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Research paper

Expression and modulation of S100A4 protein by human mast cells

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ABSTRACT

S100A4 protein is expressed in fibroblasts during tissue remodelling and in cancer stem cells and it induces the metastatic spread of tumor cells. In mast cells (MCs) S100A4 have been found in some pathological conditions, but its function in normal MCs remains to be described. The purpose of this study was to characterize the cellular localization of the S100A4 protein in MCs of human tissues with inflammatory or tumor disorders and, to determine the consequence of reducing its expression in MC response. We found that tissue resident MCs stained positive to S100A4. Both human HMC-1 cell line and resting $CD34^+$-derived MCs expressed S100A4, whose levels were differentially modulated upon MC activation. Downregulation of the S100A4 protein resulted in MC growth inhibition, enhanced apoptosis and deregulation of MMP-1 and MMP-10 production. Our results suggest that S100A4 is also playing a role in the MC life cycle and functions.

1. Introduction

The S100 protein family is a multigenic group of Ca^{2+}-binding proteins involved in the regulation of Ca^{2+} homeostasis and, as a consequence, in cell activation, proliferation and differentiation. S100 proteins have been shown to play a role during inflammatory processes of a variety of histopathological and fibrosis conditions and in metastasis development of several cancers [1,2]. In particular, S100A4, also known as fibroblast-specific protein 1 (FSP1), is expressed by fibroblasts of organs undergoing tissue remodelling and it is used as a specific marker for epithelial to mesenchymal transition (EMT) during tissue fibrogenesis [3,4]. Due to its ability to regulate angiogenesis, cell survival, motility, and invasion, S100A4 is found to be responsible for the promotion of metastasis of several cancer cells [5–7]. Indeed, in pancreatic adenocarcinoma, in colorectal, and breast cancer samples the absence of S100A4 can be used as a prognostic indicator of disease-free survival [8–10]. In some tumor cells, the nuclear fraction of S100A4 participates in the transcriptional regulation of proteins involved in invasive and metastatic processes [11]. For example, through modulation of expression of metalloproteinases (MMPs) and their tissue inhibitors (TIMPs), S100A4 can support the remodelling of the extracellular matrix [12]. Similarly, in non-cancer pathologies like rheumatoid arthritis (RA) and osteoarthritis (OA) S100A4 modulates the expression of MMPs [13,14] but, to our knowledge, information about S100A4 subcellular localization in healthy and inflammatory tissues is still scarce.

Beside fibroblast, S100A4 is found in a subset of human inflammatory macrophages of injured livers [15], in smooth muscle cells of normal and chronic obstructive pulmonary disease (COPD)-affected lungs [16], in activated T lymphocytes and in neutrophils of breast cancer specimens [17]. Moreover, it has been reported that mast cells (MCs) present in breast cancer, in RA and in idiopathic inflammatory myopathies are S100A4 positive [17–19]. Nevertheless, besides fibroblasts, the expression and function of S100A4 in normal cell types,

Abbreviations: COPD, chronic obstructive pulmonary disease; EMT, epithelial to mesenchymal transition; FICZ, 6-formylindolo[3,2-*b*]carbazole; FC, flow cytometry; FSP1, fibroblast-specific protein 1; IF, immunofluorescence; IHC, immunohistochemistry; IntDen, integrated fluorescence density; MCs, mast cells; MMPs, metalloproteinases; OA, osteoarthritis; pmaxGFP, plasmid DNA encoding for green fluorescent protein; PMA, phorbol 12-myristate 13-acetate; TIMPs, tissue inhibitors; RA, rheumatoid arthritis

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including MCs, remains to be fully characterized [6].

MCs are located in all tissues at the host–environment interface, such as skin and mucosa, where they are one of the first cell types of the immune system to interact with environmental antigens and allergens [20]. MCs can contribute to immune response to infection through multiple mechanisms such as pathogen recognition, activation, recruitment of other immune cells to the site of infection, release of inflammatory mediators, and direct bacterial killing [21,22]. MCs can be found in hypertrophic scars, where they are considered as a key stimulus for fibroblasts [23,24].

S100A4 protein could be involved in several aspects of MC biology: in the regulation of $Ca2^+$ influx observed upon cell activation [25]; in the potentiating role of MCs in skin remodelling and fibrosis [26,27] and in the modulation of allergic and non-allergic inflammatory processes [28]. Moreover, MC-derived soluble factors are able to induce EMT in different tumor settings [29–31], therefore the S100A4 molecule could play parallel roles among fibroblast and MCs cell types.

Since information about the expression of the S100A4 protein within MCs in normal and pathological conditions is only fragmentary, in this report we analyzed the cellular localization of the MC-specific marker Tryptase and the S100A4 protein in human tissues with inflammatory or tumor disorders. To find the role of S100A4 in MC biology, its expression was tested in $CD34^+$-derived human MCs and in the HMC-1 cell line where activation signals promoted S100A4 nuclear translocation. Short hairpin (sh)-mediated S100A4 gene silencing was applied to evaluate the intrinsic function of this protein to cell growth, cell cycle and apoptotic rate, as well as the effect on MMPs secretion upon cell activation.

2. Materials and methods

2.1. Tissues and cell culture

Tissue specimens were obtained from the University Hospital of Udine. This study was conducted according to the Declaration of Helsinki and with Internal Review Board approval (approval number 35/2014). Healthy tissue specimens (endometrium, urinary bladder, and colon), inflamed tissues (bladder cystitis and Crohn's disease), benign and malign tumor-affected samples (skin nevus and melanoma, respectively), and blood sample were obtained from the University Hospital of Udine. Blood samples were prospectively collected while tissue specimens (formalin fixed and paraffin embedded) were retrospectively selected from the pathologic archive of the University Hospital of Udine. Human $CD34^+$-derived MCs were obtained from 4 different donors as described in [32] and results are the mean of 4 experiments performed with each cell culture preparation. Briefly, $CD34^+$ cells were isolated and purified from discarded autologous stem cell concentrates. To this end, after centrifugation (RT, 400*g*, 10 min) the cells were resuspended in a 4 ml medium (RPMI with pen/trep, Life Technologies GmbH, Darmstadt, Germany), filtered through a 100 µm cell strainer (TPP, Trasadingen, Switzerland), and incubated (RT, 15 min) with 100 µl Fc-Block (Miltenyi, Bergisch Gladbach, Germany) plus 100 µl $CD34^+$ selection cocktail (clone QBEND/10, Stemcell Technologies, Grenoble, France). Next, the cells were incubated with 100 µl nanoparticles (RT, 15 min), followed by cell separation using an EasySep® Magnet (Stemcell Technologies) according to the manufacturer's protocol. Finally, 10^6–10^7 sorted $CD34^+$ cells were diluted with 7 ml of SFEM serum-free culture medium (Stemcell Technologies), with pen/strep (Life Technologies), 50 µg/ml human LDL solution (Stemcell Technologies), human recombinant IL-3 (100 ng/ml [3300 U/ml], Biolegend, Fell, Germany) and SCF (100 ng/ml [40 U/ml], Miltenyi). Every three to four days IL-3 and SCF were added to a final concentration of 20 ng/ml (660 U/ml IL-3 and 8 U/ml SCF). At the end of the second week of culture, 10 ng/ml SCF was added and the cells were left on IL-3-withdrawal for at least another week (resting phase). All the data presented were generated with peripheral $CD34^+$ stem cell-derived mast cells after 3 weeks of culture.

The human MCs line HMC-1 was cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO2 in complete RPMI-1640 medium supplemented with 10% FBS (Euroclone, Milano IT). To test the modulation of the S100A4 expression, HMC-1 cells ($1x10^6$ cells/ml) were cultured with 40 nM phorbol 12-myristate 13-acetate (PMA) plus 0.5 µM A23187 (Sigma-Aldrich, St. Louis, MO, USA) or 300 nM 6-formylindolo[3,2-*b*]carbazole (FICZ) (Enzo Life Sciences, Vinci-Biochem, Firenze, Italy) for 48 h. To test S100A4 nuclear translocation, 1×10^6 cells/ml of HMC-1 cells or $CD34^+$-derived MCs were stimulated with the same amount of PMA plus A23187 as above, for 30 or 60 min.

2.2. Immunohistochemistry (IHC), immunofluorescence (IF) and flow cytometry (FC)

We worked with 8 different disease tissue samples, from which we used one slide per sample for IHC and one slide for IF. IHC was performed on 3.5 µm-thick formalin-fixed paraffin-embedded slides deparaffinized and pretreated for epitope retrieval using EnVision™ FLEX citrate buffer, pH 6.0, in PT Link, then placed in an automated immunostainer Autostainer Link 48 (Dako, Glostrup, Denmark).

HMC-1 and $CD34^+$-derived MCs were cytospinned on glass slides (700 × *g*, 5 min) and fixed in 4% paraformaldehyde for 20 min at RT. Sections or fixed cells were incubated 1 h at 37 °C with primary antibodies to S100A4 (see paragraph below) and detected using EnVision™ FLEX system (Dako). Images were obtained with Leica DMD108 digital microimaging network (Leica Microsystems, Wetzlar, Germany).

For tissue IF, sections pretreated in the same way as IHC above, were incubated for 1 h and 30 min at 37 °C with the primary antibody, washed in PBS and incubated for 1 h at 37 °C with the secondary antibody. The same sections were incubated for 2 h at 37 °C with the primary antibody against MC tryptase followed by 1 h at 37 °C with the secondary antibody. Vectashield (Vector Laboratories Inc., Burlingame, CA) added with 0.1 µg/ml 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma) was used as the mounting medium.

For cellular IF, HMC-1 and $CD34^+$-derived MCs were permeabilized for 10 min at RT with 0.1% Triton X-100 (Sigma), incubated over-night at 4 °C with primary and then with secondary fluorescent antibodies. To analyze tissue distribution of S100A4 and tryptase proteins, images were digitally scanned as whole slide images at 40x using a Tissue FAXS system, and analyzed with StrataQuest (from TissueGnostics® Vienna, Austria). The area of analyzed tissues ranged from 11 to 42 mm^2 with an average size of 23 ± 11 mm^2. Software-analysis allowed graphical depiction of the cells as events in dot plots, allowing for gating strategies similar to conventional FC data assessment (Supplementary Fig. 1).

To obtain quantitative data on the nuclear translocation of S100A4 upon MC activation, images of immunofluorescent-labelled cells were acquired with a specific fully-automated imaging system (DMI6000B, Leica Microsystems or BD Pathway 850, Becton Dickinson, San Jose, CA). Quantitation of the total fluorescence of nuclear S100A4 staining was obtained by employing ImageJ software [33]. A mask to measure the average intensity of S100A4 fluorescence was created by applying a threshold to DAPI images. The mask was applied to highlight nuclear areas, which were also measured. S100A4 Integrated Fluorescence Density (IntDen) was computed for each nucleus, multiplying each nuclear area by the respective mean value of S100A4.

For FC, in vitro unstimulated or stimulated cells were fixed and permeabilized for 10 min at RT with 0.1% Saponin/1%BSA. Cells were then incubated with primary antibodies followed by 30 min incubation at RT with the Alexa 488-conjugated secondary antibody. The analysis was performed by FACS-Calibur (Becton Dickinson).

2.3. Antibodies

The primary antibodies used were: mouse anti-human monoclonal