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Inflammation Research

The C-terminus of murine S100A9 inhibits spreading and phagocytic activity of adherent peritoneal cells

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Abstract. *Objective and design:* In the present study, the effect of a synthetic peptide (H^{92}–G^{102}) identical to the C-terminus of murine S100A9 (mS100A9p) was investigated on adherent peritoneal cell function.
Materials and methods: For in vitro assays, peritoneal cells were obtained from the abdominal cavity of mice and incubated, with the different concentrations of mS100A9p, for 1 h, and then their spreading and phagocytosis activities were evaluated. For ex-vivo assays, cells obtained from animals treated for 1 h with the peptide were submitted to the mannose-receptor phagocytosis assay. Shorter homologue peptides to the C-terminus of mS100A9p were also evaluated on in vitro phagocytosis assays of *Candida albicans* particles.
Results: mS100A9p reduced both the spreading index and phagocytic activity, in vitro and ex-vivo, independent of the receptor evaluated. The homologue peptide corresponding to the H^{92}–E^{97} region of mS100A9p, the zinc-binding motif, was responsible for such an effect.
Conclusion: These results suggest a modulator effect of the C-terminus of S100A9 protein on the function of adherent peritoneal cells.

Key words: S100A9 – Adherent peritoneal cells – Spreading – Phagocytosis – Zinc

Introduction

The calcium-binding proteins S100A8 (MRP-8) and S100A9 (MRP-14) belong to the S100 protein family [1, 2], and form a complex called calprotectin [3]. The expression of these proteins is restricted to circulating granulocytes and monocytes [4, 5], representing approximately 45 and 1%, of the soluble cytosolic content of these cells, respectively [6]. S100A8 and S100A9 are translocated from cytosol to the cytoskeleton and membranes of phagocytes upon elevations of intracellular calcium concentration [7]. The complex is secreted upon protein kinase C activation, indicating its extracellular role [8]. Thus, increased plasma levels of S100A8/A9 have been found in patients suffering from a number of inflammatory disorders, including cystic fibrosis, rheumatoid arthritis, and chronic bronchitis [9, 10], making this complex a very useful biomarker of inflammatory diseases [11]. The S100A8/A9 complex binds polyunsaturated fatty acids in a calcium-dependent manner [12, 13], has zinc-dependent antimicrobial activity [14–16], and induces apoptosis in tumour cells and normal fibroblasts in a zinc-reversible manner [17–19].

Independent expression and functioning of S100A9 have also been studied [20–23]. Extracellular S100A9 regulates transendothelial migration of phagocytes, by activating β_2 integrins [24, 25] and deactivating activated peritoneal macrophages [23]. Previous investigations by our group have demonstrated that S100A9 posses a potent antinociceptive activity in an inflammatory pain model, either when injected directly in peritoneum [26] or when secreted by neutrophils in acute inflammatory peritonitis induced by glycogen or carrageenin in mice [26, 27]. We have recently observed that the C-terminus of murine S100A9 inhibits hyperalgesia and oedema induced by a metalloprotease [28]. Interestingly, the C-terminus of human S100A9 protein has sequence identity with neutrophil immobilizing factor (NIF) [29, 30] and the contact domain of high molecular weight kininogen (HMWK) [31]; besides, it is involved with zinc- and arachidonic acid-binding motif of S100A9 [32–34]. Although there is a number of hypotheses, the exact functions of either S100A9 or its C-terminus, especially in the extracellular milieu, remain unknown.

Phagocytes expressing S100A8 and S100A9 are the first cells to infiltrate in inflammatory lesions [4, 20]. The elimination of the injuring agent and cell debris present in local lesion, are undertaken by phagocytes, mainly neutrophils and macrophages [35]. Macrophages are terminally differentiated end-cells, characterized as motile, highly secretory and phagocytic cells [36, 37], and are found in several organs, tissues and cavities, where they remain quiescent as resident

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cells with low functional activities [38]. These cells play a central role for the inflammatory and immunological responses, secreting several chemistry mediators, including pro- and anti-inflammatory cytokines [39, 40], which are directly involved in the modulation of inflammatory pain response [41]. Considering these data, we decided to evaluate the effect of a synthetic peptide identical to the C-terminus of murine S100A9 (mS100A9p) on the adherent peritoneal cell function of mice. Experiments were carried out in assays of spreading and phagocytosis by using opsonized sheep erythrocytes, opsonized zymosan and non-opsonized particles of *Candida albicans* for evaluation of phagocytosis mediated by Fc-, C3b- or mannose-receptor, respectively.

Material and methods

Animals

Male Swiss mice weighing between 18–22 g were used in this study. All procedures were in accordance with the guidelines for animal experimentation, and the practices were approved by the Institutional Animal Care Committee at the Instituto Butantan (CEUAIB, protocol number 072/2002).

Synthesis of the peptides

The peptide H-E-K-L-H-E-N-N-P-R-G-H-G-H-S-H-G-K-G (H^{92}–G^{110}), identical to the C-terminus of murine S100A9 (mS100A9p), was synthesized based on the sequence published earlier [42]. Other three peptides were synthesized based on mS100A9p: H^{92}–G^{102}, H^{92}–E^{97} and E^{97}–G^{102}. Peptides were synthesized in solid phase by FMOC technique at the Department of Biophysics, Pharmacology Institute, Federal University of São Paulo, Brazil. The characterization and purification of these peptides were carried out by HPLC, and their mass evaluated by MALDI-TOF spectrometry.

Peritoneal cell preparation

Animals were killed in a CO_2 chamber and their peritoneal cavity was washed with 5 ml of cold phosphate-buffered saline (PBS), pH 7.4. After a gentle massage of the abdominal wall, the peritoneal fluid, containing resident cells, was collected. Cell viability was assessed by the Trypan blue exclusion test (>95%). Total peritoneal cells were determined in a Neubauer's chamber, and the differential counts were carried out in smears stained with a panchromatic dye [43]. For all measurements, samples of individual animals were used. The assays were always performed in duplicates.

Spreading of adherent peritoneal cells

The spreading capacity of peritoneal cells was estimated according to the method described earlier [44]. Briefly, 100 µl of cell suspension in PBS (approximately 1×10^5 of total cells) were placed onto glass cover slips and left to adhere for 15 min at room temperature. The cover slips were washed with PBS and incubated in RPMI 1640 medium (culture cell medium developed at Roswell Park Memorial Institute, USA; Gibco) at 37 °C for 1 h. Cells were fixed in a 2.5% glutaraldehyde solution and the index of spread cells was determined by examination under a phase contrast microscopy. The index of spreading activity was defined as the ratio between spread cells and 100 cells counted. Spread cells were defined as adherent peritoneal cells which changed their rounded to a flattened shape, showing a lower refractile body and a higher diameter as compared to unspread cells [44].

Phagocytic activity of adherent peritoneal cells

The cover slips containing the adherent and spread peritoneal cells were incubated with 1 ml of RPMI 1640 medium containing opsonized sheep erythrocytes, opsonized zymosan or non-opsonized *Candida albicans*, in an atmosphere containing 5% CO_2, for 1 h at 37 °C. The percentage, i.e., cells that had phagocytosed more than three particles, was determined in smears stained with a panchromatic dye [43], by examination under light microscopy.

Phagocytosis of opsonized sheep erythrocytes

A suspension of sheep erythrocytes was diluted in PBS (0.5%) and mixed (v/v) with rabbit antiserum against sheep erythrocytes and subsequently the mixture was incubated for 30 min at 37 °C. Opsonized erythrocytes were washed twice with PBS by centrifugation at 184 g for 10 min and then suspended in RPMI 1640 medium (0.5%) for the phagocytosis assay.

Phagocytosis of opsonized zymosan

Zymosan particles, obtained from yeast cell walls (Zymosan A, Sigma Chem. Co., USA) were suspended in PBS providing a concentration of particles of 5.7 mg/ ml. For opsonization, the zymosan suspension was mixed 1:1 with normal mice serum and incubated for 30 min at 37 °C [45]. Opsonized zymosan particles were then centrifuged at 184 g for 10 min washed with PBS, and suspended in RPMI 1640 medium for the phagocytosis assay. The count of particles was about 1×10^6 per cover slip.

Phagocytosis of Candida albicans

Candida albicans (ATCC Y-537) was cultured in 8% Sabouraud's dextrose broth (Microbiology and Mycology Laboratories, Department of Clinical Analyses, Faculty of Pharmaceutics Science, University of São Paulo) at 30 °C, for one day. Fungi were suspended in 3 ml of Dulbecco's PBS for determining *C. albican* count in a Neubauer's chamber, and then the particles were suspended in RPMI 1640 medium for the phagocytosis assay. Fungi viability was determined by exclusion of 0.01% methylene blue (>98%). The particle count was approximately 1×10^6 per cover slip.

Treatments in vitro

Cover slips containing the adhered peritoneal cells were incubated with (1) 1 ml of RPMI 1640 medium (control), (2) RPMI 1640 containing mS100A9p – H^{92}–G^{110} peptide (0.07, 0.15, 0.29, 0.59, 1.17, 2.35, 4.7 µM); (3) H^{92}–G^{102} peptide (0.47, 0.94, 1.87, 3.74, 7.48 µM); (4) H^{92}–E^{97} peptide (3.16, 6.31, 12.62, 25.25 µM) and (5) E^{97}–G^{102} peptide (3.65, 7.29, 14.58, 29.16 µM) at 37 °C, in an atmosphere containing 5% CO_2. After 1 h, the cover slips containing the spreading peritoneal cells were fixed to evaluate the spreading, or washed with PBS and prepared for the phagocytosis assay. Assays with H^{92}–G^{102}, H^{92}–E^{97} and E^{97}–G^{102} peptides were evaluated only in mannose-receptor mediated phagocytosis.

Treatments in vivo

Mice were inoculated by intraperitoneal route (ip.) with mS100A9p (2.35, 4.7, 18.78, 37.57 µM or 0.5, 1, 4, 8 µg) dissolved in saline (final volume 200 µl) or saline (control), before 1 h the collection of peritoneal cells for assaying mannose-receptor mediated phagocytosis. Cell viability was determined by the Trypan blue exclusion test for both in vitro and ex-vivo assays.

Statistical analysis

Comparisons between experimental and control groups were initially tested by analysis of variance (ANOVA). The alpha level (significance level related to the probability of rejecting a true hypothesis) was set at 0.05. Significant differences were then compared using Tukey's test.

Results

In vitro effect of mS100A9p on the spreading activity

mS100A9p caused a statistically significant reduction of spreading index of adherent peritoneal cells (Fig. 1). This inhibitory effect was observed with the concentrations of 0.59, 1.17 and 2.35 µM, with the percentage of inhibition 26, 21 and 32%, respectively, when compare with the percentage obtained by control group (Fig. 1).

In vitro effect of mS100A9p on phagocytosis

Adherent peritoneal cell phagocytosis was inhibited by mS100A9p, for all receptors evaluated. The concentrations of mS100A9p that induced an inhibitory effect on sheep erythrocytes phagocytosis were 0.15 (22%), 0.29 or 1.17 (30%) and 2.35 µM (41%) (Fig. 2A). The concentration of 0.59 µM of pS100A9m was evaluated in five different experiments and the results obtained were always reproduced. Phagocytosis using zymosan particles was reduced with concentrations of 0.29 (27%), 0.59 (15%) and 1.17 µM (24%) (Fig. 2B). When phagocytosis was evaluated on mannose receptors, mS100A9p induced an inhibitory effect of 43% to 0.29, 0.59 and 2.35 µM and 57% to 1.17 µM (Fig. 2C).

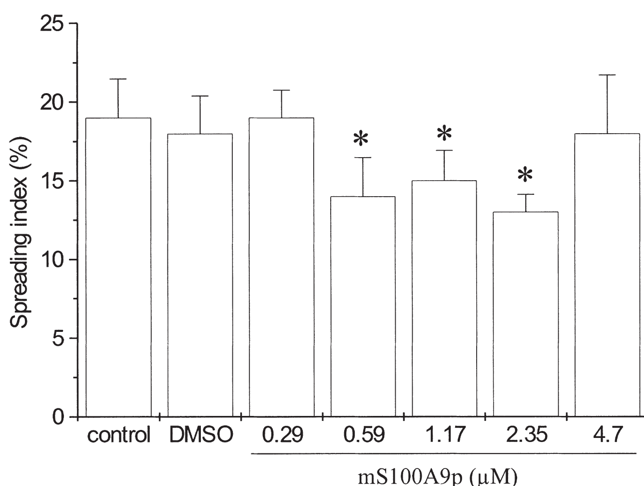

Fig. 1. In vitro effect of C-terminus of murine S100A9 (mS100A9p) on the spreading of adherent peritoneal cells. Spreading was determined in peritoneal cells obtained from naive mice and incubated during 1 h with mS100A9p or RPMI 1640 medium (control group). Results are expressed as means ± S.E.M., using six animals per group. * $P < 0.05$, significantly different from control group.

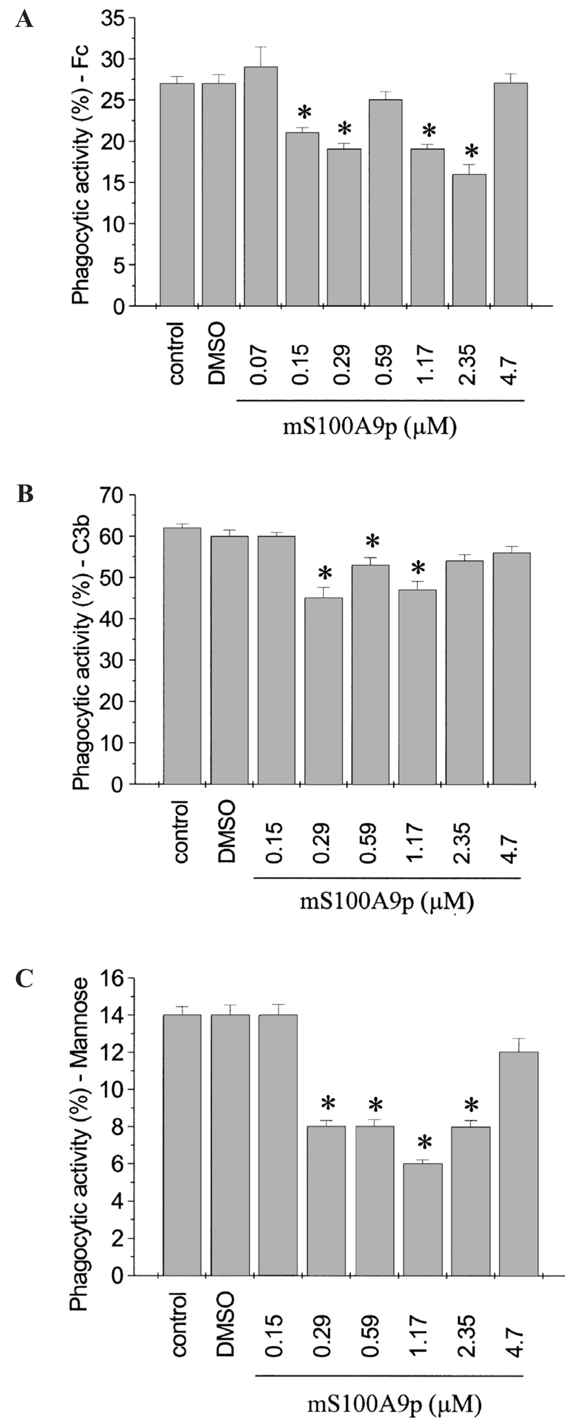

Fig. 2. Phagocytic activity of adherent peritoneal cells incubated with mS100A9p. Phagocytic activity was determined in peritoneal cells collected from naive mice and incubated with mS100A9p or RPMI 1640 medium (control) at 37 °C for 1 h. Particles of sheep erythrocytes opsonized with rabbit antiserum (A), zymosan opsonized with serum of untreated mice (B), or *C. albicans* (C) were used as phagocytic stimuli. Results are expressed as means ± S.E.M., using six animals per group. * $P < 0.05$, significantly different from control group.

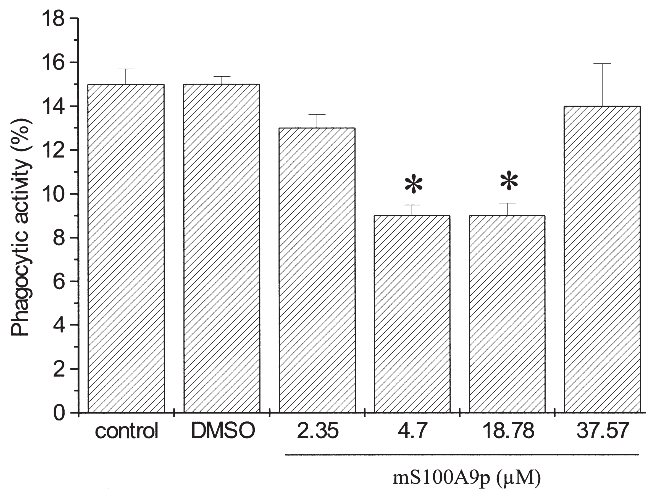

Fig. 3. Ex-vivo effect of mS100A9p on adherent peritoneal cells phagocytosis. mS100A9p (2.35, 4.7, 18.78, 37.57 µM) dissolved in saline (final volume 200 µl/mouse) or saline (control group) was injected ip. 1 h before peritoneal cells were collected. Cells obtained from the peritoneal cavity were evaluated by a phagocytosis assay using particles of *Candida albicans*. Results are expressed as means ± S.E.M., using six animals per group. $^*P < 0.05$, significantly different from control group.

Ex-vivo effect of mS100A9p on phagocytosis

For this assay, cells were obtained from the abdominal cavity after 1 h of ip. inoculation of mS100A9p, and then evaluated in an ex-vivo assay of phagocytosis via mannose receptor. mS100A9p inhibited phagocytosis of *Candida albicans* particles, similarly as observed in vitro. This effect was induced both by 4.7 and 18.78 µM of mS100A9p per mouse, causing 40% inhibition on adherent peritoneal cell phagocytosis (Fig. 3).

Effect of shorter homologue peptides of mS100A9p on phagocytosis

In order to investigate the effect of shorter sequences of mS100A9p on peritoneal cell function, the phagocytic activity via mannose receptor was assessed in cells incubated with the different concentrations of H^{92}–G^{102}, H^{92}–E^{97} and E^{97}–G^{102} peptides. The peptide corresponding with region H^{92}–G^{102} induced an inhibitory effect on phagocytosis of adherent peritoneal cells and this effect was observed with the concentrations of 0.94 (27%), 1.87 (33%), 3.74 (40%) and 7.48 µM (20%) (Fig. 4A). This inhibitory effect also was induced by H^{92}–E^{97} peptide and was 40% to 6.31 and 12.62 µM and 20% to 25.25 µM (Fig. 4B).

The peptide E^{97}–G^{102} did not interfere with phagocytosis of adherent peritoneal cells in all evaluated concentrations (Fig. 4C).

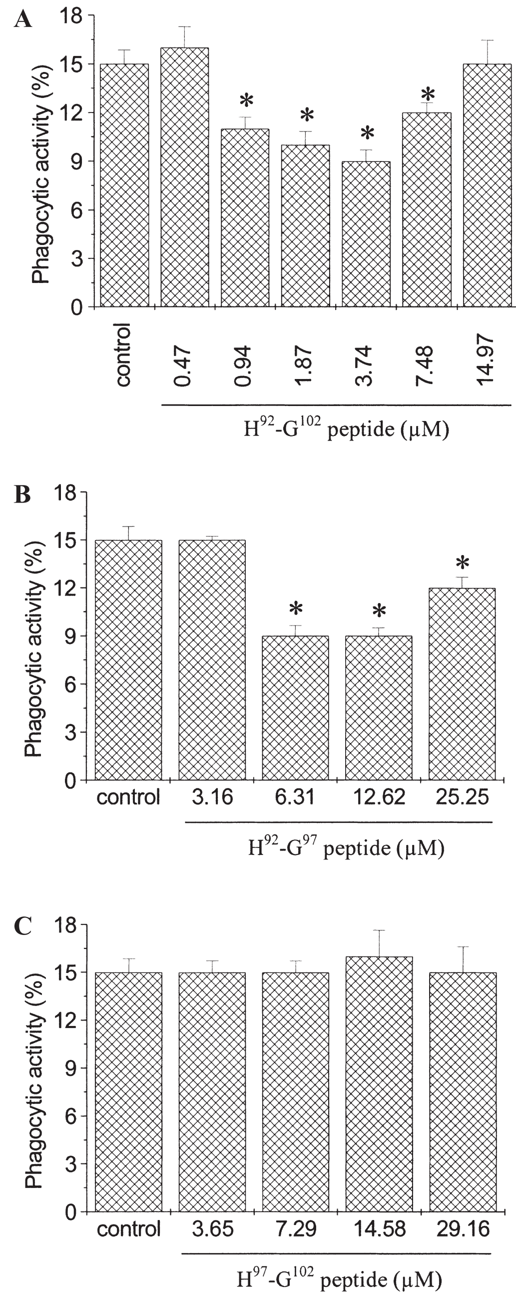

Fig. 4. In vitro effect of shorter homologue peptides to mS100A9p on phagocytosis of adherent peritoneal cells. Phagocytic activity was determined in peritoneal cells collected from naive mice and incubated with H^{92}–G^{102} (A), H^{92}–E^{97} (B) and E^{97}–G^{102} (C) peptides, or RPMI 1640 medium (control) at 37 °C for 1 h. The phagocytosis assay was evaluated on particles of *Candida albicans*. Results are expressed as means ± S.E.M., using six animals per group. $^*P < 0.05$, significantly different from control group.

Discussion

Phagocytes that express S100A8 and S100A9 proteins belong to the first group of cells that infiltrate in inflammatory sites and play a pivotal role in innate immune responses [20, 46]. These proteins attracted a special interest due to their high cytosolic concentration in phagocytes and their

high intracellular calcium-binding capacity [7]. Activated macrophages are responsible for the release of mediators involved in inflammatory pain [41]. Considering previous studies of our group, which demonstrated an inhibitory effect of either S100A9 or its C-terminus portion on inflammatory pain models [26, 28], as well as considering that S100A9 deactivates activated macrophages [23], we decided to evaluated the effect of mS100A9p on the function of adherent peritoneal cells.

Data presented herein show that the C-terminus of S100A9 (mS100A9p) protein induces a direct inhibitory effect on spreading and phagocytic activity of adherent peritoneal cells, and that such an effect does not depend on the type of the receptor involved. Inhibition was observed on phagocytosis mediated by Fc, C3b and mannose receptors, and this effect was not due to loss of membrane integrity, since cell viability, as assessed by Trypan blue exclusion test, was higher than 95%.

Other assays were performed only on mannose-receptor mediated phagocytosis, since either S100A8/A9 complex or the C-terminus of S100A9 has candidastatic properties [16, 33, 47]. mS100A9p also presents an ex-vivo effect on peritoneal cells, since inhibition of mannose-receptor mediated phagocytosis was observed in harvested cells from the peritoneal cavity of mice pre-treated with it. Furthermore, this ex-vivo effect may have a contribution of endogenous compounds generated after the treatment with the mS100A9p.

Several reports have described the association of S100A8 and S100A9 with cytoskeletal elements; however, the functional relevance of these observations has so far not been shown [7, 48]. Thus, it was proposed that both proteins are involved in the terminal differentiation of myeloid cells by inhibiting casein kinase [49], are secreted by a mechanism that involve intact microtubules [8], and induce cytoskeletal rearrangement involving the G/F-actin balance in S100A9-null neutrophils exhibiting a more organized microfilaments system [50]. Both spreading and phagocytic activities of phagocytes are related processes and require the arrangement of cytoskeletal proteins for their adequate occurrence [51–53], involving the polymerization of actin at the site of ingestion, the internalization of the particle and the phagolysosome maturation via an actin- and tubulin-based mechanism [54]. However, the intracellular pathways involved in particle ingestion during phagocytosis and the arrangement of cytoskeletal proteins on the phagosome surface depend on the phagocytic receptor involved, being these mechanisms distinct for ingestion of IgG- and complement-opsonized particles [54, 55]. The fact that this cytoskeletal organization may be responsible for the inhibitory effect induced by mS100A9p, since that the deregulation of the microfilament system is essential for the spreading and phagocytosis of these cells [56].

S100A8/A9 bind arachidonic acid (AA) in a calcium-dependent manner [12, 13, 57]. The secreted S100A8/A9-AA complex may serve as a transport protein to move AA to its target cells and the subsequent metabolism of AA into eicosanoids, representing a particular transcellular pathway for its metabolism. This mechanism may point out to an important role in the initiation and regulation of the inflammatory response [58, 59]. It was demonstrated that eicosanoids, particularly prostaglandin E_2 (PGE_2), have an inhibitory effect on phagocytic activity [60, 61]. In regard to that, PGE_2 may be involved in the inhibitory response observed with mS100A9p since the C-terminus of S100A9 contains a putative AA-binding site [34]. Other factors that may be involved with this inhibitory effect on peritoneal cells are the pro- and anti-inflammatory cytokines, which are important regulators of macrophage function, stimulating and/or inhibiting their inflammatory properties [62]. Currently, the involvement of either lipid-derived mediators or cytokines in the inhibitory effect induced by mS100A9p on the function of peritoneal cells is under investigation.

In addition to the binding of calcium, S100A9 protein has also been shown to bind zinc, apparently in a distinct and independent region of the calcium binding domains [42]. The C-terminus end of S100A9 contains a His-x-x-x-His motif, which represents the zinc binding site [33]. Its zinc binding capacity reverses the calcium-induced AA-binding capacity [57] and is responsible by antimicrobial activity [63]. It has been assumed that in some cases the biological activity of S100 proteins is regulated by zinc rather than by calcium [64].

In order to characterize the region of the C-terminus of S100A9 responsible for its inhibitory effect on the function of adherent peritoneal cells, phagocytosis assays were carried out with shorter homologue peptides to mS100A9p. The results demonstrated that the H^{92}–E^{97} region of the C-terminus of S100A9 is the portion involved in the inhibitory effect observed on phagocytosis of *Candida albicans* particles, since either H^{92}–E^{97} or H^{92}–G^{102} peptides induced the same inhibition percentage than mS100A9p. The zinc-binding site in S100A9 involves the residues in HEKMH sequence, at 91–95 [32], suggesting that this site present in C-terminus of S100A9 is involved in the inhibitory effect observed on adherent peritoneal cell phagocytosis via mannose receptor. The participation of zinc ion was shown in works evaluating S100A9 function, including antimicrobial activity, binding of AA and induction of apoptosis [46]; however it is still a matter of speculation whether zinc interferes with phagocytosis assays, since contradictory results have been reported [65]. The involvement of zinc in our model of mannose-receptor mediated phagocytosis is being undertaken and will be the issue of a future investigation.

In conclusion, the present results indicate that the C-terminus of S100A9 inhibits spreading and phagocytic activity by adherent peritoneal cells, suggesting that this peptide may have a regulatory role in phagocyte functions during inflammatory response. Once that S100A9 is expressed and secreted by phagocytes during inflammatory process, this peptide may be useful as tool to understand the mechanisms involved in this process.

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