Concise report

The metastasis-associated protein S100A4 promotes the inflammatory response of mononuclear cells via the TLR4 signalling pathway in rheumatoid arthritis

Lucie Andrés Cerezo¹, Martina Remáková¹, Michal Tomčík¹, Steffen Gay², Michel Neidhart², Eugene Lukandin³, Karel Pavelka¹, Mariam Grigorian³, Jiří Vencovsky¹ and Ladislav Šenolt¹

Abstract

Objectives. S100A4 has been implicated in cancer and several inflammatory diseases, including RA. The aim of the present study was to determine whether S100A4 can stimulate proinflammatory cytokine production in mononuclear cells.

Methods. Peripheral blood mononuclear cells (PBMCs) isolated from patients with RA were stimulated with S100A4, S100A8, S100A9 and S100A12. The production of IL-1β, IL-6 and TNF-α was measured by ELISA. Receptor for advanced glycation end products (RAGEs) and Toll-like receptor 4 (TLR4) signalling were examined. For signalling pathway blocking studies, inhibitors of myeloid differentiation primary response gene 88 (MyD88), nuclear factor kappa B (NF-κB) and the mitogen activated protein (MAP) kinases p38, extracellular signal-regulated kinase 1/2 (ERK1/2) and Jun N-terminal kinase (JNK) were used. MAP kinase activation was evaluated by western blotting.

Results. Stimulation of PBMCs with S100A4 significantly up-regulated IL-1β, IL-6 and TNF-α production compared with unstimulated cells (P < 0.001). Importantly, the production of these cytokines was markedly enhanced in response to S100A4 compared with S100A8 and S100A12; however, it was less pronounced compared with S100A9. Furthermore, enhanced production of proinflammatory cytokines in S100A4-stimulated PMBCs was at least partly mediated via TLR4, but not RAGEs, and by activation of the transcription factor NF-κB and the MAP kinases p38 and ERK1/2.

Conclusion. This is the first study to demonstrate that S100A4 can induce an inflammatory response mediated by TLR4 and by the activation of NF-κB and the kinases p38 and ERK1/2 in mononuclear cells from patients with RA. Therefore S100A4 may be a potential therapeutic target for immune-mediated diseases.

Key words: S100A4, Toll-like receptor 4, RAGE, pro-inflammatory cytokines, rheumatoid arthritis.

Introduction

RA is a chronic, systemic, autoimmune disease characterized by persistent inflammation of synovial joints, leading to progressive joint destruction. Immune cells such as T and B cells, macrophages and dendritic cells are recruited to affected joints where they, together with synovial fibroblasts, produce a wide range of cytokines, inflammatory mediators and matrix-degrading enzymes, contributing to the development of adjacent bone erosion and cartilage destruction [1].

S100A4 protein, also referred to as mts1, FSP1, CAPL, calvasculin, p9Ka, pEL-98, 18A2 and 42A, belongs to the S100 family of structurally related calcium binding proteins. It was originally described as a protein differentially regulated in metastatic tumours and was established as a metastasis-promoting factor associated with poor survival rates in a variety of cancers [2]. However, we and others...
have recently shown enhanced S100A4 expression in several chronic inflammatory conditions [3–7]. S100A4 is increased at local sites of inflammation and in the blood circulation of patients with RA [4]. Additionally, it correlates with disease activity [5] and has both proinflammatory and destructive properties [3, 5]. Moreover, S100A4 has been shown to be up-regulated and actively involved in the pathogenesis of other immune-mediated diseases such as inflammatory myopathies and psoriasis [6, 7].

Nevertheless, the exact mechanism leading to S100A4-induced inflammatory reactions has not been identified. S100A4 has been shown to bind to receptor for advanced glycation end products (RAGEs) and to enhance MMP-13 expression via activation of nuclear factor kappa B (NF-kB) in human chondrocytes [8]. Independent of RAGEs, S100A4 can induce neurite outgrowth via interactions with heparin sulphate proteoglycans at the cell surface [9], stimulate angiogenesis through interaction with annexin II [10] and regulate tumour progression by interacting with the epidermal growth factor receptor [11]. Another S100 family member, S100A12, has been reported to bind RAGEs and thus activate proinflammatory and tissue-destructive mediators [12]. Recent studies have provided novel insight into the role of S100 proteins by showing that both S100A8/9 and S100A12 exert proinflammatory effects via interaction with Toll-like receptor 4 (TLR4) [13, 14]. Very recently, Björk et al. [15] revealed that S100A4 and S100A9 proteins show an overlapping reactivity with TLR4 and RAGEs and that these proteins can form heterodimers with reactivity to these receptors. Our aim was to investigate the involvement and functional role of S100A4 in the immune response of RA mononuclear cells and the potential receptors and cell signalling pathways involved in this process.

Materials and methods

Cell isolation

Peripheral blood samples were obtained from 31 patients with RA who fulfilled the 1987 ACR criteria for the classification of RA. Detailed patient characteristics are given in supplementary Table S1, available at *Rheumatology* Online. The study was approved by the Ethical Board of the Institute of Rheumatology and all patients provided informed consent. Peripheral blood mononuclear cells (PBMCs) were isolated from pooled peripheral blood samples by Ficoll-Paque density gradient centrifugation. PBMC suspensions were adjusted at a density of 5.0 × 10^5 cells per 1 ml of Advanced RPMI-1640 culture medium (Invitrogen) and were incubated in six-well plates and were incubated for 30 min at 37 °C with 5% CO2 in a humidified atmosphere.

Cell cultures and stimulatory experiments

Cells were treated with S100A4 multimer (1 μg/ml), S100A8 (1 μg/ml), S100A9 (1 μg/ml) or S100A12 (1 μg/ml) for 24 h. The S100 proteins were obtained from recombinant His6-tagged protein by gel filtration as described previously for S100A4 [16]. The purity of the S100 proteins was checked by adding a potent endotoxin/lipopolysaccharide (LPS) inhibitor polymyxin B sulphate in the experiments (n = 4) along with the S100 proteins (see supplementary Fig. S1, available at *Rheumatology* Online). The dose and time course were selected in accordance with our previous experiments [3, 4, 6, 16]. For inhibition studies, cells were pretreated for 30 min with inhibitors prior to incubation with S100A4. The tested inhibitors included the following: 10 μg/ml abRAGE (R&D Systems, Minneapolis, MN, USA); 100 μg/ml AGE-BSA (BioVision, Mountain View, CA, USA), which is a competitive RAGE ligand; 10 μg/ml abTLR-4 (BioLegend, San Diego, CA, USA); inhibitors of mitogen activated protein (MAP) kinases, including extracellular signal-regulated kinase (ERK) (PD98059; 2 μM), p38 (SB203580; 50 μM) and Jun N-terminal kinase (JNK) (SP600125; 40 nM) (Calbiochem, San Diego, CA, USA); and an inhibitor of NF-κB, 500 μM caffeic acid phenethyl ester (Tocris Bioscience, Ellenville, MI, USA). After a 24 h incubation, conditioned medium was collected and stored at −80 °C.

ELISA

The levels of TNF-α, IL-1β and IL-6 were measured in the cell culture supernatants using ELISA kits according to the manufacturer’s protocol (Ray Biotech, Norcross, GA, USA). The analyses were performed using a SUNRISE ELISA reader (Tecan, Salzburg, Austria) at 450 nm.

Protein isolation and western blotting

PBMC suspensions adjusted to a density of 20 × 10^6 cells per 4 ml of Advanced RPMI-1640 culture medium (Invitrogen) were treated with an S100A4 multimer (1 μg/ml) for 15, 30 and 60 min, centrifuged and washed with PBS. For protein isolation, cell pellets were resuspended in lysis buffer [20 mM Tris-HCl, pH 7.4, 300 mM NaCl, 0.2 mM dithiothreitol (DTT)] and lysed for 2 h at 4 °C. Protein extracts (40 μl) were denatured at 70 °C in 2× Laemmli sample buffer at a 1:1 ratio, separated by SDS-PAGE electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). Blots were blocked for non-specific binding and incubated with rabbit anti-human antibody overnight at 4 °C, followed by 2 h at room temperature with secondary horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody. Protein bands were visualized using the DAB Enhanced Liquid Substrate System (Sigma, St Louis, MO, USA). The following antibodies were used: α-tubulin, dilution 1:1000 (Cell Signaling Technology, Danvers, MA, USA; 2125); phospho-SAPK/JNK (Thr183/Tyr185), dilution 1:1000 (Cell Signaling, 9251); phospho-p44/p42 MAPK (ERK1/2) (Thr202/Tyr204), dilution 1:1000 (Cell Signaling, 9101); phospho-p38 MAP kinase (Thr180/Tyr182), dilution 1:1000 (Cell Signaling, 9211); anti-rabbit IgG-HRP-linked antibody (Cell Signaling, 7074).
Statistical analyses
S100A4 protein concentrations are expressed as the mean (s.e.m.). The Wilcoxon test was used for comparisons between two variables. For all statistical evaluations, P-values < 0.05 were considered to be statistically significant.

Results
S100A4 up-regulates proinflammatory cytokines in PBMCs
To evaluate the proinflammatory effects of S100A4, we stimulated PBMCs (n = 8 experiments) with the S100A4 multimer for 24 h and analysed the levels of TNF-\(\alpha\), IL-1\(\beta\) and IL-6 in the supernatants. PBMCs treated with S100A4 significantly up-regulated the synthesis of TNF-\(\alpha\) [763.5 pg/ml (s.d. 80.6) vs 34.95 (8.029), P < 0.01], IL-1\(\beta\) [161.4 pg/ml (s.d. 16.72) vs 3.256 (0.699), P < 0.01] and IL-6 [845.2 pg/ml (s.d. 24.42) vs 9.957 (1.546), P < 0.01] compared with unstimulated cells (Fig. 1A). In addition, S100A4 exerted a significantly stronger effect on cytokine production in contrast to S100A8 and S100A12, but a lesser effect when compared with S100A9 (Fig. 1A-C).

S100A4 inflammatory response is mediated via TLR4 and activation of NF-\(\kappa\)B, p38 and ERK1/2 kinases
As previously reported, the proinflammatory effects of several members of the S100 family are mediated via RAGEs [12] and TLR4 [13, 14]. In the present study, PBMCs (n = 3-5 experiments) pretreated with the antibody against RAGE or AGE-BSA (an alternative ligand for RAGE) prior to S100A4 or S100A12 stimulation did not significantly alter cytokine production compared with PBMCs with no pretreatment (supplementary Fig. S3, available at Rheumatology Online).

Discussion
In the present study we investigated the proinflammatory role of S100A4. We demonstrated for the first time that S100A4 protein induces the production of proinflammatory cytokines such as TNF-\(\alpha\), IL-1\(\beta\) and IL-6 in mononuclear cells. S100A4 acted through TLR4 to activate MyD88, the transcription factor NF-\(\kappa\)B and the tyrosine kinases ERK1/2 and p38. These results support the hypothesis that S100A4 is involved in the activation of the immune response and inflammation in immune-mediated diseases such as RA.

Although S100A4 has been primarily studied in cancers [2], its potential roles in chronic inflammatory diseases have recently been discovered [3-7]. It has been demonstrated that several proinflammatory cytokines, in addition to the chemokine CCL5 [regulated on activation, normal T cell expressed and secreted (RANTES)], are responsible for the induction of S100A4 release from different cell types [17, 18]. Our previous studies have demonstrated that S100A4 accumulates in the joints of patients with RA and identified synovial fibroblasts and immune cells as major sources of S100A4 [3, 4]. Furthermore, S100A4 expression was localized at sites of joint invasion. We have also demonstrated that synovial fibroblasts are responsive to the S100A4 multimer, which regulates apoptosis and the production of matrix-degrading enzymes [3, 4]. In agreement with our data, S100A4 has been shown to be responsible for myeloid cell recruitment to sites of inflammation in vivo [19] and thereby for the acceleration of inflammatory processes.

Here we demonstrate S100A4 as a mediator of innate immune response and show a notable difference in the proinflammatory effect within studied S100 proteins. S100A4, S100A8 and S100A9 strongly up-regulated production of proinflammatory cytokines in PBMCs, whereas the proinflammatory effect of S100A12 on PBMCs was
**Fig. 1** PBMCs treated with S100A4 demonstrated significantly up-regulated production of TNF-α, IL-1β and IL-6 compared with unstimulated cells (n = 8). The production of these cytokines was markedly enhanced in response to S100A4 compared with S100A8 and S100A12 but lower than S100A9. Blocking TLR4 (n = 5) or the MyD88 (n = 4–5) adaptor protein significantly down-regulated the S100A4-induced secretion of (D) IL-1β, (E) IL-6 and (F) TNF-α in PBMCs. The protein levels in cell culture supernatants were measured after 24 h. All results are expressed as the mean (S.E.M.). *P < 0.05, **P < 0.01, ***P < 0.001.

(A–C) The production of these cytokines was markedly enhanced in response to S100A4 compared with S100A8 and S100A12 but lower than S100A9. Blocking TLR4 (n = 5) or the MyD88 (n = 4–5) adaptor protein significantly down-regulated the S100A4-induced secretion of (D) IL-1β, (E) IL-6 and (F) TNF-α in PBMCs. The protein levels in cell culture supernatants were measured after 24 h. All results are expressed as the mean (S.E.M.). *P < 0.05, **P < 0.01, ***P < 0.001.
Fig. 2 S100A4 activates nuclear factor κB (NF-κB) and the kinases p38 and ERK1/2 in mononuclear cells from patients with RA.

(A–C) Inhibition of NF-κB (n = 5), p38 and ERK1/2 kinases (n = 8) resulted in significant down-regulation of TNF-α production, whereas (D) inhibition of the JNK MAP kinase (n = 8) did not alter the levels of TNF-α released by PBMCs treated with S100A4. (E) The treatment of PBMCs with S100A4 multimer induced phosphorylation of p38 and ERK1/2, but not JNK, with significant increases observed at 15 and 30 min, respectively. Protein levels in cell culture supernatants were measured after 24 h. All results are expressed as the mean (s.e.m.). *P < 0.05, **P < 0.01.
not clearly demonstrated. However, there are conflicting reports of either elevated, reduced or unchanged production of cytokines in different cell types treated by S100A12 [12, 14]. More importantly, we show that S100A8, and particularly S100A4 and S100A9, share similar proinflammatory properties. It is well established that S100A9 is commonly expressed as a heterodimer together with S100A8, however, recent data have revealed that S100A9 can also heterodimerise with S100A4 [15]. The heterodimers as well as homodimers can interact with the TLR4 or RAGE receptors [15]. According to Björk et al. [15], S100A4 homodimer interacts with the TLR4 at a lower response level and with a slightly slower off-rate in contrast to S100A9. We have demonstrated that the TLR4 receptor is engaged in the S100A4-mediated immune response, however, the proinflammatory effect of S100A4 is somewhat lower when compared with S100A9. In this context we can speculate that this can be attributed to the fact that S100A9 binds to TLR4 with a signal that is higher than the response from S100A4. Our data on the S100A4/TLR4 proinflammatory axis are in line with the work of Vogl et al. [13], who showed that S100A8/9 up-regulated TNF-α production in monocytes via TLR4. Furthermore, we demonstrated that blocking S100A4 binding to RAGEs has no significant effect on inflammatory cytokine production in PBMCs, as previously documented for some S100 proteins [8, 12]. Consistently S100A12 was recently described as a TLR4 ligand that induces monocyte activation, whereas the engagement of RAGEs is not essential for S100A12-induced monocyte activation [14].

In addition, we have demonstrated that the inhibition of TLR4 signalling in S100A4-stimulated mononuclear cells reduced the production of several cytokines by 22–55%, whereas inhibition of the adaptor protein MyD88 decreased cytokine production by 90%. Because signalling through most TLR family members and the IL-1 receptor depends on the MyD88 signalling adaptor [20], it can be hypothesized that some other receptors are involved in S100A4-induced inflammation.

Furthermore, consistent with S100A8/9 [13], we demonstrated that S100A4 induced TNF-α release from PBMCs via activation of NF-κB and the MAP kinases ERK1/2 and p38. This is in agreement with a previous study that demonstrated phosphorylation of these MAP kinases and downstream activation of NF-κB by S100A4 in chondrocytes [8]. Our findings suggest that extracellular S100A4 binds to TLRs and activates MAP kinase signalling pathways and the transcription factor NF-κB to stimulate the production of crucial proinflammatory cytokines in mononuclear cells.

Conclusion

Taken together, our results suggest a possible mechanism through which S100A4 mediates the innate immune response. We demonstrated that mononuclear cells were responsive to the extracellular S100A4 multimer. Our results suggest that S100A4 is a novel TLR-4 ligand that activates intracellular proinflammatory signalling pathways. Therefore, targeting S100A4 may be a potential therapy for immune-mediated diseases.

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**Supplementary data**

Supplementary data are available at Rheumatology Online.

**References**


