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S100A8 Triggers Oxidation-sensitive Repulsion of Neutrophils

H. Y. Sroussi^{1*}, J. Berline², P. Dazin³, P. Green⁴, and J. M. Palefsky²

¹Department of Oral Medicine and Diagnostic Sciences, University of Illinois at Chicago, College of Dentistry (M/C 838), 801 S. Paulina St., Room 556, Chicago, IL 60612-7213, USA

²Department of Medicine, University of California at San Francisco, San Francisco, CA, USA

³Howard Hughes Medical Institute, University of California at San Francisco, San Francisco, CA, USA

⁴Department of Oral and Maxillofacial Surgery, University of California at San Francisco, San Francisco, CA, USA

Abstract

The inflammatory response to tissue injury is a multi-faceted process. During this process, neutrophils migrate in the extravascular spaces, directed to the site of injury by chemical gradients generated by chemotactic molecules. S100A8, a protein associated with a wide variety of inflammatory conditions, is heavily over-expressed in association with inflammation. We hypothesized that human S100A8 possesses neutrophil-repelling properties that result in an anti-inflammatory effect *in vivo*. The chemotactic activity of S100A8 on neutrophils was tested in Transwell chemotaxis assays. Analysis of the data indicates that S100A8 causes a repulsion of peripheral neutrophils, an activity that S100A8 loses upon its oxidation. Using a mutant of S100A8 resistant to oxidation and consistent with the *in vitro* findings, we demonstrated that S100A8 causes a strong anti-inflammatory effect in the rat air-pouch model of inflammation *in vivo*. These data highlight a naturally occurring novel anti-inflammatory pathway and provide potential molecular targets for the development of novel anti-inflammatory therapeutics.

Keywords

S100A8; anti-inflammatory; fugetaxis; oxidation; neutrophil

INTRODUCTION

Integrated signals from a variety of chemokines regulate leukocyte movement in a complex process in which each chemokine, secreted proteins with a signature cysteine structure, act in coordination with other chemokines (Butcher, 1991; Foxman *et al.*, 1997). The effects of chemokines are concentration-dependent. Whereas chemokines attract leukocytes at low concentrations, exponentially greater concentrations may also inhibit this migratory process or repel leukocytes (Zlatopolskiy and Laurence, 2001). Poznansky *et al.*, (2002) have termed this process 'fugetaxis', to describe retrograde cellular movements driven by chemokines.

Infectious lesions of the mucosa and skin are generally associated with the recruitment of an acute and/or chronic inflammatory leukocytic infiltrate. In contrast, hairy leukoplakia, a benign oral lesion associated with Epstein-Barr virus, is characterized by the absence of leukocyte infiltrates (Daniels *et al.*, 1987). S100A8, a small acidic protein with reported chemotactic effect (Ryckman *et al.*, 2003), is heavily over-expressed in the lesion (J. Palefsky, unpublished observation). It is possible that high levels of the S100A8 protein repel or inhibit the recruitment

*corresponding author, sroussih@uic.edu.

of leukocytes. Consistent with this hypothesis, the S100A8 null mouse is associated with lethal infiltration of the S100A8 null fetus with heterozygous maternal cells (Passey *et al.*, 1999).

S100A8 and its hetero-dimerization partner S100A9 are members of the S100 family of proteins. This family is comprised of low-molecular-weight protein members of the EF-hand endowed superfamily of calcium-binding proteins. S100A8 and S100A9 lack a leader sequence or a transmembrane region and are secreted by a novel secretory pathway (Rammes *et al.*, 1997). S100A8 and S100A9 are detected at high levels in a wide variety of inflammatory conditions (Gabrielsen *et al.*, 1986), both locally in epithelium and in the saliva and circulation (Muller *et al.*, 1994; Luger *et al.*, 1995a,b). They are expressed in cells of myeloid lineage, representing up to 45% of neutrophil cytosolic protein weight (Edgeworth *et al.*, 1991), in monocytes and tissue macrophages, and in epithelial cells in diseases such as psoriasis (Nagpal *et al.*, 1996). Normal buccal mucosal epithelium expresses S100A8 and S100A9 constitutively at low levels (Wilkinson *et al.*, 1988), whereas keratinized epithelium expresses the two proteins only under pathologic conditions (Marionnet *et al.*, 2003).

The murine homologue of S100A8 is a strong chemoattractant for peripheral monocytes and neutrophils at a very low concentration (10^{-13} M) (Lackmann *et al.*, 1993). Studies of the chemoattractant properties of human S100A8 have yielded variable results. Whereas Ryckman *et al.* (2003) reported chemotactic activity for human S100A8, others have been unable to demonstrate a similar effect (Lackmann *et al.*, 1993). This discrepancy could be attributed to differences in methodology used to produce and assay the proteins. Thus, the effect of S100A8 on leukocyte migration remains controversial.

In this study, we used *in vitro* and *in vivo* assays to address this issue. We hypothesized that the human S100A8 protein possesses chemotactic properties *in vitro*, and that these may correspond to an anti-inflammatory effect *in vivo*.

MATERIALS & METHODS

Recombinant and Mutant S100 Proteins

S100A8 proteins were produced, purified, and cleaved from a GST fusion protein expressed in a pGEX-2T GST vector (Amersham, Piscataway, NJ, USA) in Top-10 F' *E. coli* (Invitrogen, Carlsbad, CA, USA), according to the standard protocol recommended by the manufacturer and as previously described (Tugizov *et al.*, 2005). The endotoxin level in the recombinant protein was below 1 ng/μg of proteins, as measured by limulus amoebocyte lysate assay (LAL) (Associates of Cape Cod, Falmouth, MA, USA). The protein was kept at -20°C in a solution consisting of 10^{-2} M TRIS (pH 7.5), 0.1% cholate, 10^{-3} M EDTA, and 10^{-3} M beta-mercaptoethanol (BME) to prevent their oxidation. The ala⁴²S100A8 mutation was generated from wild-type (WT) S100A8, with the pGEX-2T GST vector containing the WT S100A8 sequence as a template through a cut-and-paste strategy. The resulting mutation and the WT sequence were confirmed with standard sequencing methods. Protein concentrations were determined through a Bradford assay. For use in experiments as oxidized S100 proteins, WT and mutated S100A8 were treated with 10^{-5} M sodium hypochlorite on ice for 30 min.

Transwell Migration Assays of Peripheral Neutrophils

The assays were adapted from methods previously described by others (Wu *et al.*, 2001; Hanson and Quinn, 2002; de Coupade *et al.*, 2004). Blood was obtained according to a protocol approved by the Committee on Human Research of the University of California, San Francisco (UCSF), with informed consent.

Peripheral neutrophils were isolated from heparinized blood on a Ficoll gradient from healthy adult volunteers. A 100-μL quantity of RPMI containing 100,000 neutrophils was placed in

the upper chamber of a Transwell apparatus (6.5 mm, 3- μ m pore, polycarbonate membrane, Corning Costar Inc., Corning, NY, USA). The Transwells were incubated at 37°C, 5% CO₂ for 3 hrs. The cells that migrated through the upper chamber's filter to the lower chamber were collected and counted by flow cytometry and/or a hemocytometer. For the pertussis toxin (PTX) inhibition experiments, cells were pre-incubated with different concentrations of *Bordetella pertussis* toxin (Sigma, St. Louis, MO, USA) for 30 min at room temperature.

***In vivo* Chemotaxis Assay in the Air-pouch Model**

Assays were conducted according to a protocol that was approved by the Committee on Animal Research at UCSF. Air-pouch rats were anesthetized with 2–3% isoflurane in oxygen. Their backs were shaved and swabbed with 70% ethanol, and a 20-mL quantity of sterile air (passed through a 0.2- μ m filter) was injected subcutaneously to form an air-pouch. Three days later, this procedure was repeated, except that only a 10-mL quantity of sterile air was injected. Rats were used for assessment of leukocyte recruitment 3 days following the second air injection.

Leukocyte Harvesting—Rats were injected with 300 μ L LPS (30 ng) or sterile PBS into the seven-day-old air-pouch. Three hrs later, the rats were anesthetized with pentobarbital (65 mg/kg), and cells were collected by the injection of 5 mL of sterile PBS into the pouch. Cell-containing fluid was aspirated, placed into sterile culture tubes, and centrifuged at 1500 rpm for 10 min (25°C). Supernatant was aspirated and cells re-suspended in 1 mL PBS containing 1% bovine serum albumin at room temperature. For separation of nucleated cells from red blood cells, we added 10 μ L of 1 mg/mL Hoechst 33342 (bisbenzimidazole) (Serotec, Raleigh, NC, USA) to flow cytometry tubes for at least 40 min in the dark for antibody labeling (see below); we used 100 μ L of sample. A 1- μ L quantity of 1 mg/mL propidium iodide (Sigma, St. Louis, MO, USA) was added immediately before flow cytometry, so that nucleated cell viability could be assessed.

Flow Cytometry—We performed leukocyte quantitation using nuclear content, forward-scatter, and side-scatter patterns obtained from excitation at 488-nM and 354/63-nM wavelengths. We used FITC-labeled rabbit anti-rat polymorphonuclear leukocyte (PMN) antibody (Accurate, Berkshire, UK) to quantify the number of PMNs recruited to the air-pouch. Log fluorescence was measured for 30 sec at constant pressure for each sample, by means of a triple laser Vantage SE cell sorter (Becton Dickinson, San Jose, CA, USA). Data acquisition was performed with CellQuest Pro software, version 4.01 (Becton Dickinson), and off-line analysis was performed with FlowJo, version 4.5 (Tree Star, Inc., Ashland, OR, USA).

Data Analysis

Data are presented as mean $\pm$ SD. Data were analyzed with a one-way ANOVA analysis with Statview 5.0.1. For the statistical analysis, ANOVA (with repeated measures) and Scheffé constants were used for comparison of more than two variables for determination of significance.

RESULTS

S100A8 Acts Not as a Chemoattractant But as a Repellant

The addition of S100A8 to the lower chamber of the Transwell did not result in a chemotactic effect (Fig. 1A) at any of the concentrations tested. Conversely, IL-8 displayed a dose-dependent chemotactic effect. We then added S100A8 to the upper chamber of the Transwell assay, to test the potential fugetactic effect of S100A8 on neutrophils, a method previously described (Poznansky *et al.*, 2002). The addition of S100A8 to the upper well of the Transwell apparatus increased the number of neutrophils that migrated to the lower well, with a peak concentration of 10⁻⁹ M (Fig. 1A). To differentiate between a chemokinetic and a repellant effect down a

gradient (fugetaxis), we conducted a partial checkerboard experiment at a concentration of S100A8 that had a maximal effect (10^{-9} M). Analysis of the data showed that the increased migration to the lower well was not observed when the neutrophils were placed in an equimolar concentration of S100A8 in the upper and lower wells (Fig. 1B), supporting a directional fugetactic activity exerted by S100A8 on peripheral neutrophils *in vitro*. Further experiments in the Transwell system demonstrated that the fugetactic effect of S100A8 protein on neutrophils was pertussis-toxin-sensitive (Fig. 1C).

A Species-conserved Cysteine Residue of S100A8 Confers Functional Sensitivity to Oxidation

S100A8 had a fugetactic effect on neutrophils at concentrations substantially lower than the concentration in normal human serum (Lugering *et al.*, 1995b), which suggests that S100A8 functions are tightly regulated and at least partly controlled by post-translational modifications. Previous reports have indicated that murine S100A8 chemotaxis, observed *in vitro* at 10^{-13} M, was negatively regulated by oxidative modification of a species-conserved cysteine residue in the hinge region (Lackmann *et al.*, 1993; Harrison *et al.*, 1999).

To determine if human S100A8-mediated fugetaxis was similarly regulated, we mutated the cysteine 42 residue to an alanine (ala⁴²S100A8). The mutant protein was unable to form a covalently bound homodimer of S100A8, even after treatment with 10^{-5} M sodium hypochlorite for 15 min (Fig. 2A). Monoclonal antibodies directed against WT S100A8 detected ala⁴²S100A8 in a Western blot (Fig. 2B). Ala⁴²S100A8 displayed a fugetactic effect similar to that observed with the WT S100A8 protein on peripheral neutrophils. However, in contrast to WT S100A8, ala⁴²S100A8 fugetaxis was dose-dependent, but oxidation-resistant (Fig. 2C). We also noted a shift in the maximal fugetactic effect between WT S100A8 and ala⁴²S100A8, from 10^{-9} M to 10^{-8} M, respectively, similar to the effect on chemotaxis observed by investigators using mutated murine S100A8 (Harrison *et al.*, 1999).

Ala⁴²S100A8 Acts as an Anti-inflammatory Agent in the Rat Air-pouch Model

To address the physiological relevance of S100A8 activity, we tested the *in vivo* effect of S100A8 on LPS-driven inflammation in a rat air-pouch model. Because inflammatory processes are associated with an oxidative burst that could potentially inhibit the function of the recombinant S100 proteins, we studied the *in vivo* activity of the oxidation-insensitive mutant of S100A8 (ala⁴²S100A8). Consistent with the *in vitro* data, ala⁴²S100A8 displayed a potent anti-inflammatory effect by reducing leukocyte recruitment to the air-pouch. Inflammation generated by the injection of 30 ng of LPS into the air-pouch was abrogated by ala⁴²S100A8 in the 10^{-5} M range (Fig. 3A). The anti-inflammatory effect of ala⁴²S100A8 was further shown to be dose-dependent (Fig. 3B).

The effects of small quantities of endotoxin on the results in the air-pouch assay were evident when a GST control, prepared according to a suboptimal endotoxin removal method, was used (data not shown). Similarly, ala⁴²S100A8 purified according to a suboptimal endotoxin removal protocol also increased neutrophil recruitment. This effect was lost and, instead, fugetaxis in the air-pouch assay was seen when recombinant protein ala⁴²S100A8 purified according to our optimized endotoxin removal protocol was used.

DISCUSSION

In this study, the effect of S100A8 on neutrophil movement was tested in the Transwell system. S100A8 was found to have a repellent activity on neutrophils that could be distinguished from a chemokinetic effect in a partial checkerboard experiment. While our experiments did not directly address the mechanism of fugetaxis, we showed that pertussis toxin inhibited fugetaxis,

similar to the inhibition of function of other S100 proteins, such as S100L (Komada *et al.*, 1996), S100A9 (Newton and Hogg, 1998), and the murine homologue of S100A8 (Cornish *et al.*, 1996). Pertussis toxin is known to inhibit the activity of most classic chemotactic cytokines (chemokines), which suggests that activation of an α_i trimeric GTPase pathway may play a role in S100-mediated fugetaxis.

Murine S100A8 activity has been shown to be regulated by oxidation, and we explored the role of oxidation in modulating human S100A8 activity. S100A8 activity was abrogated by oxidation, and, conversely, we were able to create a mutant of S100 resistant to oxidation by mutating the cysteine residue at position 42 to alanine. The ensuing molecule (ala⁴²S100A8) displayed a fugetactic effect similar to that of the wild-type S100A8 except for its resistance to inhibiting oxidation. This finding not only supported a role for oxidation in the regulation of S100A8 fugetaxis, but also offered a potential strategy for efficient anti-inflammatory intervention.

This strategy was explored in a set of experiments in which ala⁴²S100A8 displayed a potent and dose-dependent anti-inflammatory effect in an LPS-driven inflammation in a rat air-pouch model *in vivo*. This model, widely used for the *in vivo* study of synovial arthritis (Dransfield *et al.*, 1992; Perretti *et al.*, 2002), was used to test the effect of S100A8 on the recruitment of neutrophils *in vivo*. Using this model, we showed that ala⁴²S100A8 had a potent anti-inflammatory effect, abrogating the ability of endotoxins to recruit neutrophils to the pouch.

The results presented in our paper differ from previous chemotaxis reports (Ryckman *et al.*, 2003). One explanation for this discrepancy is that previous investigators reported chemotaxis at concentrations several logs below physiological concentrations of S100A8 and S100A9, thus raising the question as to its biologic relevance (Roth *et al.*, 2003).

Overall, our findings support an important role for S100A8 in the regulation of inflammatory processes, and suggest that S100A8 may create a chemical barrier to inflammatory infiltrates, possibly explaining the paucity of inflammatory responses in hairy leukoplakia. Future studies should determine the level of expression and the oxidation status of S100A8, since this may be a potential local controlling factor in mucosal inflammatory conditions. We propose a model in which oxidative modification of S100A8 by neutrophil enzymes secreted during acute inflammation may attenuate this barrier, allowing for leukocyte translocation and migration to extravascular spaces.

Finally, our findings may have important therapeutic implications, and our results indicate the feasibility and effectiveness of the therapeutic anti-inflammatory effect of ala⁴²S100A8. The concentration of ala⁴²S100A8 required to achieve fugetaxis in the air-pouch model was low and likely to be pharmacologically achievable for therapeutic purposes. Further studies will be needed to establish the mechanisms and the utility of the findings described in this report.

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Abbreviations

EDTA, ethylene diamine tetraacetic acid; LAL, limulus amoebocyte lysate assay; PTX, pertussis toxin; FSC, forward scatter; IL-8, Interleukin-8; fMLP, formyl-Met-Leu-Phe; MCP1, monocyte chemotactic protein 1.

Figure 1.

Transwell migration of peripheral neutrophils. (A) The data are the mean of a representative experiment conducted in triplicate ($\pm$ SD) with cells isolated from different healthy adult volunteers. IL-8 was introduced in the lower chamber. S100A8 was introduced in the lower or upper chamber of the Transwells. Analysis of the data indicated a dose-dependent increase in neutrophil migration to the lower chambers of the Transwells when IL-8 and S100A8 were added to the lower and upper chambers, respectively. The addition of S100A8 to the lower wells did not have any measurable effect at the concentrations and conditions tested ($\#p < 0.05$; $*p < 0.01$ when compared with control with no compound added). (B) The data represent the average of 4 experiments performed in duplicate ($\pm$ SD) with cells isolated from different

adult volunteers. S100A8 was introduced in the lower, upper, or both chambers of the Transwells at a concentration of 10^{-9} M. Analysis of the data indicated that the increased migration of neutrophils observed when S100A8 was added to the upper well was inhibited by the addition of S100A8 at an equimolar concentration in the lower well ($^*p < 0.01$ when compared with control with no compound added). (C) The data represent the average of 2 experiments performed in duplicate ($\pm$ SD) with cells isolated from different adult volunteers. S100A8 was added to the upper chamber of the Transwell at a concentration of 10^{-9} M. IL-8-mediated chemotaxis served as a positive control for the effect of pertussis toxin and was added to the lower chamber of the Transwell at a concentration of 10^{-9} M. The cells were incubated with different concentrations of pertussis toxin for 30 min at room temperature before the beginning of the assay. Analysis of the data indicated that the fugetactic effect of S100A8 was pertussis-toxin-sensitive. IL-8-mediated chemotaxis displayed a similar sensitivity ($^*p < 0.01$ when compared with the migration ratio with no pertussis toxin for IL-8 and S100A8 separately).

Figure 2.

Non-reducing gel electrophoresis and chemotaxis assays of recombinant wild-type (WT) S100A8 and ala⁴²S100A8 exposed to 10⁻⁵ M hypochlorite (OCl). (A) Coomassie-blue-stained gel (1) WT S100A8 and (2) ala⁴²S100A8. ala⁴²S100A8 did not form covalently bound dimers. (B) Western blot analysis of WT S100A8 and mutated S100A8 with monoclonal antibodies to S100A8. (1) WT S100A8; (2) ala⁴²S100A8. The antibodies recognized both WT S100A8 and ala⁴²S100A8. (C) The data represent the average of 3 experiments performed in duplicate ($\pm$ SD) with cells isolated from different adult volunteers. Transwell fugetaxis assays of neutrophils with WT S100A8 and ala⁴²S100A8 oxidized by 10⁻⁵ M OCl on ice for 30 min. The fugetactic effect of ala⁴²S100A8 was resistant to oxidation, whereas the WT S100A8 protein's fugetactic effect was inhibited by oxidation ($\#p < 0.05$; $*p < 0.01$ when compared with control with no compound added). We calculated the migration ratio by dividing the number of cells that migrated to the lower well in each experiment by the number of cells in the lower well in the untreated control wells.

Figure 3.

Effect of ala⁴²S100A8 on lipopolysaccharide (LPS)-induced recruitment of neutrophils in a rat air-pouch model. The data represent a mean ($\pm$ SD). **(A)** Air-pouches were injected with 300 μ L of a PBS control, 30 ng LPS in PBS, and 30 ng LPS in PBS containing 10⁻⁵ M of ala⁴²S100A8. Co-injection of ala⁴²S100A8 inhibited the inflammatory response induced by LPS, as indicated by the number of neutrophils recruited by LPS into the air-pouch (* p < 0.01 when compared with PBS-only control). **(B)** Dose response of the inhibitory effect of ala⁴²S100A8 on the recruitment of neutrophils induced by LPS in the air-pouch model. All the air-pouches were injected with 30 ng LPS in 300 μ L PBS containing different concentrations of ala⁴²S100A8. ala⁴²S100A8 displayed dose-dependent inhibition of the recruitment of

neutrophils ($\#p < 0.05$; $*p < 0.01$ when compared with LPS-only control; n indicates the number of rats in each treatment group). PMN, polymorphonuclear leukocyte.