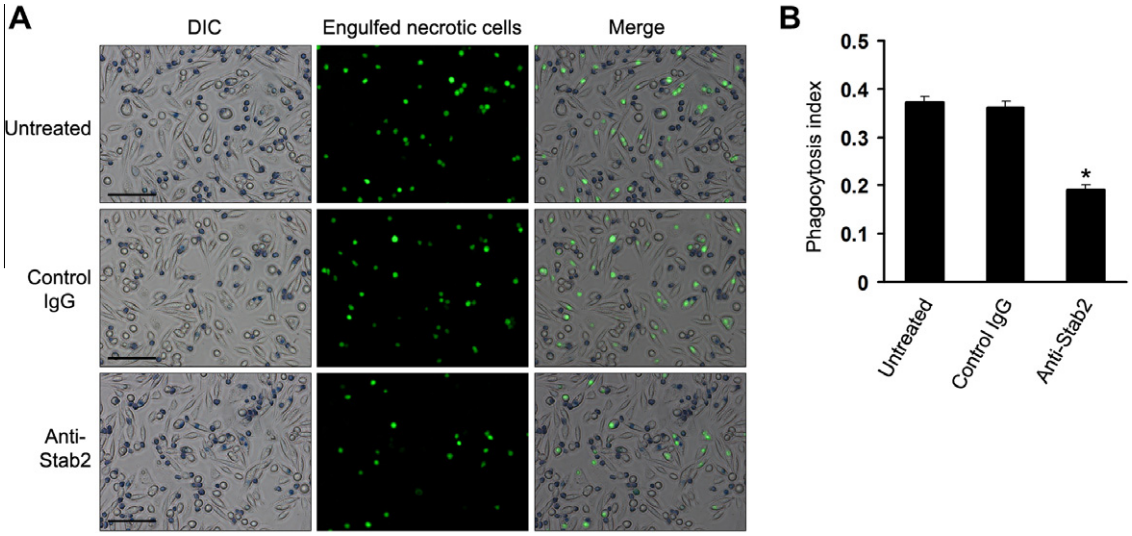

Fig. 2. Stabilin-2 was found to be involved in necrotic cell phagocytosis. (A) Representative images of necrotic cell engulfment by L/Stab2 cells following pretreatment with anti-stabilin-2 antibody or isotype-matched control IgG. Scale bar, 100 μm. (B) Microscopic quantification of necrotic cell phagocytosis in L/Stab2 cells pretreated with anti-stabilin-2 antibody or isotype-matched control IgG. Phagocytic indices were calculated. Results are means ± SD of three independent experiments. Students *t*-test: $^{*}P < 0.01$ versus untreated control.

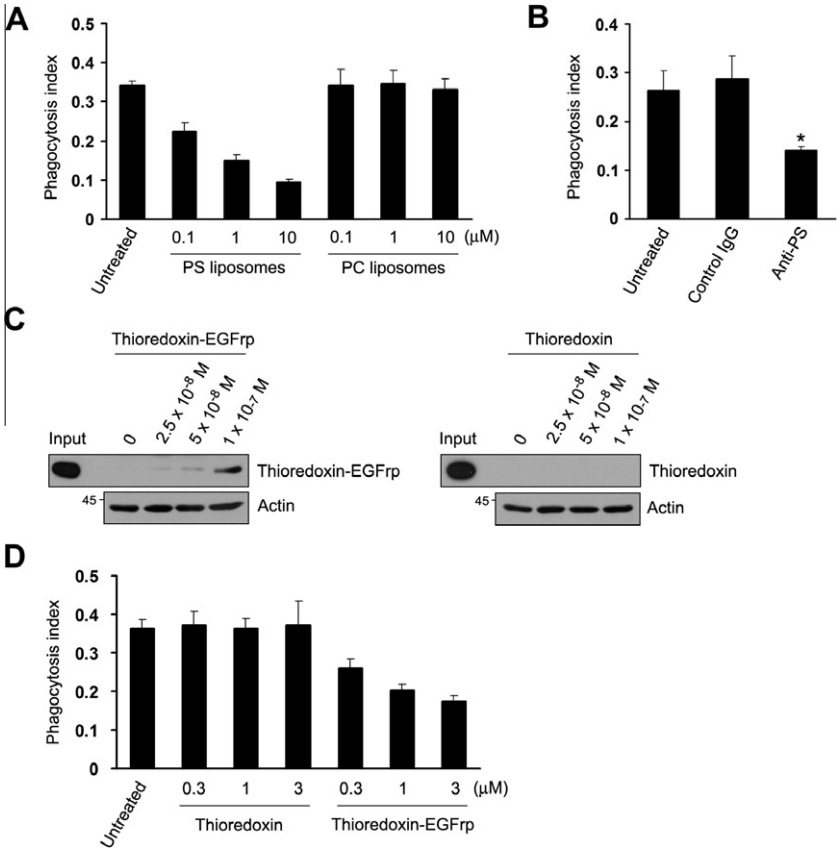

Fig. 3. Stabilin-2 mediated the engulfment of primary necrotic cells in a PS-dependent manner. (A) L/Stab2 cells were incubated with necrotic cells in the presence of PS or PC liposomes at various concentrations, and phagocytic indices were calculated. Results are expressed as means ± SD of three independent experiments. (b) L/Stab2 cells were incubated with necrotic cells pretreated with anti-PS antibody or isotype-matched control IgG, and phagocytic indices were determined. Results are means ± SD of three independent experiments. Students *t*-test: $^{*}P < 0.01$ versus untreated control. (C) Heat-killed necrotic Jurkat cells were incubated with the indicated amount of thioredoxin-tagged EGFrp or thioredoxin protein for 1 h at 4 °C. Thioredoxin protein was used as a control. After lysis, the amount of thioredoxin-tagged EGFrp protein associated with necrotic cells was determined by immunoblotting with HRP-conjugated anti-His antibody. A representative image of three independent experiments is shown. (D) Effect of EGFrp proteins on the engulfment of necrotic Jurkat cells by L/Stab2 cells. Necrotic cells were pre-incubated with three different concentrations of EGFrp protein and then added to L/Stab2 cells. After incubation for 1 h, the phagocytosis indices were calculated. Results are means ± SD of three independent experiments.

necrotic cells, phagocytosis assays were performed in the presence of EGFrp proteins. It was found that EGFrp proteins impeded the engulfment of necrotic Jurkat cells in a dose-dependent manner, whereas control thioredoxin proteins had no effect (Fig. 3D). These results demonstrate that heat-killed primary necrotic targets, like PS-exposed RBCs and apoptotic cells, are also recognized and engulfed via a similar mechanism, where exposed PS is engaged as a recognition ligand by stabilin-2 receptor.

3.3. Stabilin-2-mediated engulfment of primary necrotic cells did not induce an anti-inflammatory effect

Stabilin-2 receptor is known to have an anti-inflammatory effect, namely, the production of the anti-inflammatory cytokine TGF-β, in phagocytes presented with cell corpses [12]. To determine whether stabilin-2 induces similar signals in L/Stab2 cells in response to necrotic cells, TGF-β production was evaluated in L/Stab2 cells incubated with viable, apoptotic, and necrotic Jurkat cells. The results showed that only apoptotic Jurkat cells had the potential to evoke the production of TGF-β in L/Stab2 cells, whereas exposure to viable and necrotic Jurkat cells did not (Fig. 4A). This result suggests although stabilin-2 can direct the efficient clearance of both apoptotic and primary necrotic targets via PS recognition that the immune responses induced differ. One possible explanation for this is that the anti-inflammatory signal induced by stabilin-2 is abrogated by proinflammatory mediators released by the heat-killed necrotic cells. To examine effect of necrotic cells on the activation of NF-κB p65 (a mediator of inflammatory responses), the nuclear translocation of NF-κB p65 was examined after treatment of apoptotic and necrotic cells. As shown in Fig. 4B, necrotic cells, but not apoptotic cells, induced the nuclear translocation of p65 in L/Stab2 cells (Fig. 4B, left panels). Furthermore, whereas apoptotic cells inhibited p65 translocation in LPS-stimulated L/Stab2 cells, necrotic cells had no effect (Fig. 4B, right panels). To elucidate the signal transduction initiated in response to necrotic cells, the phosphorylation of Erk1/2, p38 MAPK, and JNK were examined in L/Mock and L/Stab2 cells treated with necrotic cells. The phosphorylation of Erk1/2 was increased in L/Stab2 and L/Mock cells by necrotic cells (Fig. 4C), indicating that necrotic cells induced Erk1/2 activation irrespective of stabilin-2. In addition, treatment with U0126 (an Erk1/2 inhibitor) prevented the nuclear translocation of NF-κB p65 by necrotic cells (Fig. 4D), indicating that primary necrotic cells induce proinflammatory signal via Erk1/2 activation. These results suggest that proinflammtory signal via Erk1/2 activation may abrogate anti-inflammatory signals resulting from PS recognition by stabilin-2.

Growing evidences indicate that the externalization of PS serves as an 'eat-me' signal for apoptotic and necrotic cell clearance [7–10]. However, the molecular mechanism responsible for PS-dependent clearance of necrotic cells remains to be investigated. The present study provides evidence that stabilin-2 utilizes PS as a ligand for the efficient engulfment of primary necrotic targets. First, PS liposomes, but not PC liposomes, were found to block necrotic cell engulfment by stabilin-2-expressing cells. Second, blockade of PS on the surfaces of primary necrotic cells with anti-PS antibody caused a significant reduction in the engulfment of necrotic cells. Third, EGFrp proteins directly interacted with necrotic cells and inhibited stabilin-2-mediated necrotic cell clearance. These results indicate that irrespective of type of death, stabilin-2-mediated corpse clearance is predominantly dependent on PS exposure by dying targets. However, the blockade of stabilin-2 or PS on cell surfaces did not completely inhibit necrotic cell clearance, which suggests the existence of a PS-independent mechanism(s) of necrotic cell clearance. Considering that our experiments were conducted in the presence of 10% FBS, it is possible that another mechanism of necrotic cell engulfment also exist in L/Stab2 cells. Recently, we reported that cross talk between stabilin-2 and integrin αvβ5 orchestrates the engulfment of PS-exposed RBCs in L/Stab2 cells [24]. Given that integrin αvβ5 is involved in apoptotic cell clearance via its binding to the opsonin MFG-E8 [25], it is possible that the integrin αvβ5–stabilin-2 complex recognizes some opsonin, and thereby, mediates necrotic cell engulfment.

In this study, although stabilin-2 was found to interact directly with the PS of heat-killed necrotic cells, stabilin-2 failed to invoke an anti-inflammatory effect. This result is consistent with previous

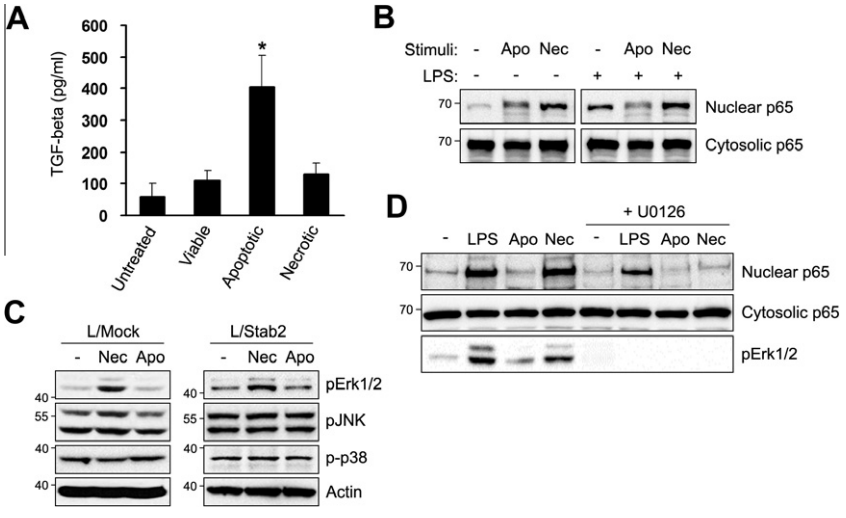

Fig. 4. Stimulation of Stabilin-2 by necrotic or apoptotic Jurkat target cells, elicited differential signals. (A) L/Stab2 cells were challenged with viable, apoptotic, or necrotic Jurkat cells. Supernatants were collected 18 h later and the concentrations of TGF-β released were determined by ELISA. Results are means ± SD of at least three independent experiments. Students *t*-test: $^{*}P < 0.01$ versus untreated control. (B) L/Stab2 cells were stimulated with necrotic (Nec) or apoptotic (Apo) Jurkat cells in the absence or presence of LPS (5 μg/mL) for 1 h. Cytosolic and nuclear fractions of cell lysates were probed with anti-NF-κB p65. The image shown is representative of three independent experiments. (C) L/Mock and L/Stab2 cells were stimulated with necrotic (Nec) or apoptotic (Apo) Jurkat targets for 15 min. Non-ingested necrotic targets were removed by thorough washing. Cell lysates were probed with anti-phospho-Erk1/2, anti-phospho-JNK, anti-phosopho-p38, or anti-actin antibodies. The image shown is representative of three independent experiments. (D) L/Stab2 cells were preincubated with U0126 (10 μM) for 2 h and then stimulated with LPS (5 μg/mL), necrotic cells (Nec), or apoptotic cells (Apo) for 1 h. Non-ingested necrotic targets were removed by thorough washing. Cytosolic and nuclear fractions of cell lysates were probed with anti-NF-κB p65. The image shown is representative of three independent experiments.

observations that heat-killed necrotic cells did not induce an anti-inflammatory effect in macrophages, such as, the inhibition of pro-inflammatory cytokine production [7,9,26]. In the present study, we found that primary necrotic cells induced proinflammatory signals via Erk1/2 activation, irrespective of stabilin-2, which suggests that anti-inflammatory signals from PS recognition by stabilin-2 may be abrogated by proinflammatory signals from necrotic cells. However, it is unclear at present whether stabilin-2 activates different signal transductions by recognizing PS on apoptotic or necrotic cells. Further study is required to identify the various signaling molecules released by necrotic cells, as these might interact with stabilin-2 receptor and influence recognition, engulfment, and immunomodulatory signals.

Summarizing, our results show that stabilin-2 acts as a PS-receptor during the PS-dependent clearance of primary necrotic cells. We hope that our findings will facilitate the elucidation of the molecular mechanism responsible and signaling pathway involved in necrotic cell clearance via PS recognition.

Acknowledgments

This study was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) (2010-0029206); by the Converging Research Center Program through the Ministry of Education, Science, and Technology (2010K001054); by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science, and Technology (2011-0016102); and by the Brain Korea 21 Project.

References

[1] L.M. Stuart, R.A. Ezekowitz, Phagocytosis: elegant complexity, Immunity 22 (2005) 539–550.
[2] M.O. Hengartner, Apoptosis: corralling the corpses, Cell 104 (2001) 325–328.
[3] R.A. Schlegel, P. Williamson, Phosphatidylserine, a death knell, Cell Death Differ. 8 (2001) 551–563.
[4] M.R. Elliott, K.S. Ravichandran, Clearance of apoptotic cells: implications in health and disease, J. Cell Biol. 189 (2010) 1059–1070.
[5] W.X. Zong, C.B. Thompson, Necrotic death as a cell fate, Genes Dev. 20 (2006) 1–15.
[6] I.K. Poon, M.D. Hulett, C.R. Parish, Molecular mechanisms of late apoptotic/necrotic cell clearance, Cell Death Differ. 17 (2010) 381–397.
[7] R.E. Cocco, D.S. Ucker, Distinct modes of macrophage recognition for apoptotic and necrotic cells are not specified exclusively by phosphatidylserine exposure, Mol. Biol. Cell 12 (2001) 919–930.
[8] G. Brouckaert, M. Kalai, D.V. Krysko, X. Saelens, D. Vercammen, M.N. Ndlovu, G. Haegeman, K. D'Herde, P. Vandenabeele, Phagocytosis of necrotic cells by macrophages is phosphatidylserine dependent and does not induce inflammatory cytokine production, Mol. Biol. Cell 15 (2004) 1089–1100.
[9] U.A. Hirt, M. Leist, Rapid, noninflammatory and PS-dependent phagocytic clearance of necrotic cells, Cell Death Differ. 10 (2003) 1156–1164.
[10] A. Bottcher, U.S. Gaipl, B.G. Furnrohr, M. Herrmann, I. Girkontaite, J.R. Kalden, R.E. Voll, Involvement of phosphatidylserine, alphavbeta3, CD14, CD36, and complement C1q in the phagocytosis of primary necrotic lymphocytes by macrophages, Arthritis Rheum. 54 (2006) 927–938.
[11] M. Falkowski, K. Schledzewski, B. Hansen, S. Goerdt, Expression of stabilin-2, a novel fasciclin-like hyaluronan receptor protein, in murine sinusoidal endothelia, avascular tissues, and at solid/liquid interfaces, Histochem. Cell Biol. 120 (2003) 361–369.
[12] S.Y. Park, M.Y. Jung, H.J. Kim, S.J. Lee, S.Y. Kim, B.H. Lee, T.H. Kwon, R.W. Park, I.S. Kim, Rapid cell corpse clearance by stabilin-2, a membrane phosphatidylserine receptor, Cell Death Differ. 15 (2008) 192–201.
[13] B. Zhou, J.A. Weigel, L. Fauss, P.H. Weigel, Identification of the hyaluronan receptor for endocytosis (HARE), J. Biol. Chem. 275 (2000) 37733–37741.
[14] H. Adachi, M. Tsujimoto, FEEL-1, a novel scavenger receptor with in vitro bacteria-binding and angiogenesis-modulating activities, J. Biol. Chem. 277 (2002) 34264–34270.
[15] Y. Tamura, H. Adachi, J. Osuga, K. Ohashi, N. Yahagi, M. Sekiya, H. Okazaki, S. Tomita, Y. Iizuka, H. Shimano, R. Nagai, S. Kimura, M. Tsujimoto, S. Ishibashi, FEEL-1 and FEEL-2 are endocytic receptors for advanced glycation end products, J. Biol. Chem. 278 (2003) 12613–12617.
[16] E.N. Harris, J.A. Weigel, P.H. Weigel, The human hyaluronan receptor for endocytosis (HARE/Stabilin-2) is a systemic clearance receptor for heparin, J. Biol. Chem. 283 (2008) 17341–17350.
[17] S.Y. Park, S.Y. Kim, M.Y. Jung, D.J. Bae, I.S. Kim, Epidermal growth factor-like domain repeat of stabilin-2 recognizes phosphatidylserine during cell corpse clearance, Mol. Cell. Biol. 28 (2008) 5288–5298.
[18] S. Kim, D.J. Bae, M. Hong, S.Y. Park, I.S. Kim, The conserved histidine in epidermal growth factor-like domains of stabilin-2 modulates pH-dependent recognition of phosphatidylserine in apoptotic cells, Int. J. Biochem. Cell Biol. 42 (2010) 1154–1163.
[19] S.J. Lee, S.Y. Park, M.Y. Jung, S.M. Bae, I.S. Kim, Mechanism for phosphatidylserine-dependent erythrophagocytosis in mouse liver, Blood 117 (2008) 5215–5223.
[20] S. Chung, T.L. Gumienny, M.O. Hengartner, M. Driscoll, A common set of engulfment genes mediates removal of both apoptotic and necrotic cell corpses in *C. elegans*, Nat. Cell Biol. 2 (2000) 931–937.
[21] S.Y. Park, M.Y. Jung, S.J. Lee, K.B. Kang, A. Gratchev, V. Riabov, J. Kzhyshkowska, I.S. Kim, Stabilin-1 mediates phosphatidylserine-dependent clearance of cell corpses in alternatively activated macrophages, J. Cell Sci. 122 (2009) 3365–3373.
[22] M.Y. Jung, S.Y. Park, I.S. Kim, Stabilin-2 is involved in lymphocyte adhesion to the hepatic sinusoidal endothelium via the interaction with {alpha}M{beta}2 integrin, J. Leukoc. Biol. 82 (2007) 1156–1165.
[23] S.Y. Park, D.J. Bae, M.J. Kim, M.L. Piao, I.S. Kim, Extracellular low pH modulates phosphatidylserine-dependent phagocytosis in macrophages by increasing stabilin-1 expression, J. Biol. Chem. 287 (2012) 11261–11271.
[24] S. Kim, S.Y. Park, S.Y. Kim, D.J. Bae, J.H. Pyo, M. Hong, I.S. Kim, Cross talk between engulfment receptors stabilin-2 and integrin alphavbeta5 orchestrates engulfment of phosphatidylserine-exposed erythrocytes, Mol. Cell. Biol. 32 (2012) 2698–2708.
[25] S. Akakura, S. Singh, M. Spataro, R. Akakura, J.I. Kim, M.L. Albert, R.B. Birge, The opsonin MFG-E8 is a ligand for the alphavbeta5 integrin and triggers DOCK180-dependent Rac1 activation for the phagocytosis of apoptotic cells, Exp. Cell Res. 292 (2004) 403–416.
[26] V.A. Fadok, D.L. Bratton, L. Guthrie, P.M. Henson, Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases, J. Immunol. 166 (2001) 6847–6854.