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Suppression of MIP-2 or IL-8 production by annexins A1 and A4 during coculturing of macrophages with late apoptotic human peripheral blood neutrophils

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ABSTRACT

Annexin A1 (ANXA1) is a well-known anti-inflammatory protein that is expressed on the surface of apoptotic cells. Annexin A4 (ANXA4) is also recruited from the nucleus to the cytoplasm in apoptotic cells, although it is not known whether or not ANXA4 is expressed on the surface of apoptotic cells. In this study, we obtained rabbit anti-human ANXA1 and ANXA4 antibodies, and then examined whether or not ANXA1 and ANXA4 are expressed on the surface of early and late human apoptotic cells. ANXA1 and, to a lesser extent, ANXA4 were detected on late but not early apoptotic HeLa cells, whereas ANXA1 and a small amount of ANXA4 were detected on both early and late apoptotic human neutrophils. We then examined the effects of the anti-human ANXA1 and ANXA4 antibodies on the mouse or human macrophage response to human apoptotic cells. Upon coculturing of mouse or human macrophages with late apoptotic human neutrophils, anti-human ANXA1 antibodies and, to a lesser extent, anti-human ANXA4 antibodies increased MIP-2 or IL-8 production significantly, suggesting that ANXA1 and ANXA4 suppress MIP-2 or IL-8 production by macrophages in response to late apoptotic human neutrophils.

1. Introduction

Apoptotic cells are cleared by phagocytes as soon as they appear in vivo. If phagocytosis is impaired for some reason or if apoptotic cells outnumber phagocytes, apoptotic cells become late apoptotic or secondary necrotic ones, and then secrete their intracellular contents, thereby causing an inflammatory response, such as neutrophil infiltration [1]. Consistent with this notion, we previously showed that coculturing of human and mouse macrophages with late apoptotic cells induced the production of IL-8 and MIP-2, a mouse homolog of IL-8, one of the chemokines specific to neutrophils [2,3]. This is also true in vivo, because whole body X-irradiation induces massive apoptosis in the thymus, thereby causing MIP-2 production by macrophages and neutrophil infiltration into the thymus [4].

Apoptotic cells express phosphatidylserine (PS) on their surface, through which phagocytes recognize apoptotic cells [5]. PS is thus the key molecule for the clearance of apoptotic cells by phagocytes. In support of this, it has been reported that annexin V inhibits the phagocytosis of apoptotic cells by blocking PS on the apoptotic cells [6]. Phagocytes recognize apoptotic cells either directly via a PS receptor such as TIM-4 [7] or indirectly via bridging molecules such as milk fat globule epidermal growth factor 8 [8].

Annexin A1 (ANXA1), also called lipocortin, is a member of the annexin family that binds to PS in a calcium-dependent manner and is known as an anti-inflammatory molecule [9]. Recently, it was reported that ANXA1 is expressed on the surface of apoptotic cells and assists in the clearance by macrophages [10–12]. ANXA1 consists of two domains, a core domain exhibiting high affinity toward PS and an N terminal one exhibiting high affinity toward the formyl peptide receptor [13]. It is thus hypothesized that ANXA1 is best suited for facilitating phagocytosis because of these two domains. However, there has been another study showing that ANXA1 is expressed on late apoptotic cells but does not facilitate clearance by macrophages [14]. Annexin A4 (ANXA4), another member of the annexin family, is reported to show a change in subcellular localization from the nucleus to the cytoplasm during apoptosis [15], although it is not known whether or not ANXA4 is expressed on apoptotic cells. Moreover, the roles of ANXA1 and ANXA4 in a pro-inflammatory response during coculturing of macrophages with apoptotic cells remain largely unexplored except for one study in which the effect of phagocytosis of late apoptotic cells on LPS-induced production of inflammatory cytokines was examined [14].

In this study, we therefore examine whether or not ANXA1 and ANXA4 are expressed on the surface of apoptotic cells, and whether or not ANXA1 and ANXA4 suppress MIP-2 or IL-8 production during coculturing of macrophages with apoptotic cells.

Abbreviations: PS, phosphatidylserine; ANXA1, annexin A1; ANXA4, annexin A4; GST, glutathione-S-transferase; DTT, dithiothreitol; PI, propidium iodide

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2. Materials and methods

2.1. Preparation of human ANXA1 and ANXA4

The glutathione-S-transferase (GST) fusion protein expression vectors, pGEX-6P-1-human ANXA1 and pGEX-6P-1-human ANXA4, were provided by Dr. Isamu Matsumoto of Ochanomizu University. Each vector was introduced into *E. coli* JM109 cells. The transformed cells were cultivated in 500 ml of LB broth (Invitrogen) containing 50 μg/ml of ampicillin. When the absorbance of the cell culture at 600 nm reached approximately 1.0, expression was induced by the addition of 1 mM isopropyl-ß-D-thiogalactopyranoside. The cells were cultured overnight at 37 °C with constant shaking, and then harvested as a pellet by centrifugation. The pellet was resuspended in PBS containing 2 mM EDTA, 0.1 mM dithiothreitol (DTT), 1 μg/ml aprotinin, 1 μg/ml α-anti trypsin, 0.01 μg/ml soybean trypsin inhibitor, 1 μg/ml leupeptin and 1 μg/ml pepstatin A. After Triton X-100 had been added to 0.75%, the cells were solubilized by freezing–thawing (twice) and sonication (ten times). After centrifugal separation, the supernatant was collected as the soluble fraction and mixed with Sepharose 4B beads (GE Healthcare), which had been equilibrated with PBS. This mixture was rotated for 6 h at 4 °C, followed by centrifugation. The supernatant was then mixed with glutathione-Sepharose 4B beads (GE Healthcare), which had been equilibrated with PBS. This mixture was rotated at 4 °C overnight. The beads were then washed with PBS five times, and resuspended in 50 mM Tris–HCl (pH 7.0) containing 150 mM NaCl, 1 mM EDTA and 1 mM DTT (cleavage buffer). This suspension was then loaded onto a Poly-Prep chromatography column (Bio-Rad), followed by the addition of the cleavage buffer containing PreScission protease and incubation for 4 h at 4 °C. After centrifugal separation, the supernatant was collected and dialyzed against PBS.

2.2. Immunization and preparation of antibodies

Japanese White rabbits, which had been purchased from Sankyo Lab Service (Tokyo), were immunized with 500 μg aliquots of antigen preparations in Freund's complete adjuvant (Difco). Four weeks later, booster immunizations were performed with 500 μg of antigen in Freund's incomplete adjuvant (Difco) every two weeks. Whole blood was obtained and then subjected to incubation at 37 °C for 1 h and at 4 °C overnight, followed by centrifugation. Serum was mixed with an equal volume of 60 mM sodium acetate buffer (pH 4.0) and a 0.04 volume of octanoic acid, followed by stirring for 30 min at r.t. and centrifugation. The supernatant was then precipitated with ammonium sulfate.

2.3. Western blotting analysis

The recombinant antigen proteins and HeLa cell lysates were subjected to 0.1% SDS–12.5% polyacrylamide gel electrophoresis and

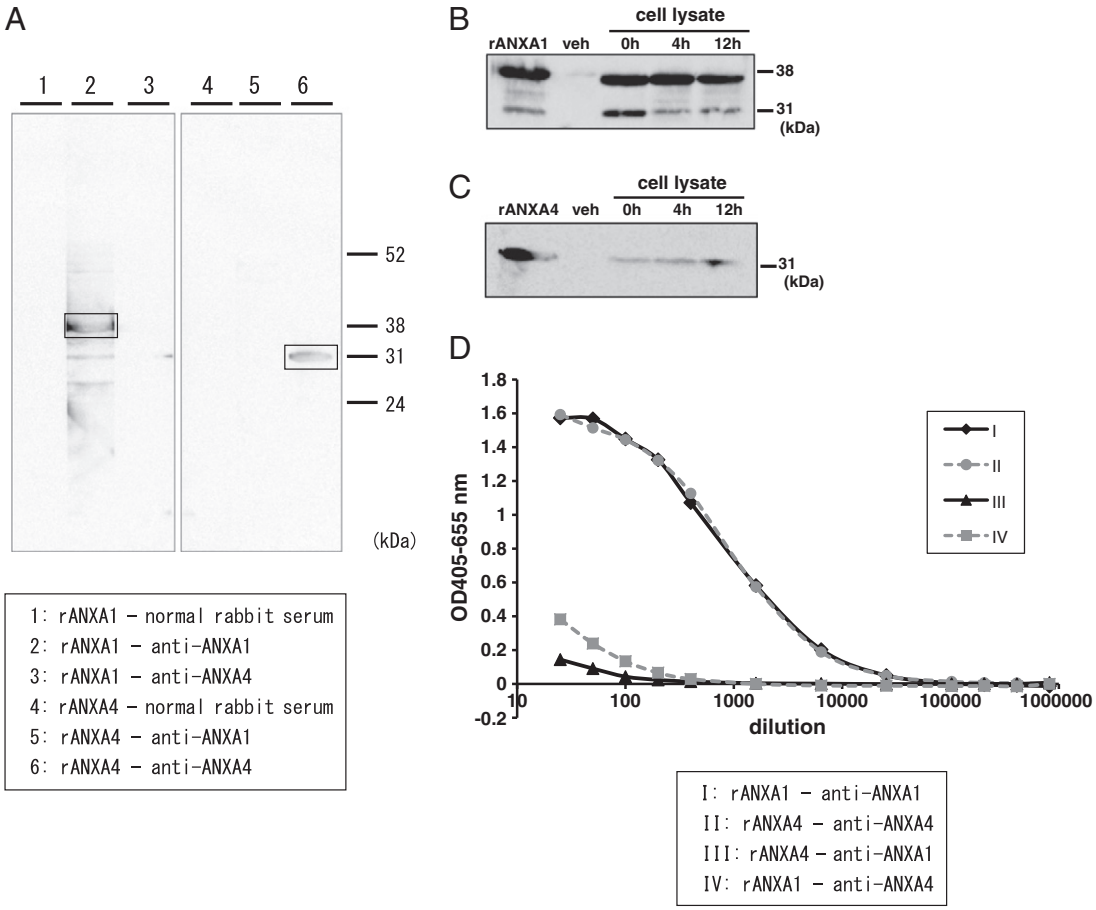

Fig. 1. Characterization of anti-human ANXA1 and anti-human ANXA4 antibodies. (A) Recombinant human ANXA1 and ANXA4, 0.5 μg each, were subjected to SDS PAGE, followed by Western blotting with anti-human ANXA1 and anti-human ANXA4 antibodies. Boxes show intact human ANXA1 and human ANXA4, respectively. Other bands in lane 2 presumably represent fragments of human ANXA1. (B, C) HeLa cells, at a cell density of 2×10^5 cells/ml, were irradiated with UV at 230 mW/cm^2 (total dose of 50 mJ/cm^2), followed by culturing for 4 or 12 h and preparation of cell lysates with a lysis buffer containing 20 mM Tris, 4 mM EDTA, 2 mM EGTA and 1% NP-40 (pH. 7.4). The cell lysates from 25×10^4 cells were subjected to SDS PAGE, followed by Western blotting with anti-human ANXA1 and anti-human ANXA4 antibodies. veh, buffer control. (D) Recombinant human ANXA1 and ANXA4 at 100 ng/ml were added to a 96 well microplate, followed by addition of serially diluted anti-human ANXA1 and anti-human ANXA4 antibodies whose original concentration was 1.3 mg/ml. Then HRP-conjugated goat anti-rabbit IgG was added to each well, followed by addition of the substrate.

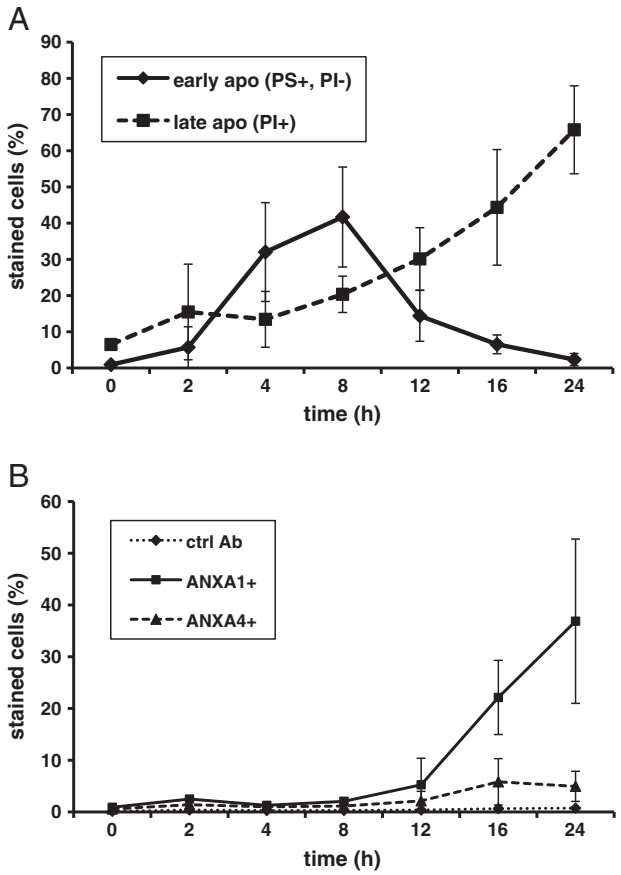

Fig. 2. Surface expression of PS, ANXA1 and ANXA4 during culturing of UV-irradiated HeLa cells. HeLa cells, at a cell density of 2×10^5 cells/ml, were irradiated with UV at 230 mW/cm^2 (total dose of 50 mJ/cm^2), followed by culturing. Surface expression of PS (A), ANXA1 and ANXA4 (B) was analyzed with a flow cytometer according to the methods described under Materials and Methods. The results in (A) are expressed as the percentages of early (PS^+, PI^-) or late (PI^+) apoptotic cells, and all the results are expressed as means ± standard error. n = 3–6.

then transferred to PVDF membranes (Hybond-P; Amersham Bioscience). The membranes were treated with a 2% skim milk solution. After washing of the membranes with 0.05% Tween-PBS, the antigens were detected with rabbit anti-human ANXA1 or ANXA4 antibodies, HRP-conjugated goat anti-rabbit IgG (Invitrogen), and ECL Western blotting detection reagents (Amersham Bioscience).

2.4. Induction of apoptosis

HeLa cells were seeded onto a plastic dish at a cell density of 5×10^4 cells/ml, and then incubated overnight. After washing with PBS three times, the cells were irradiated with UV at a dose of 230 mW/cm^2 for 217 s (50 mJ/cm^2). The cells were then incubated at 37 °C for various times.

Human peripheral blood neutrophils were isolated from heparinized venous blood from volunteers with informed consent according to the standard method. Briefly, peripheral blood was mixed with Dextran solution at a final concentration of 1%, followed by centrifugation and hemolysis. The cells were then subjected to Ficoll density gradient. The cells were suspended in RPMI1640 medium containing 7% FCS at a cell density of 5×10^5 cells/ml, and then incubated at 37 °C for various times.

2.5. Flow cytometric analysis

The HeLa cells or human neutrophils were centrifuged, and the cell pellet was incubated with FITC-labeled annexin V and propidium iodide (PI) for 5 min at r.t. After washing with PBS, the cells were suspended in a sheath solution (Iso Flow) (Beckman Coulter) and filtered through a mesh, followed by flow cytometric analysis with FACSCalibur.

The HeLa cells or human neutrophils were centrifuged and the cell pellet was incubated with human Fc receptor blocking reagent (Clear Back) (MBL, Nagoya) for 5 min at r.t. The cells were then incubated with 0.5 μg anti-human ANXA1 antibodies, anti-human ANXA4 antibodies, or control normal IgG (TOYOBO) for 30 min on ice, followed by washing with PBS containing 0.5% BSA and 0.01% NaN_3 (staining buffer) twice and further incubation with 0.5 μg biotinylated goat anti-rabbit IgG F(ab′)$_2$ fragment (American Qualex). The cells were then washed, and incubated with streptavidin-PE for 30 min on ice. After washing with the staining buffer, the cells were suspended in the sheath solution (Iso Flow) and filtered through a mesh, followed by flow cytometric analysis with FACSCalibur.

2.6. Coculturing of mouse peritoneal macrophages or human macrophages with apoptotic cells

Mouse peritoneal cells were obtained from CDF1 mice (Sankyo Lab Service) by peritoneal lavage, and then centrifuged. The cells were then suspended in RPMI1640 medium containing 7% FCS, allowed to adhere to each well of a 96 well plate at 2×10^5 cells/well, incubated for 3 h at 37 °C, and washed with PBS three times. Approximately half of the cells were obtained as macrophages, as judged on staining with a FITC-labeled anti-F4/80 monoclonal antibody.

Human macrophages were obtained from peripheral blood monocytes according to the method [16] by using recombinant human M-CSF (PeproTech, USA) instead of human urine-derived M-CSF.

Apoptotic HeLa cells or human neutrophils were then added to mouse peritoneal macrophages or human macrophages in the ratio of 1:1, followed by incubation for 24 h at 37 °C. The supernatants were then harvested, and stored at −30 °C until assaying. The MIP-2 or IL-8 levels in the supernatants were determined with specific ELISAs (PeproTech, USA).

2.7. Confocal microscopy

Apoptotic HeLa cells were fixed with neutral buffered 2% formaldehyde (Nakarai, Kyoto) for 20 min at r.t. The cells were then washed with the staining buffer, followed by permeabilization with PBS containing 0.2% digitonin, 0.5% BSA and 0.01% NaN_3 for 5 min on ice and washing with the staining buffer twice. The cells were then stained with anti-human ANXA1 or ANXA4 antibodies and further incubated with biotinylated goat anti-rabbit IgG F(ab′)$_2$ fragment. The cells were then incubated with streptavidin-FITC for 30 min on ice, and cytospun onto a slide glass, followed by examination under a confocal microscope (FluoView FV1000) (Olympus, Tokyo).

3. Results

3.1. Surface expression of PS, ANXA1 and ANXA4 during culturing of UV-irradiated HeLa cells

We prepared recombinant human ANXA1 and ANXA4 to raise antibodies against these proteins in rabbits. We then characterized the antibodies as shown in Fig. 1. Each antibody was specific to human ANXA1 or ANXA4, respectively, as demonstrated with recombinant human ANXA1 and ANXA4 as well as HeLa cell lysates (Fig. 1A, B and C). Moreover, these antibodies possessed fairly high titers to each antigen (Fig. 1D).

We then examined cell surface expression of PS, ANXA1 and ANXA4 on apoptotic HeLa cells by flow cytometry according to the methods described under Materials and methods (Fig. 2). For this purpose, apoptotic HeLa cells were obtained by UV irradiation and subsequent incubation for various times. As shown in Fig. 2A, PS was transiently expressed, the peak being at 8 h. In contrast, ANXA1 and, to a lesser

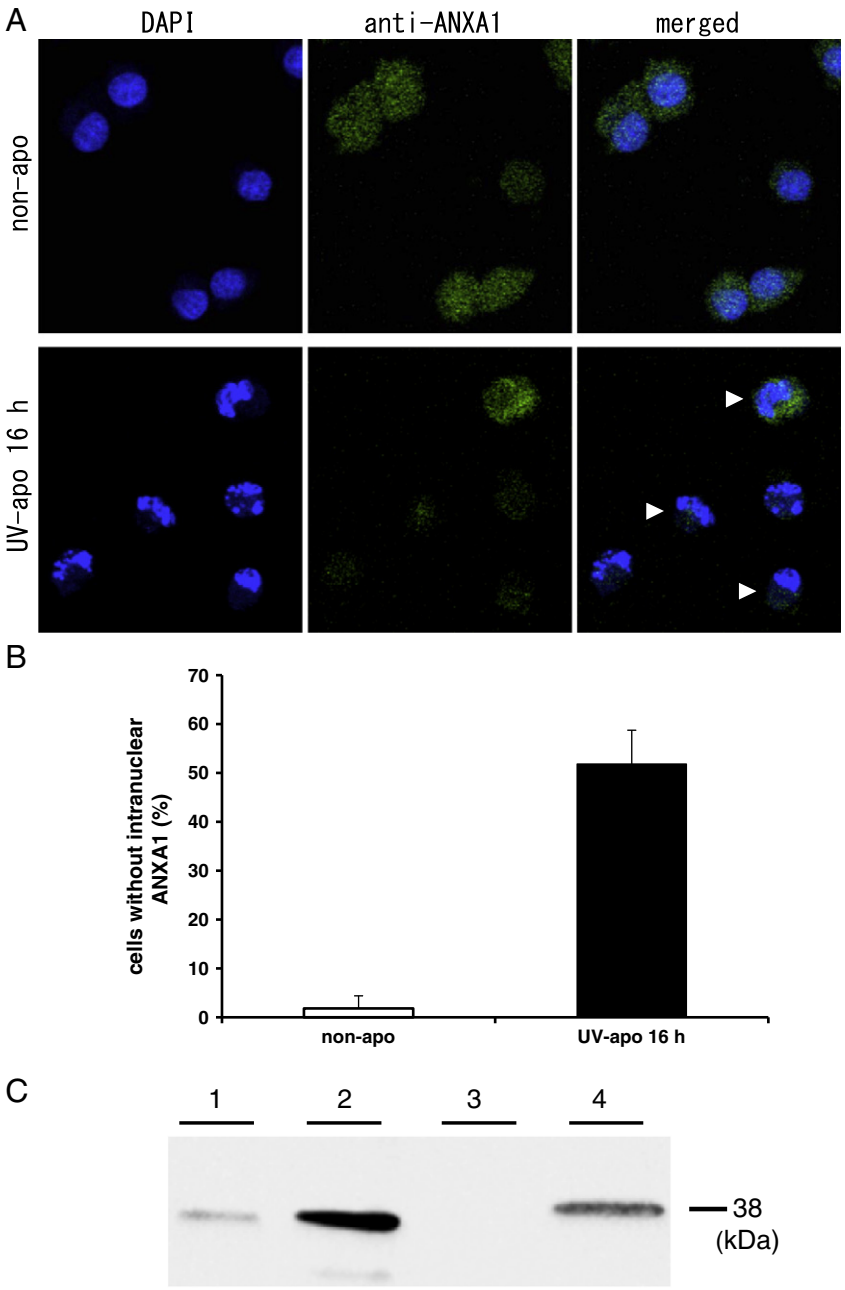

Fig. 3. A change in the subcellular localization of ANXA1 during culturing of UV-irradiated HeLa cells. (A) Either untreated HeLa cells (non-apo) or HeLa cells cultured for 16 h after UV exposure (UV-apo 16 h) were permeabilized with 0.2% digitonin, followed by staining with anti-human ANXA1 antibodies. The nuclei were stained with 300 nM DAPI in PBS for 5 min. The cells were then examined under a confocal microscope. Triangles showed translocation of ANXA1 from nuclei. Original magnification: ×400. (B) The percentage of the HeLa cells without intranuclear ANXA1 was determined in randomly chosen four different fields, and the results are expressed as means ± standard error. n = 2. (C) Either untreated HeLa cells or ones cultured for 16 h after UV exposure (1.5×10^6 cells) were separated into the nuclear and cytoplasmic fractions according to the method in the reference [27], followed by SDS PAGE and Western blotting. 1 untreated HeLa nuclei, 2 untreated HeLa cytoplasm, 3 apoptotic HeLa nuclei, and 4 apoptotic HeLa cytoplasm.

extent, ANXA4 were expressed at 16 and 24 h when the cells were positive for PI (Fig. 2B), indicating that the cell surface expression of ANXA1 and ANXA4 is associated with late apoptotic HeLa cells.

We also determined the change in the subcellular localization of ANXA1 and ANXA4 under a confocal microscope. ANXA1 was detected throughout untreated HeLa cells (Fig. 3A, upper panels). In contrast, in late apoptotic HeLa cells, ANXA1 appears to be translocated from the nucleus to the cytoplasm (Fig. 3A, lower panels). The percentage of the cells without intranuclear ANXA1 in apoptotic HeLa cells was over 50%, being much higher than that in untreated HeLa cells (Fig. 3B). We confirmed the change in the subcellular localization of ANXA1 by Western blotting after separation into the nuclear and cytoplasmic fractions (Fig. 3C, see Discussion). ANXA4 was hardly detected under the confocal microscope even in untreated HeLa cells (data not shown).

ANXA1 and ANXA4 were expressed on the surface of late apoptotic HeLa cells minimally positive for PS, suggesting that ANXA1 and ANXA4 bind to molecule(s) other than PS. Because annexins bind to PS in a calcium-dependent manner, there is a possibility that the cell surface expression of ANXA1 and ANXA4 is not dependent on calcium. We therefore examined the effect of EGTA treatment. To our surprise, EGTA treatment significantly decreased the cell surface expression of

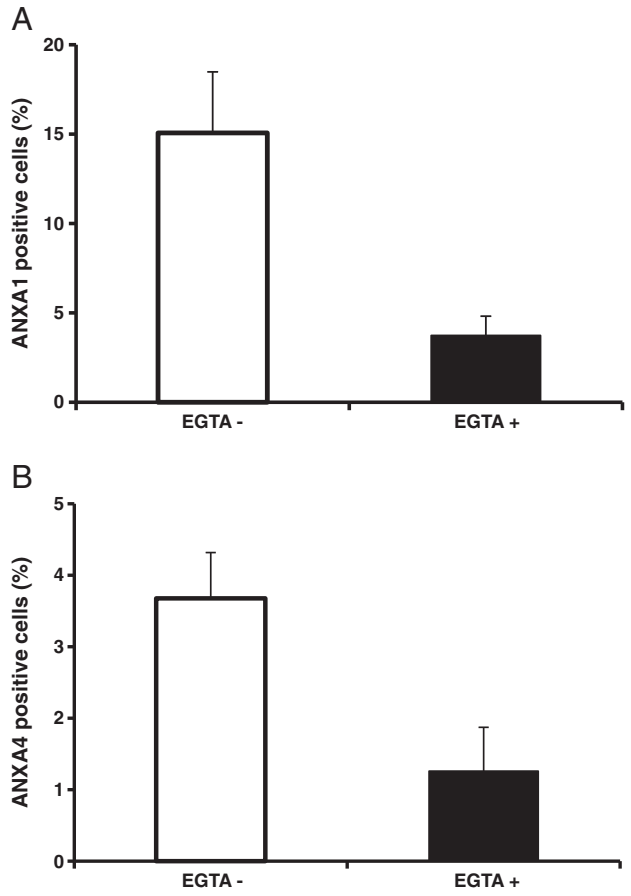

Fig. 4. Decrease in cell surface ANXA1 and ANXA4 caused by EGTA. HeLa cells cultured for 16 h after UV exposure were washed with buffer in the presence or absence of 10 mM EGTA, followed by staining with anti-human ANXA1 (A) or anti-human ANXA4 (B) antibodies in the presence or absence of 10 mM EGTA and analysis with a flow cytometer. The results were expressed as means ± standard error. n = 3, * $P<0.05$ as compared with EGTA.

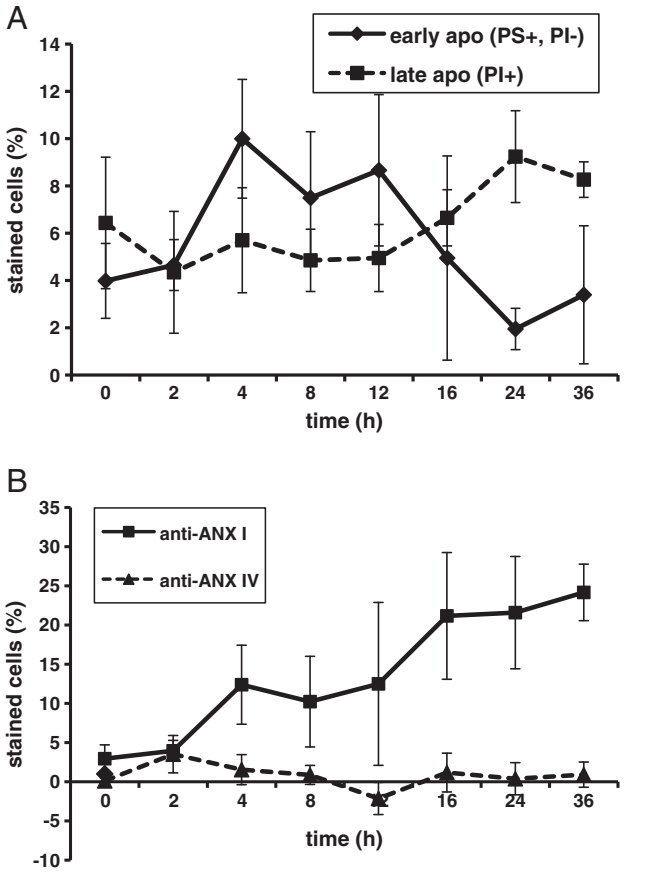

Fig. 5. Surface expression of PS, ANXA1 and ANXA4 during culturing of human peripheral blood neutrophils. Human peripheral blood neutrophils, at a cell density of 5×10^5 cells/ml, were cultured for various times. Surface expression of PS (A), ANXA1 and ANXA4 (B) was then analyzed with a flow cytometer according to the methods described under Materials and methods. The results in (A) are expressed as the percentages of early (PS^+, PI^-) or late (PI^+) apoptotic cells, and all the results are expressed as means ± standard error. n = 3–6.

ANXA1 and ANXA4 (Fig. 4), suggesting that ANXA1 and ANXA4 bind to molecule(s) other than PS in a calcium-dependent manner.

3.2. Surface expression of PS, ANXA1 and ANXA4 during culturing of human neutrophils

We then obtained second apoptotic cells by culturing human neutrophils, and examined the cell surface expression of PS, ANXA1 and ANXA4 by flow cytometry. PS was transiently expressed, the peak being at 4 h (Fig. 5A). The cell surface expression of ANXA1 was detected at 4 h and then gradually increased (Fig. 5B). Therefore, unlike in the case of HeLa cells, the cell surface expression of ANXA1 on apoptotic human neutrophils was associated with not only early apoptosis but also late apoptosis. Only a small amount of ANXA4 was detected at all times.

3.3. Effect of anti-human ANXA1 or ANXA4 antibodies on MIP-2 production on coculturing of mouse macrophages with apoptotic human neutrophils

We previously reported that IL-8 or MIP-2 is produced on coculturing of human or mouse macrophages with late apoptotic cells, such as IL-2-dependent CTLL-2 cells, P388 cells and human neutrophils [2,3,16]. We therefore examined whether or not late apoptotic HeLa cells and human neutrophils induced MIP-2 production after coculturing with mouse peritoneal macrophages. Late apoptotic HeLa cells hardly induced MIP-2 production (data not shown), whereas late apoptotic human neutrophils induced MIP-2 production (Fig. 6). It is not surprising that late apoptotic HeLa cells did not induce MIP-2 production, because we previously reported that late apoptotic thymocytes induce a much smaller amount of MIP-2 than late apoptotic CTLL-2 cells and P388 cells did [17].

We then examined whether or not anti-human ANXA1 or ANXA4 antibodies affect MIP-2 production by coculturing mouse macrophages with early or late apoptotic human neutrophils in the absence or presence of IFN-γ, because our recent study demonstrated that IFN-γ modulates cytokine production of mouse macrophages in response to either early apoptotic mouse neutrophils or late apoptotic ones [18]. In the absence of IFN-γ, both anti-human ANXA1 antibodies and anti-human ANXA4 antibodies increased MIP-2 production in response to late apoptotic human neutrophils but not early apoptotic ones significantly, as compared with control antibodies (Fig. 6A). In the presence of IFN-γ, however, both antibodies increased MIP-2 production in response to not only late apoptotic human neutrophils but also early apoptotic ones (Fig. 6B).

3.4. Cross-reactivity of anti-human ANXA1 antibodies to mouse ANXA1

Although our rabbit anti-human ANXA1 antibodies were obtained by immunization with human ANXA1, there is a possibility that anti-human ANXA1 antibodies cross-react with mouse ANXA1. Unstimulated mouse macrophages were negative for staining by anti-human ANXA1 antibodies, whereas IFN-γ-stimulated ones were positive (Fig. 7, top vs. middle), suggesting that our preparation of anti-human ANXA1 antibodies cross-reacts with mouse ANXA1. Moreover, late apoptotic neutrophils-fed macrophages were also positive for staining by

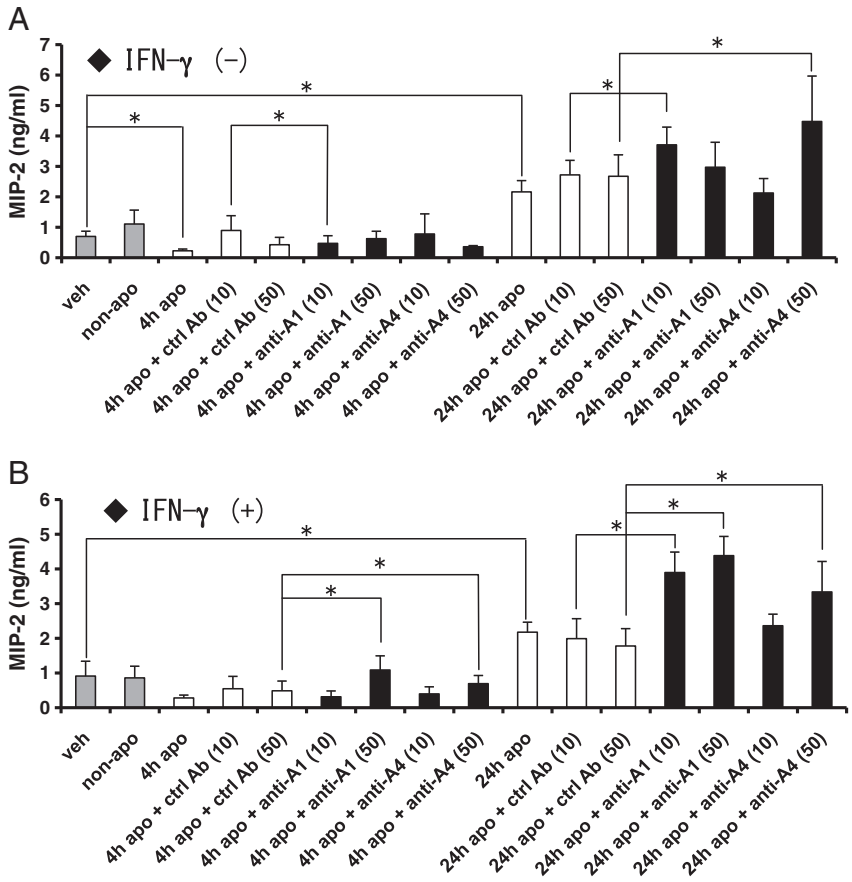

Fig. 6. Effect of anti-human ANXA1 or anti-human ANXA4 antibodies on MIP-2 production on coculturing of mouse macrophages with apoptotic human peripheral blood neutrophils. Human peripheral blood neutrophils were cultured for 4 or 24 h, followed by the addition of 10 or 50 μg/ml of anti-human ANXA1 antibodies, anti-human ANXA4 antibodies or normal rabbit IgG. After incubation on ice for 30 min, the apoptotic human neutrophils were added to the mouse peritoneal macrophages in the ratio of 1 to 1 in the absence (A) or presence (B) of 100 ng/ml IFN-γ. Culture supernatants were then harvested, followed by analysis by MIP-2 ELISA. Three independent experiments were carried out and the results are expressed as means ± standard error. * $P<0.05$.

anti-human ANXA1 antibodies (Fig. 7, bottom). Because the macrophages were washed several times to remove free and bound apoptotic neutrophils, it is likely that the signals were derived from mouse macrophages but not human neutrophils.

3.5. Effect of anti-human ANXA1 or ANXA4 antibodies on IL-8 production on coculturing of human macrophages with apoptotic human neutrophils

We also examined whether or not late apoptotic human neutrophils induced IL-8 production after coculturing with human macrophages. Late apoptotic human neutrophils induced IL-8 production (Fig. 8). We then examined whether or not anti-human ANXA1 or ANXA4 antibodies affect IL-8 production by coculturing human macrophages with late apoptotic human neutrophils. Both anti-human ANXA1 antibodies and anti-human ANXA4 antibodies increased IL-8 production in response to late apoptotic human neutrophils significantly, as compared with control antibodies (Fig. 8).

4. Discussion

It has been reported that ANXA1 is expressed on either early [9,10] or late apoptotic cells [14]. In this study, ANXA1 and, to a lesser extent, ANXA4 were detected on the surface of late apoptotic HeLa cells, whereas ANXA1 and a small amount of ANXA4 were detected on the surface of both early and late apoptotic human neutrophils. These results suggest that the stage of apoptosis when ANXA1 and ANXA4 are expressed on the cell surface depends on the cell type, although the underlying mechanism is currently unknown. The reason why the level of surface ANXA4 was much smaller than that of ANXA1 in HeLa cells may be related to that, as compared with ANXA1, a smaller amount of ANXA4 was detected in untreated HeLa cells (Fig. 1B). ANXA1 was found to change in subcellular localization from the nucleus to the cytoplasm in late apoptotic HeLa cells, as evidenced by staining with both anti-human ANXA1 antibodies and DAPI (Fig. 3A). Of note is that the total level of ANXA1 in apoptotic HeLa cells is smaller than that in untreated HeLa cells (Figs. 1B and 3C), raising the alternative possibility that ANXA1 in the nuclear region is decreased below the detection limit. Although it is not known how ANXA1 is expressed on the cell surface, identification of the cell surface molecule(s) to which ANXA1 binds in a calcium-dependent manner may provide a clue as to the underlying mechanism. Calcium binding to ANXA1 causes protein re-organization, with exposure of the N-terminus [19], which may be required for binding to the receptor.

Although ANXA1 is known as an anti-inflammatory protein, its role in the inflammatory response during coculturing of macrophages with apoptotic cells remains largely unexplored. In this study, we found that, in the absence of IFN-γ, anti-human ANXA1 antibodies and, to a lesser extent, anti-human ANXA4 ones increased MIP-2 production during coculturing of macrophages with late apoptotic human neutrophils, but not early apoptotic ones. The combination of these two antibodies may further increase MIP-2 production, although we did not examine it. The exact reason why anti-human ANXA1 antibodies are more effective than anti-human ANXA4 ones is not known at present, although it may be related to the difference in the levels of cell surface ANXA1 and ANXA4. Moreover, the anti-inflammatory activities of ANXA1 and ANXA4 may suggest the role of the core domain conserved

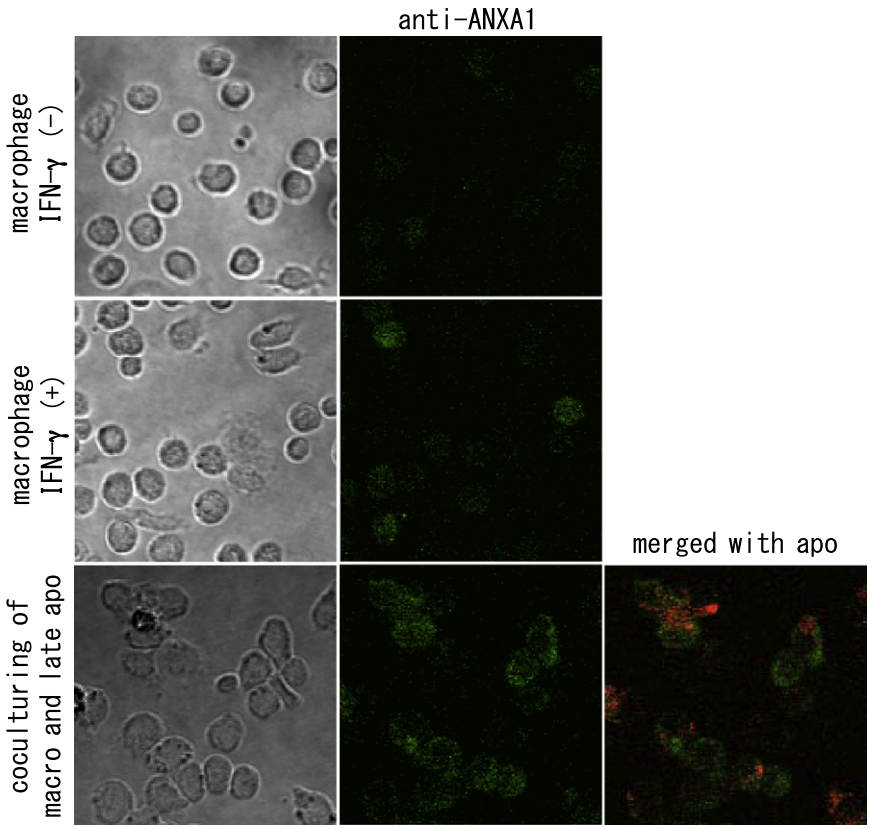

Fig. 7. Cross-reactivity of anti-human ANXA1 antibodies with mouse ANXA1. Mouse peritoneal macrophages were cultured for 3 h in the absence or presence of 100 ng/ml IFN-γ. Alternatively, mouse peritoneal macrophages were fed for 3 h with human peripheral blood neutrophils cultured for 24 h and stained with PKH26-Red, followed by extensive washing to remove free or attached apoptotic human neutrophils. The macrophages were then fixed with 2% formaldehyde, followed by staining with anti-human ANXA1 antibodies without permeabilization and examination under a confocal microscope. Original magnification, ×400. Green: ANXA1, Red: apoptotic cells.

in ANXA1 and ANXA4, although this contrasts with the notion that the N terminal domain is responsible for the anti-inflammatory function of ANXA1 [13]. Alternatively, the anti-inflammatory activities of ANXA1 and ANXA4 may be different from each other. If the latter possibility is correct, there is the possibility that FPR2 is responsible for ANXA1-mediated suppression of MIP-2 production, because ANXA1 activates

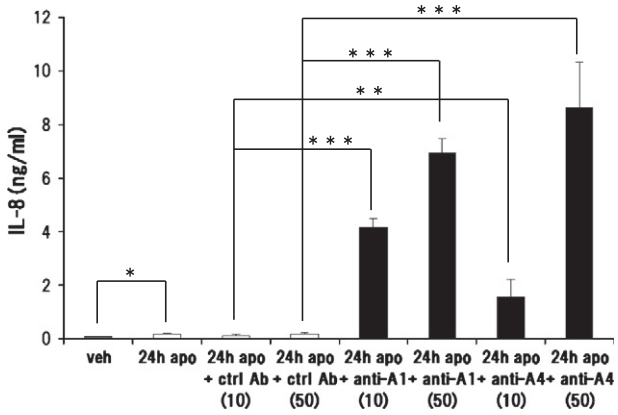

Fig. 8. Effect of anti-human ANXA1 or anti-human ANXA4 antibodies on IL-8 production on coculturing of human macrophages with apoptotic human peripheral blood neutrophils. Human peripheral blood neutrophils were cultured for 24 h, followed by the addition of 10 or 50 μg/ml of anti-human ANXA1 antibodies, anti-human ANXA4 antibodies or normal rabbit IgG. After incubation on ice for 30 min, the apoptotic human neutrophils were added to the human macrophages in the ratio of 1 to 1. Culture supernatants were then harvested, followed by analysis by IL-8 ELISA. Three independent experiments were carried out and the results are expressed as means ± standard error. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

FPR2 to promote phagocytosis [20]. Under the condition we used, however, anti-ANXA1 antibodies did not suppress phagocytosis, suggesting that as yet unknown receptor other than FPR2 may be involved in ANXA1-mediated suppression (data not shown). In agreement with this, a previous study reported that ANXA1 regulates macrophage cytokine production via "glucocorticoid-induced leucine zipper" protein and that it does not require ANXA1 binding to FPR family members [21]. In this study, we also found that, in the presence of IFN-γ, both antibodies increased MIP-2 production during coculturing of macrophages with not only late apoptotic human neutrophils but also early apoptotic ones. It is not known at present, however, why the effect on MIP-2 production in response to early apoptotic human neutrophils in the presence of IFN-γ differs from that in the absence of IFN-γ. The difference may be due to either enhanced production of ANXA1 in IFN-γ-stimulated macrophages (Fig. 7) or modulation of cytokine production by IFN-γ [18].

Both anti-human ANXA1 antibodies and anti-human ANXA4 ones increased IL-8 production during coculturing of macrophages with late apoptotic human neutrophils. This further supports the anti-inflammatory role of ANXA1 and ANXA4 upon an encounter of macrophages to late apoptotic neutrophils.

What mechanism is responsible for the suppressive effect of ANXA1 on MIP-2 production in response to late apoptotic cells? Recently it was reported that LPS-induced cytokine expression was increased in $ANXA1^{-/-}$ macrophages, and that it was accompanied by significantly increased LPS-induced activation of ERK and JNK MAPK [21]. In contrast, exogenous ANXA1 reportedly activates ERK in another study [22]. Although it is difficult to reconcile these results, the former result [21] is consistent with our previous report that ERK activation is critical in the MIP-2 production by macrophages in response to late apoptotic

cells [23], raising the possibility that ERK activation is suppressed by ANXA1, thereby leading to a decrease in MIP-2 production.

TGF-β is responsible for the suppressive effect of early apoptotic cells on the inflammatory response of macrophages in response to LPS and zymosan, although TGF-β is produced by macrophages in response to both early and late apoptotic cells [24,25]. It is not known, however, whether or not TGF-β suppresses the response of macrophages to early apoptotic cells. In contrast, ANXA1 and ANXA4 suppress the inflammatory response of mouse macrophages to not only late apoptotic human neutrophils but also early apoptotic ones in the presence of IFN-γ.

In this study we did not examine whether or not cell-to-cell contact is required for MIP-2 production. Since our previous study demonstrated that culture supernatant of late apoptotic mouse neutrophils induced TNF-α production by IFN-γ-stimulated macrophages but not untreated ones [18], it is likely that MIP-2 production in response to late apoptotic neutrophils in the absence of IFN-γ is dependent on cell-to-cell contact.

We found that our preparation of rabbit anti-human ANXA1 antibodies cross-reacts with ANXA1 on the surface of activated mouse macrophages. Other researchers have also reported the cell surface expression of ANXA1 on activated macrophages that had been obtained after injection of thioglycollate broth [11], and up-regulation of ANXA1 in activated microglia in Alzheimer's disease [26]. It is thus suggested that ANXA1 and, presumably, ANXA4 on the surface of both late apoptotic human neutrophils and activated mouse macrophages are involved in the suppression of MIP-2 production.

Overall, our study suggests that ANXA1 and ANXA4 down-regulate the inflammatory response when late apoptotic neutrophils are generated upon chemotherapy and X-irradiation without an infection.

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