Increased expression of S100 calcium binding protein A8 in GM-CSF-stimulated neutrophils leads to the increased expressions of IL-8 and IL-16

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Abstract

Objectives

In our previous proteomic surveillance, we found that at least 11 proteins in neutrophils were increased more than 2.5-fold by the stimulation of GM-CSF. In this paper, focusing on one of the 11 proteins, S100 calcium binding protein A8 (S100A8), we tried to elucidate the effect of S100A8 and the cooperative effect of S100A8 and GM-CSF on production and secretion of cytokines of neutrophils.

Methods

S100A8 in neutrophil was detected by western blotting, and concentrations of S100A8 in synovial fluid (SF) from patients with rheumatoid arthritis (RA) and osteoarthritis (OA) were measured by ELISA. Cytokine levels in the culture medium of neutrophils incubated with and without S100A8 were measured by an antibody array. IL-8 and IL-16 levels in the culture medium of neutrophils stimulated with S100A8, GM-CSF, and the combination of S100A8 and GM-CSF were measured by ELISA. The mRNA levels of IL-8 and IL-16 in the stimulated neutrophils were analysed by real-time PCR.

Results

The western blotting analysis confirmed that S100A8 is up-regulated in neutrophil by the stimulation of GM-CSF. Furthermore, the ELISA analysis confirmed that S100A8 was significantly elevated in SF of patients with RA compared to SF of patients with OA. S100A8 induced mRNA expression and secretion of IL-8 and IL-16. S100A8 further enhanced production of IL-8 by GM-CSF but not that of IL-16.

Conclusions

These data suggest that S100A8 may be involved in the exacerbation of RA, and that S100A8 may be a therapeutic target of RA.

Key words

rheumatoid arthritis, neutrophil, GM-CSF, S100A8, cytokine

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterised by the infiltration of T cells, B cells, macrophages, and neutrophils into the synovial lining and fluid. In synovial fluid (SF) from patients with RA, the most abundant cells are neutrophils, which comprise as much as 80% of all the infiltrating cells (1, 2). This characteristic is quite different from SF from patients with osteoarthritis (OA) of a non-inflammatory arthritis, which contains a rather small number percents (26.7%) of neutrophils (3). Neutrophils are activated in patients with RA (4, 5). It is representatively supported by the expression of CD69. CD69, located inside of inactivated neutrophils, translocates rapidly onto the cell surface by stimulation, for example, that with N-formyl-methionyl-leucyl-phenylalanine or phorbol 12-myristate 13-acetate (4). In fact, CD69 is expressed on neutrophils in synovial fluid and peripheral blood from the patients with RA (6). Such activated neutrophils are thought to contribute to severe inflammation of the joints in RA through the secretion of high amounts of some proinflammatory cytokines and chemokines including tumour-necrosis factor (TNF) α, interleukin (IL) 1β, and IL-8 (7).

Several cytokines are involved in the differentiation of neutrophils from hematopoietic stem cells (8-10). In particular, granulocyte-colony stimulating factor (G-CSF) maintains neutrophils production at a steady-state and increases production of neutrophils at emergency situations (11, 12). On the other hand, granulocyte-macrophage colony-stimulating factor (GM-CSF) sustains viability of neutrophils and activates their functions. In fact, GM-CSF-stimulated neutrophils expressed CD69 on their surface (13). GM-CSF was reported to be produced at high levels by synoviocytes collected from the patients with RA *in vitro* (14, 15). Clinically, GM-CSF was found more frequently in SF from the patients with RA than that in SF from the patients with OA (16). Further, in a RA animal model of collagen-induced arthritis, the arthritis was exacerbated by the injection of GM-CSF (17) and was ameliorated by the injection of anti-GM-CSF antibodies (18). Therefore, GM-CSF is suggested to play critical roles in pathogenesis of RA in the context of neutrophils activation. However, effects of GM-CSF on neutrophils have not been fully understood.

Previously, using proteomic surveillance (19, 20), we tried to elucidate novel effects of GM-CSF on neutrophils. We compared proteins extracted from GM-CSF-stimulated neutrophils to that from unstimulated neutrophils by the analysis using matrix-assisted laser desorption ionisation-time-of-flight mass spectrometer (MALDI-TOF MS). We detected 33 peptide peaks whose expression was up-regulated by more than 2.5-fold in the GM-CSF-stimulated neutrophils and identified 11 proteins out of the 33 peptides using MALDI-TOF/TOF MS analysis and protein database search (21).

Next, we wanted to know whether a therapeutic target of RA is involved in the identified proteins. As the first step, we selected S100 calcium binding protein A8 (S100A8). S100A8 is a member of S100 family (22, 23). S100A8, secreted by neutrophils and monocytes, forms a Ca-dependent heterodimer (S100A8/A9) with S100A9. S100A9 has been reported to stimulate neutrophil adhesion to fibrinogen. This function of S100A9 was negatively regulated by the formation of S100A8/A9, although S100A8 itself had no effect on neutrophil adhesion (24). On the other hand, it is reported that S100A8 in cervico-vaginal secretions stimulates HIV replication in human monocytic infected cells (25) and that S100A8 has chemotactic activity for periodontal ligament cells (26). However, the role of S100A8 in neutrophils remains obscure.

In this paper, we confirmed the previous proteomic surveillance that the GM-CSF-stimulated neutrophils increased the production of S100A8 and revealed that, in SF from the patient with RA, S100A8 concentration was significantly elevated. Furthermore, S100A8 induced the secretion and mRNA expression of IL-8 and IL-16 in neutrophils. S100A8 further enhanced the interleukin-inducing activity of GM-CSF on IL-8 but not on IL-16. Thus, together with GM-

CSF, S100A8 would be involved in the pathogenesis of RA and also be a potential therapeutic target of RA.

Materials and methods

Clinical samples

Human neutrophils were separated from the peripheral blood obtained from healthy donors. SF samples were obtained from 13 patients with RA (13 women, 0 men; ages 59–84 years, mean 70.7 years), 16 patients with OA (10 women, 6 men; ages 55–89 years, mean 68.6 years). The patients were diagnosed according to the respective classification criteria for each of the two diseases (27, 28). All the samples were obtained with their informed consent and this study was approved by the local institutional ethics committee.

Purification of neutrophils

Human neutrophils were obtained by dextran sedimentation and Ficoll-Hypaque (GE Healthcare Bio-Science Corp., Piscataway, NJ) density-gradient centrifugation (29). Briefly, a heparinised blood sample was mixed with an equal volume of a 0.9% NaCl solution containing 3% dextran T-500 (GE Healthcare Bio-Science), and followed by incubation in the upright position for 20 minutes at room temperature. The upper layer was collected and centrifuged at 1000 rpm (250 × g) for 10 minutes, and the resulting pellet cells were re-suspended in a 0.9% NaCl solution. Then 10 ml of Ficoll-Hypaque solution was layered beneath the cell suspension. After centrifugation for 40 minutes at 1400 rpm (400 × g) at 20ºC, pelleted neutrophils were collected, from which residual erythrocytes were removed by hypotonic lysis using a 0.2% NaCl solution. Then, the purified neutrophils were re-suspended in phosphate-buffered saline (PBS), pH 7.4 and cell concentration was determined. More than 98% of the purified cells consisted of viable neutrophils as assessed by morphology and a trypan blue exclusion test.

Cell culture and stimulation

The purified neutrophils were re-suspended in RPMI 1640 containing 10% FBS, 100 U/ml penicillin, 100mg/ml streptomycin, and 2 mM glutamine. To activate the neutrophils, the cells were cultured in the presence or absence of 10 ng/ml recombinant human GM-CSF (Millipore, Billerica, MA) under 5% CO_2 at 37ºC for 18 hours (13, 21). To determine the effects of S100A8 on cytokine production, we used the sufficient concentration of S100A8 to exert its activity in the cell culture condition (24, 30-32). Cells were cultured in a medium alone, or that containing 500 ng/ml S100A8 (Abnova, Taipei, Taiwan), 10 ng/ml GM-CSF, and both under 5% CO_2 at 37ºC for 1, 3, and 6 hours.

Western blotting

The cultured neutrophils were lysed in a buffer containing 30 mM Tris-HCl (pH 8.5), 4% 3-((3-cholamidopropyl)dimethylammonium)-1-propanesulfonate, 7 M urea, and 2 M thiourea. After centrifugation for 30 minutes at 15000 rpm (14000 × g), the supernatants 20μg protein for each lane were loaded onto 16% SDS-polyaclylamide gel electrophoresis (PAGE). The separated proteins were then transferred to nitrocellulose membranes. After blocking for 1 hour in PBS containing 1% BSA and 0.1% Tween 20, the membranes were incubated for 1 hour with a mouse monoclonal anti-human S100A8 antibody (IgM; HyCult biotechnology, Uden, Netherlands), followed by incubation with horseradish peroxidase-conjugated rabbit anti-mouse IgM antibodies. Immunoreactive bands were detected by using 3,3'-diaminobenzidine and H_2O_2.

Quantification of S100A8, IL-8 and IL-16 by ELISA

Concentrations of S100A8 in SF of patients with RA and OA were measured by a commercially available ELISA kit (BMA Biomedical, Augst, Switzerland) according to the instruction of the manufacturer. Concentrations of IL-8 and IL-16 in the medium from the cultured neutrophils were measured by a commercially available ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction.

Detection of cytokine by a cytokine antibody array

Cytokine levels in the medium from the cultured neutrophils were analysed using a Proteome Profiler kit (R&D Systems), according to the instruction of the manufacturer. The analysed cytokines were C5a, CD40L, G-CSF, GM-CSF, GROα, I-309, sICAM-1, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL12 p70, IL-13, IL-16, IL-17, IL-17E, IL-23, IL-27, IL-32α, IP-10, I-TAC, MCP-1, MIF, MIP-1α, MIP-1β, Serpin E1, RANTES, SDF-1, TNF-α, and sTREM-1. The bound cytokines were visualised by ECL Plus, and the intensity of each cytokine spot was measured. The intensities were normalised to that of positive control spots. Relative cytokine secretion levels were compared with those of unstimulated ones.

Real-time PCR

Total RNA was isolated from neutrophils cultured with 500 ng/ml S100A8 and 10 ng/ml GM-CSF by RNeasy Mini Kit (Qiagen, Valencia, CA) according to the instruction of the manufacturer. Reverse-transcription of mRNA was performed using random primers and Superscript II (Invitrogen, Carlsbad, California). The produced cDNA were used as a template for PCR amplification. PCR was performed using 2×UniversalMasterMix (Applied Biosystems, Foster City, CA) and 20×TaqMan Gene Expression Assay primer/probe mixture (Applied Biosystems) for IL-8, IL-16, or GAPDH. Each PCR was carried out in triplicate. PCR reaction was initiated by a step at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The expressions of mRNA for IL-8 and IL-16 were normalised by those of GAPDH and the values are expressed as a ratio of IL-8 or IL-16 and GAPDH.

Statistical analysis

Statistical significance was calculated using Student's *t*-test. A *p*-value of <0.05 was considered to be statistically significant.

Results

The increase of the entire S100A8 in neutrophils stimulated with GM-CSF and in SF from patients with RA

In the previous proteome surveillance of neutrophils stimulated by GM-CSF

(21), we found that S100A8 was increased in the cytosolic fraction of the stimulated neutrophils. Specifically we analysed the trypsin-digested peptides from the cytosolic fraction of the stimulated neutrophils, and found that the peptide (m/z 963) derived from S100A8 increased its intensity. Thereby we first confirmed that GM-CSF up-regulates the expression of entire S100A8 in neutrophils. For this aim, S100A8 in whole neutrophil lysate of the GM-CSF stimulated neutrophils was detected by SDS-PAGE and the following western blotting with antibodies to human S100A8. As a result, we demonstrated that the protein expression of entire S100A8 in the GM-CSF stimulated neutrophils was increased (Fig. 1a). Densitometry quantification of the bands indicates that GM-CSF increased the expression of S100A8 by 4-fold.

The concentration of S100A8 in SF from patients with RA is reported to be significantly higher than that in that with OA (30). To confirm the report, we also measured the concentrations of S100A8 in SF from patients with RA and OA by ELISA. As a result, the levels of S100A8 were significantly elevated by more than 15-fold in SF from patients with RA compared with SF from patients with OA (p<0.01). The mean values of the S100A8 concentrations in SF from patients with RA and OA were 41.8±55.8 and 2.7±3.9 ng/ml, respectively (Fig. 1b).

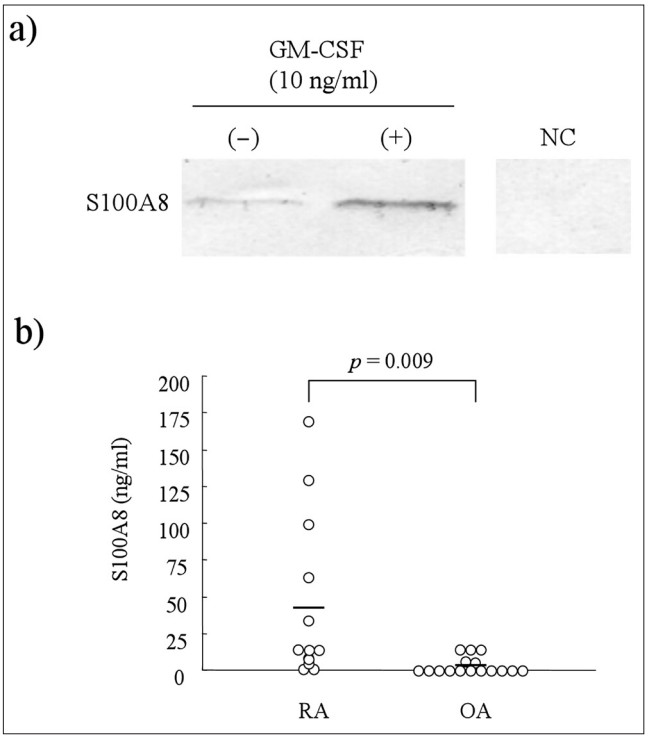

Fig. 1. Immunodetection of S100A8 in neutrophils and in SF from patients. (**a**) The increase of S100A8 in the GM-CSF stimulated neutrophils was confirmed by western blotting. Neutrophils were stimulated with 10 ng/ml GM-CSF for 18 hours. The cell lysates were separated by 16% SDS-PAGE gel. NC, no primary antibody was added. (**b**) Concentrations of S100A8 in SF from RA patients (n=13) and from OA patients (n=16) were measured by ELISA. The horizontal bars indicate the mean values. Each open circle indicates a concentration of S100A8 in SF from individual patients.

Effects of S100A8 on the cytokine secretion

To elucidate effects of S100A8 on neutrophils, we stimulated neutrophils with S100A8 *in vitro* and then analysed the secreted cytokine profile using a cytokine antibody array. As shown in Figure 2a, out of the 36 cytokines measured, we found that the secretion of two cytokines, IL-8 and IL-16, both of which function as a chemoattractant, were increased by the stimulation with S100A8. Measurement of the spot intensities of the cytokines indicates that the secretions of IL-8 and IL-16 were increased upto 1.3-fold compared to the unstimulated control (Table I).

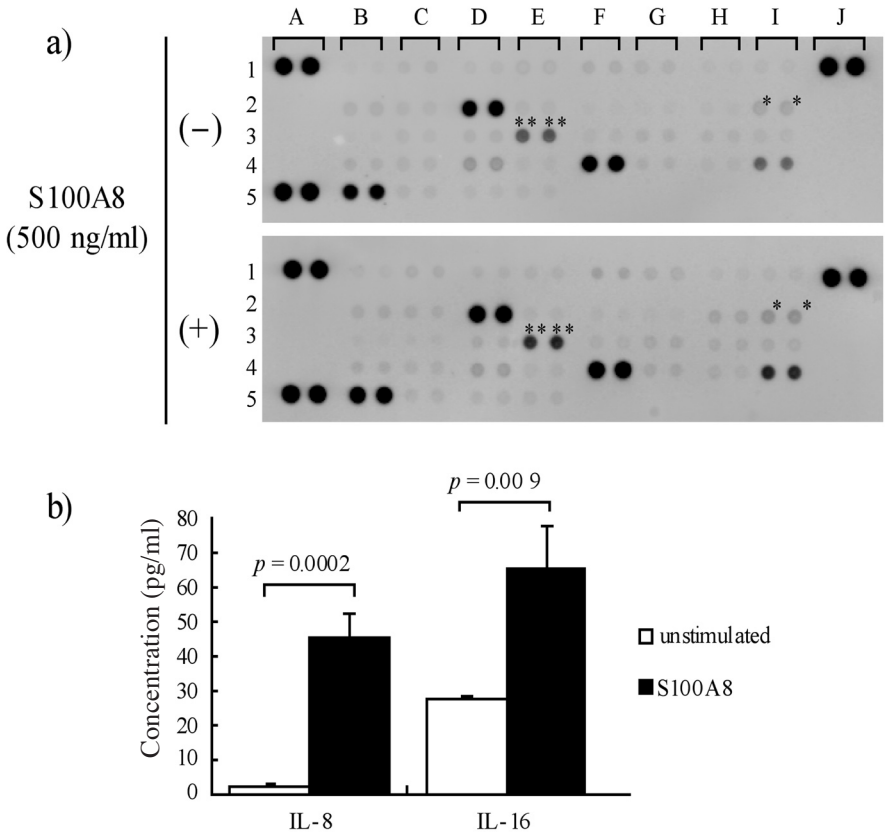

Fig. 2. Effect of S100A8 on the cytokine secretion. (**a**) Cytokine antibody array, blotted 36 different anti-cytokine antibodies, demonstrates levels of cytokines secreted from the neutrophils cultured in a medium with or without 500 ng/ml S100A8 for 6 hours. Relative levels of cytokines were compared with those of unstimulated ones. Spots were visualised by ECL Plus, and each of signal intensity was measured. *, IL-8 spot; **, IL-16 spot. (b) Concentrations of IL-8 and IL-16 in medium of the neutrophils stimulated or unstimulated with 500 ng/ml S100A8 for 6 hours were measured by ELISA.

Table I. The position on the cytokine array and the fold-increase of the spot density.

	A	B	C	D	E	F	G	H	I	J
1	control 1.00	C5a 1.08	CD40L 1.03	G-CSF 0.93	GM-CSF 0.94	GROα 1.15	I-309 1.20	sICAM-1 1.08	IFN-γ 1.02	control 1.00
2	—	IL-1α 0.92	IL-1β 0.97	IL-1ra 1.12	IL-2 1.02	IL-4 1.10	IL-5 1.18	IL-6 1.22	IL-8 1.31*	—
3	—	IL-10 0.96	IL12 p70 1.01	IL-13 1.04	IL-16 1.33 * *	IL-17 1.04	IL-17E 1.05	IL-23 1.04	IL-27 0.98	—
4	—	IL-32α 0.99	IP-10 0.97	I-TAC 0.83	MCP-1 1.05	MIF 1.00	MIP-1α 1.10	MIP-1β 1.03	Serpin E1 1.20	—
5	control 1.00	RANTES 1.03	SDF-1 0.98	TNFα 1.12	sTREM-1 1.16	—	—	—	—	—

Bar indicates no spotting area.

Confirmation of the increase of IL-8 and IL-16

Next, to confirm the increase of the secretions of IL-8 and IL-16 by S100A8 by the array analysis, we precisely measured their concentration in the medium by ELISA. Neutrophils were incubated with S100A8 for 6 hours. Then, the cells and the medium were separated by centrifugation. As shown in Figure 2b, S100A8 significantly increased the secretions of IL-8 and IL-16. The secretion levels of IL-8 and IL-16 were increased to 29.3-fold ($p<0.01$) and 2.4-fold ($p<0.01$), respectively, compared to the unstimulated control.

Effects of co-stimulation with S100A8 and GM-CSF on neutrophils

Furthermore, we examined the synergistic effect of combination of S100A8 and GM-CSF on the secretion and the mRNA expression of IL-8 and IL-16. The neutrophils were incubated with GM-CSF together with S100A8 for 6 hours. As shown in Figure 3a, the combination of S100A8 and GM-CSF significantly enhanced the secretion of IL-8 than S100A8 or GM-CSF alone. The secretion levels were increased 4.7-fold ($p<0.01$) and 1.8-fold ($p<0.01$) compared with S100A8 alone and GM-CSF alone, respectively. The mRNA expression level of IL-8 mRNA was also significantly enhanced by the combination of S100A8 and GM-CSF (Fig. 3b). The mRNA expressions were up-regulated to 9.4-fold ($p<0.01$) and 1.4-fold ($p<0.01$) compared to those with S100A8 alone and GM-CSF

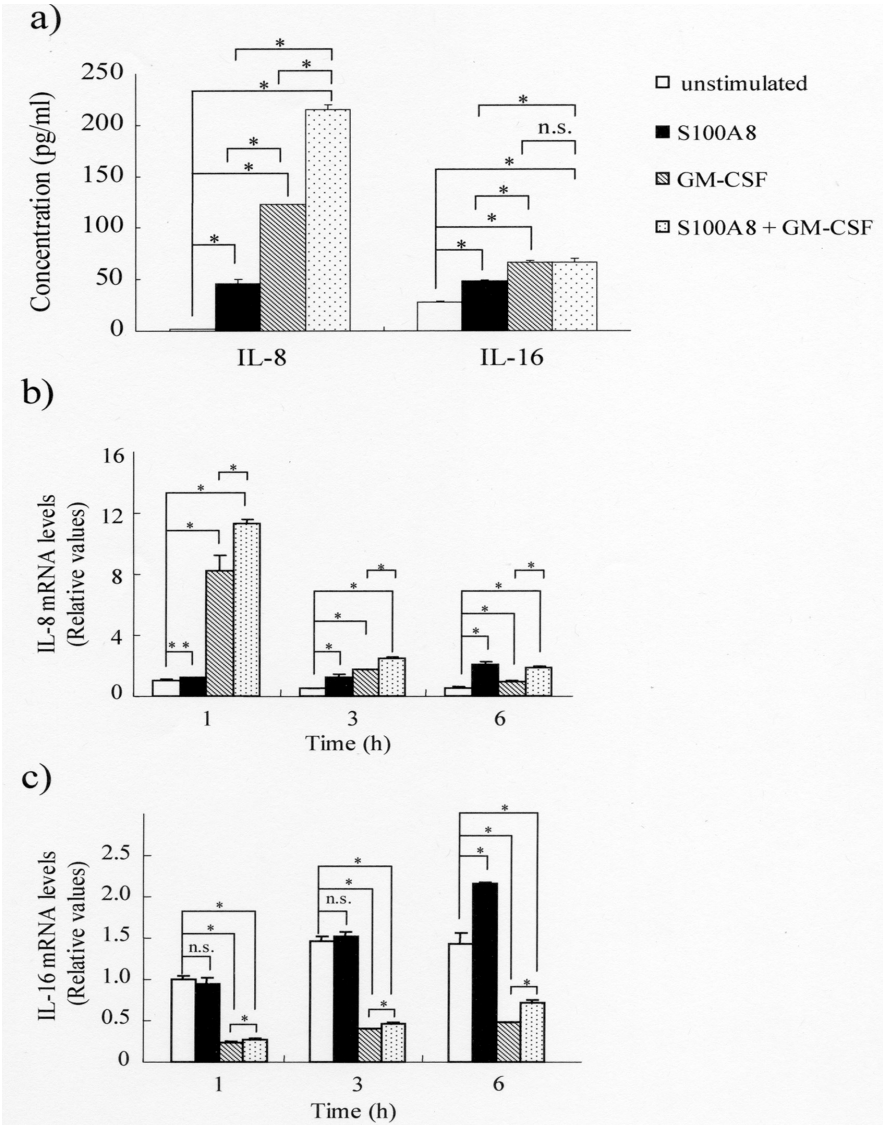

Fig. 3. Effect of co-stimulation of GM-CSF and S100A8 on the secretion and the mRNA expression of IL-8 and IL-16. Neutrophils were cultured in a medium containing 500 ng/ml S100A8, 10 ng/ml GM-CSF, or both. (a) Concentrations of IL-8 and IL-16 were measured by ELISA. *$p<0.05$. n.s., not significant. (b and c) mRNA levels of IL-8 and IL-16 were analysed by real-time PCR. The expression levels were shown as relative values compared with that of unstimulated ones.

ever, interestingly, GM-CSF strongly reduced the mRNA expression of IL-16 in neutrophils, and S100A8 partially overcame the reduction of the IL-16 mRNA expression by GM-CSF (Fig. 3c). However, as shown in Figure 3a there was no difference in the secretion levels of IL-16 between GM-CSF alone and the combination of S100A8 and GM-CSF.

Discussion

In the previous study, we investigated effects of GM-CSF on neutrophils by proteomic approach to understand roles of neutrophils in RA. We have revealed that GM-CSF up-regulated the expression of S100A8 in neutrophils. As mentioned, S100A8 is reported to form a heterodimer with S100A9. Further, the levels of S100A8, S100A9, and S100A8/A9 are reported to be high in serum and SF from patients with RA (30). Therefore, neutrophils activated by GM-CSF possibly bring about formation of S100A8/A9, which pathway would lead to invasion of immune cells (31), the production of proinflammatory cytokines such as TNF-α (32), and degradation of cartilage (33). Thus, the main function of S100A8 known upto now would be formation of S100A8/A9. The function of S100A8 alone remains to be elucidated. Thereby we addressed to find other effects of S100A8 on neutrophils. By cytokine antibody array, we revealed that S100A8 increased the secretion and the mRNA expression of IL-8 and IL-16 in neutrophils.

IL-8 is a member of the CXC chemokine family. This chemokine is one of the major mediators of the inflammatory responses. IL-8 has been reported to be produced in synovial tissue macrophages (34), fibroblasts (35), and chondrocytes (36). IL-8 has been reported to function as a strong inducer of neutrophil recruitment into joints in RA (37), and also reported to be a potent angiogenic factor in RA (38). The expression of IL-8 in rheumatoid synovial tissue has been reported to be associated with disease activity (35). Further IL-8 is elevated in SF from patients with RA compared to that from patients with OA (39). We here showed that S100A8 can induce quickly the secretion and the mRNA expression of IL-8, and that the combination of S100A8 and GM-CSF can induce IL-8 mRNA expression more effectively than S100A8 and GM-CSF alone, respectively. The combination may cause the high concentration of IL-8 in SF from patients with RA.

IL-16 has been reported to be produced by $CD4^+$ and $CD8^+$ T cells (40), eosinophils, mast cells (41), and dendritic cells (42). IL-16 possesses a variety of pro-inflammatory functions including chemotaxis of $CD4^+$ T cells (43), monocytes, and eosinophils (44), expansion of memory effector T cells in concert with other cytokines (45), and activation of antigen-presenting functions in monocytes (46). IL-16 is significantly elevated in SF from patients with RA, as compared to that from patients with non-arthritis and OA (43). We showed that the mRNA expression of IL-16 were induced by S100A8, and strongly reduced by GM-CSF. The mRNA expression of IL-16 was previously reported to be down-regulated

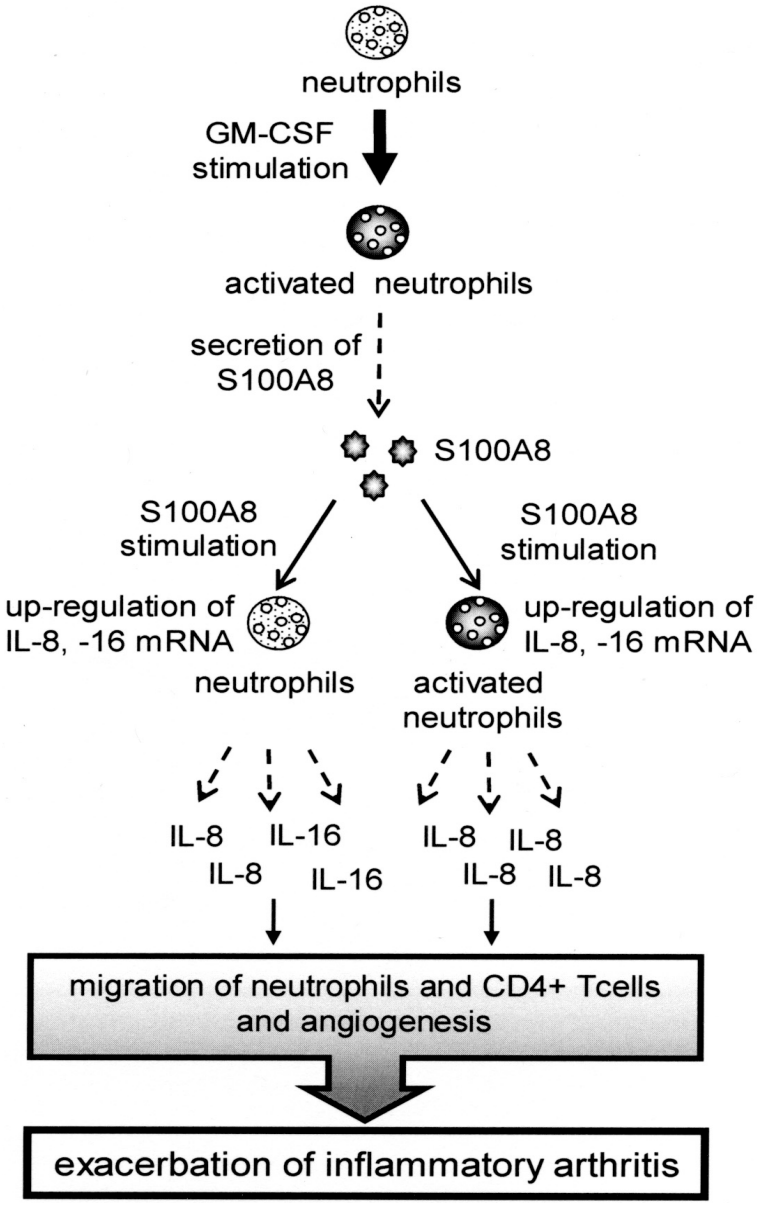

Fig. 4. Schematic representation of role of S100A8 on neutrophils. S100A8 secreted from the GM-CSF stimulated neutrophils accumulates in SF of RA patient. S100A8 induces the expressions and the secretions of IL-8 and IL-16 in both GM-CSF stimulated neutrophils and unstimulated ones. The cytokines induce the infiltration of leukocytes, such as neutrophils and $CD4^+$ T cells, and angiogenesis into joint. The leukocytes infiltrated by IL-8 and IL-16 further release pro-inflammatory cytokine, which may result in the exacerbation of inflammatory arthritis.

in activated T cells. In T cells, IL-16 is synthesised as an 80-kDa precursor, pro-IL-16 (47). The 121-aa C terminus of the precursor is cleaved by caspase-3 and then secreted (48) as a biologically active immunomodulatory cytokine that binds to CD4 (49). Resting T cells contains pro-IL-16 abundantly in both their nucleus and cytoplasm (50). Upon activation, T cells decrease mRNA for pro-IL-16 (51, 52), and pro-IL-16 drastically by unknown mechanisms. In this study, the expression of IL-16 mRNA was also reduced in neutrophils activated by GM-CSF. This indicates that a similar pro-IL-16 regulation pathway to T cells may exist in neutrophils.

The reduction of the mRNA expression of IL-16 by GM-CSF was slightly but significantly recovered by S100A8, however, there was no difference in the secretion level of IL-16 between GM-CSF alone and the combination of S100A8 and GM-CSF for 6 hours. These indicate that the secretion of IL-16 by the combination of S100A8 and GM-CSF may gradually be induced.

These data represent the first demonstration that S100A8 induced the secretion and the mRNA expression of IL-8 and IL-16, and that the combination of S100A8 and GM-CSF strongly enhanced the secretion and the mRNA expression of IL-8. The leukocytes infiltrated into joints by IL-8 and IL-16 release pro-inflammatory cytokine such as IL-1β and TNF-α. Taken together, S100A8 possibly contributes to an exacerbation of the inflammatory arthritis immunologically and/or cell-biologically through infiltration of leukocytes by IL-8 and IL-16.

In conclusion, our results show that GM-CSF induced the protein expression of S100A8 in neutrophils, and that the secreted S100A8 was accumulated in SF of patients with RA. S100A8 induced the secretion and the mRNA expression of IL-8 and IL-16 in neutrophils. The combination of S100A8 and GM-CSF strongly enhanced the secretion and mRNA expression of IL-8. Thus, GM-CSF may induce the infiltration of T cells and neutrophils through the up-regulation of S100A8 in neutrophils, which leads to an exacerbation of the inflammatory arthritis (Fig. 4). S100A8 may be a crucial pathogenic factor in RA and also a therapeutic target of RA.

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