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Macrophage suppression following phagocytosis of apoptotic neutrophils is mediated by the S100A9 calcium-binding protein

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ABSTRACT

The clearance of apoptotic cells by phagocytes is a fundamental process during tissue remodeling and resolution of inflammation. In turn, the phagocytosis of apoptotic cells generates signals that suppress pro-inflammatory activation of macrophages. These events occur during the resolution phase of inflammation and therefore the malfunctioning of this process may lead to inflammation-related tissue damage. Here, we demonstrate that the calcium-binding protein S100A9, normally abundant in the cytoplasm of neutrophils and also released by apoptotic neutrophils, is involved in the suppression of macrophages after the uptake of apoptotic neutrophils. Both, spontaneous and induced production of inflammatory species (nitric oxide, hydrogen peroxide and TNF-α) as well as the phagocytic activity were inhibited when macrophages were in presence of apoptotic neutrophils, conditioned medium from neutrophil cultures or a peptide corresponding to the C-terminal region of S100A9 protein. On the other hand, macrophages kept in the conditioned medium of neutrophils that was previously depleted of S100A9 were shown to resume the activated status. Finally, we demonstrate that the calcium-binding property of S100A9 might play a role in the suppression process, since the stimulation of intracellular calcium release with ionomycin significantly reversed the effects of the uptake of apoptotic neutrophils in macrophages. In conclusion, we propose that S100A9 is a novel component of the regulatory mechanisms of inflammation, acting side-by-side with other suppressor factors generated upon ingestion of apoptotic cells.

Introduction

Neutrophils play an important role in antibacterial and antifungal defense mechanisms. These leukocytes migrate to inflamed tissues early in acute inflammatory responses, where they eliminate pathogens by phagocytosis and release of toxic mediators (Borregaard and Cowland 1997; Hampton et al. 1998). Subsequently, neutrophils undergo apoptosis and are phagocytosed by macrophages, a sequence of events that ensures the removal of apoptotic neutrophils from injured tissues and prevents the release of hazardous intracellular contents from dying cells (Erwig and Henson 2007; Savill and Fadok 2000).

Phagocytosis of apoptotic neutrophils not only prevents the release of toxic and immunogenic intracellular factors but also leads macrophages to express an anti-inflammatory or deactivated phenotype (Fadok et al. 1998). The intake of apoptotic neutrophils decreases the ability of macrophages to further phagocytose cells upon a second challenge with apoptotic neutrophils (Erwig et al. 1999). Thus, considering that phagocytic clearance of apoptotic neutrophils plays a role in the resolution of inflammation and restoration of homeostasis, the identification of regulators of this process poses clear implications for the understanding of and intervention on the inflammatory response (Gilroy et al. 2004).

Abbreviations: MDF, macrophage deactivating factor; ROS, reactive oxygen species; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; SSZ, sulfasalazine; NO, nitric oxide; H_2O_2, hydrogen peroxide; (TNF)-α, tumor necrosis factor alpha; PBS, phosphate buffer saline; IFN-γ, interferon gamma; PGE2, prostaglandine E2; TGF-α, tumor growth factor alpha; NF-κβ, nuclear factor kappa beta

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S100A8 (MRP-8) and S100A9 (MRP-14) are the members of the S100 protein family (Kerkhoff et al. 1998; Schafer and Heizmann 1996) expressed by monocytes and granulocytes such as neutrophils (Roth et al. 1993). These two calcium-binding proteins interact to form a complex called calprotectin (Steinbakk et al. 1990), which is detected in high concentrations in patients with inflammatory disorders like cystic fibrosis, rheumatoid arthritis, and chronic bronchitis suggesting conceivable extracellular roles for these proteins (Kerkhoff et al. 1998). Independent functioning of S100A9 has also been documented indicating marked anti-inflammatory effects in a rat model of arthritis (Brun et al. 1995) and anti-nociception activity in a model of acute peritonitis in mice, either when injected directly in the peritoneum (Giorgi et al. 1998) or when secreted by neutrophils (Giorgi et al. 1998; Pagano et al. 2002, 2006).

Epithelioid macrophages found in the tuberculosis granuloma in mice selectively express S100A9, and a close homology of S100A9 with the macrophage deactivating factor (MDF), a factor also produced by epithelioid cells, has been suggested (Aguiar-Passeti et al. 1997). In fact, findings in our laboratory demonstrated that S100A9 inhibits the production of reactive oxygen species (ROS) by activated macrophages both *in vivo* and *in vitro* (Aguiar-Passeti et al. 1997). In addition, it was demonstrated that a peptide corresponding to the C-terminus region of murine S100A9 inhibits the spreading and phagocytic activities of adherent peritoneal cells (Pagano et al. 2005). However, the mechanism by which S100A9 downreagulates macrophages remains unclear.

Considering the data above, the aim of the present study is to verify whether the suppression of macrophages following phagocytosis of apoptotic neutrophils could be mediated by S100A9 present in neutrophils cytoplasm. Here, we show that the uptake of apoptotic neutrophils by macrophages leads to suppression of pro-inflammatory activation of the latter *in vitro* and that S100A9 is a mediator of this suppressor mechanism. In addition, we show that the modulation of intracellular calcium by S100A9 is likely to be a key component of this inhibitory phenomenon.

Material and Methods

Animals

Swiss mice 6 to 8 weeks of age were obtained from the animal facility at Federal University of São Paulo. All procedures were in accordance with the current guidelines for animal experimentation.

Reagents

Lipopolysaccharide (LPS) from *E. coli* (055:B5), ionomycin and phorbol 12-myristate 13-acetate (PMA) were from Sigma (Saint Louis, MO, USA). The rat monoclonal antibody (IgG1) anti-murine S100A9 (anti-MRP-14, EPM1B4D3) was produced in our laboratory. The peptide HEKLHENNPRGHGHSHGKG (H92-G110), corresponding to the C-terminal region of murine S100A9 (mS100A9p) (Voganatsi et al. 2001), was kindly provided by Dr. Luiz Juliano Neto from Federal University of São Paulo, Brazil. An irrelevant peptide with the sequence CRGSGAFSC was used as a control for the peptide H92-G110.

Macrophage cultures

Macrophages were obtained from mice by washing the peritoneal cavity with RPMI1640 culture medium (Sigma). Cells were resuspended (2×10^6 cells/ml) in an RPMI1640 medium supplemented with 10% fetal bovine serum (complete medium) and 100 μl of the cell suspension was dispensed on glass coverslips in 24-well-plates following incubation for 1 h at 37 °C. The culture supernatant containing non-adherent cells was discarded, medium was replaced and the culture was maintained at 37 °C for 72 h when further treatments were performed.

Preparation of apoptotic neutrophils

Mice were injected intraperitoneally with 500 μl of zymosan suspension (Zymosan A, Sigma, 2 mg/ml) to recruit neutrophils to the peritoneal cavity. Neutrophils were harvested 4 h later by peritoneal wash with RPMI1640. Induction of apoptosis was carried out by treating neutrophils with 1 μM Sulfasalazine (SSZ, a gift from Apsen Farmaceutica, SP, Brazil) (Akahoshi et al. 1997) in complete medium in siliconized tubes for the indicated times. Further cells were washed with RPMI1640 medium, resuspended in complete medium and added to the macrophage cultures prepared as described above.

DNA extraction and analysis

Neutrophils in culture with SSZ were harvested after the indicated times and washed with PBS. Genomic DNA was extracted as described before (Jeanpierre 1987). DNA samples were resuspended in deionized water containing 20 μg/ml of RNAse and incubated for 15 min at 37 °C. The DNA content was determined spectrophotometrically by reading the absorbance at 260 nm. To confirm DNA integrity and apoptosis of neutrophils, the samples were analyzed by electrophoresis in agarose gels as described previously (Fliss and Gattinger 1996). DNA bands were visualized under UV light after staining with ethidium bromide (Sigma) and images were digitally acquired.

Phagocytosis assay

Macrophages grown on coverslips in 24-well-plates as described above were co-cultured with apoptotic neutrophils (1:4 ratio) for 24 h. Subsequently, 1×10^5 zymosan particles were added and the cultures were incubated for 1 h at 37 °C. The culture medium was then renewed and cells were further incubated for 2 h to allow completion of particles uptake. Next, the cultures were rinsed with PBS, stained with hematoxilin and examined by light microscopy. The score used to evaluate the phagocytosis was 0, 1–4, 5–9 and 10 or more particles ingested by macrophages.

Nitric oxide (NO) and hydrogen peroxide (H_2O_2) detection

NO production by macrophages was indirectly determined by measuring the nitrite content in culture supernatants by the Saville–Griess assay as described before (Eu et al. 2000). Both spontaneous and PMA-induced production of H_2O_2 by cells was measured as described by Pick and Mizel (1981) and adapted by Russo et al. (1989).

Tumor necrosis factor (TNF)-α detection

TNF-α release by macrophages was assessed by cytotoxicity assays. Murine fibroblasts L929 (ATCC) were kept in complete medium. For toxicity assays, these cells were distributed in 96-well-plates at 4×10^5 cells/well in the conditioned culture medium of macrophages cultured in presence or absence of apoptotic neutrophils as described above. Actinomycin-D (Sigma, 8 μg/ml) was then added to the culture and cells were incubated