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S100 to receptor for advanced glycation end-products binding assay: Looking for inhibitors

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ABSTRACT

Secreted by tumor and stromal cells, S100 proteins exert their biological functions via the interaction with surface receptors. The most described receptor is the receptor for advanced glycation end-products (RAGE), thereby participating in the S100-dependent cell migration, invasion, tumor growth, angiogenesis and metastasis. Several approaches have been described for determining this interaction. Here we describe an easy, specific and highly reproducible ELISA-based method, by optimizing several parameters such as the binding and blocking buffer, interaction time and concentrations, directed to screen chemical and biological inhibitors of this interaction for S100A4, S100A7 and S100P proteins. The efficiency of the protocol was validated by using well described neutralizing agents of the RAGE receptor and of the S100A4 activity. The methodology described here will allow future works with other members of the S100 protein family and their receptors.

1. Introduction

Malignant and stromal cells communicate each other through cell–cell and cell–matrix interactions and by the secretion of soluble factors, supporting tumor survival and progression [1,2]. There are growing evidences that the expression of the S100 proteins is altered in many tumors, often in association with tumor development and malignance, and that these small calcium binding proteins play a crucial role in the crosstalk between the tumor cell components contributing to the development of the disease [3,4]. It has been described that S100A4, when secreted into the microenvironment, contributes to tumor progression enhancing tumor cells migration and invasion, which results in a higher metastatic potential, and promotes tumor vascularization [5,6]. S100A7 protein has also been related with tumor angiogenesis and metastasis, as well as with immune cell infiltration into the tumor [7], whereas S100P has been associated to tumor cell invasion, proliferation and chemoresistance [8]. The extracellular implication of these S100 proteins, in tumor progression, makes them exciting candidates to be blocked in cancer therapy.

One of the most described receptors for S100 proteins is RAGE, through which the different members of the family activates survival, proliferative and inflammatory pathways [9]. RAGE is a member of the immunoglobulin superfamily of cell surface molecules, consisting of three Ig-domains (V, C1 and C2), a transmembrane region and a cytosolic tail required for RAGE-mediated intracellular signaling [10]. Besides S100/calgranulin family, there are many other ligands which have been described to bind to RAGE, such as AGEs (advanced glycation end-products), amyloid fibrils and amphoterin [11,12]. However, it has been described that some members of the S100 protein family are not ligands for RAGE [13], but exert their activities via other cell surface receptors, such as Toll like receptors (TLRs), scavenger receptors and G-protein-coupled receptors [4].

Some approaches have been directed to block the extracellular interaction between S100 proteins and RAGE. Using surface-plasmon resonance (SPR) analysis, it has been demonstrated that Tasquinimod, a small molecule used in clinical phases for castration-resistant prostate cancer [14], blocks the interaction between S100A9 and its receptors RAGE and TLR4 [15]. In addition, it has been shown that oxyclozanide is able to block the interaction of both S100A4 and S100A9 to RAGE and TLR4 [16]. Using co-immunoprecipitation studies, it was demonstrated that cromolyn was able to block the interaction between S100P and RAGE [17]. Finally, a previous work of our group demonstrated, using SPR analyses, that a mAb against S100A4 was able to block the interaction with RAGE [6].

Abbreviations: ELISA, enzyme-linked immunosorbent assay; RAGE, receptor for advanced glycation end-products; BSA, bovine serum albumin; HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; SPR, surface-plasmon resonance.

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The need for a fast and accurate assay to screen S100 inhibitors, directed to block the extracellular interaction between the protein and the receptor RAGE, lead us to develop an *in vitro* assay to select the best drugs in an easy way. Based on a previous work of Liu and collaborators [18], we have optimized an ELISA assay to make this approach. We demonstrated that the interaction of RAGE to S100 proteins (S100A4, S100A7 and S100P) was time and dose-dependent, and we obtained promising results validating the method by using specific small molecules and mAbs against S100A4 protein.

2. Material and methods

2.1. Recombinant proteins and reagents

To generate the human S100A4, mouse S100A4, human S100A7 and human S100P recombinant proteins, a cDNA encoding the full-length sequence of each protein was obtained by RT-PCR from mRNA of the cell lines HCT-116 (human colon adenocarcinoma), NIH3T3 (mouse embryonic fibroblast), MDA-MB-468 (human breast adenocarcinoma) and BxPC3 (human pancreatic adenocarcinoma), respectively. The primers used in the PCR reaction are sown in Table 1. Sequences were cloned into the NdeI site of the bacterial expression vector pET28a(+) (Novagen) and transformed into *Escherichia coli* Tuner™ (DE3) Competent Cells (Novagen). Recombinant proteins were purified by using HisTrap™ Chelating affinity columns (Amersham).

Chimeric recombinant proteins, human RAGE-Fc, mouse RAGE-Fc and human EGFR-Fc were purchased from R&D Systems, tagged with a C-terminal human IgG1.

For blocking experiments, the following materials were used: goat polyclonal antibody anti-human RAGE (R&D Systems), oxyclozanide (Sigma) and cromolyn (Sigma). Mouse mAbs anti-human S100A4 (LEITAT Technological Center) were obtained as described [6].

2.2. ELISA-based binding assay

Flat-bottom 96-well plates (Maxisorb, NUNC) were coated with different concentrations of human recombinant proteins S100A7, S100A4 or S100P in PBS-Ca^{2+}/Zn^{2+}/Mg^{2+} buffer containing 1 mM $CaCl_2$, 10 μM $ZnCl_2$ and 0.5 mM $MgCl_2$, overnight at 4 °C. Plates were washed 3 times with PBS and were blocked with 1% BSA (w/v) in PBS-Ca^{2+}/Zn^{2+}/Mg^{2+} buffer for 2 h at room temperature. Then, different concentrations of human RAGE-Fc, mouse RAGE-Fc or human EGFR-Fc in blocking buffer were added, and incubation continued during 24, 48 or 72 h at 4 °C. EGFR-Fc was used as a negative control. Unbound receptors were removed by washing 3 times with PBS. To detect the bound receptor, a goat anti-human HRP conjugate (Calbiochem; at a 1/25,000 dilution) in PBS-Ca^{2+}/Zn^{2+}/Mg^{2+} buffer + 0.2% BSA was added and incubated for 2 h at 4 °C. After washing, the tetramethylbenzidine substrate (TMB; Sigma) was added and plates were read at 450 nm using a Multiskan Ascent spectrophotometer (Thermo Corporation). The specific binding was calculated by removing the OD due to the unspecific binding of the receptor (coating-free wells for each condition).

2.3. Blocking assays

2.3.1. S100 protein competition

Human S100A4 protein was used as an inhibitor to determine if S100A7 and S100P interact with the same region of the human RAGE. Specifically, 30 nM of RAGE-Fc was preincubated with the recombinant protein S100A4 at different concentrations in 1% BSA/PBS-Ca^{2+}/Zn^{2+}/Mg^{2+} buffer for 4 h at 37 °C. This combination or the receptor alone was added to previously S100-coated wells. After 48 h, the specific binding was determined as described above.

2.3.2. Anti-RAGE antibody

Goat polyclonal antibody anti-human RAGE (R&D Systems) was used to neutralize the interaction. Specifically, 30 nM of RAGE-Fc was preincubated with the antibody at different concentrations in 1% BSA/PBS-Ca^{2+}/Zn^{2+}/Mg^{2+} buffer for 4 h at 37 °C. This combination or the receptor alone was added to previously S100-coated wells. After 48 h, the specific binding was determined as described above.

2.3.3. Chemical entities

Two small molecules, oxyclozanide and cromolyn were used to test their blocking ability. Human S100A4 (1 μM) was coated in the presence or absence of several concentrations of the correspondent chemical molecule in PBS-Ca^{2+}/Zn^{2+} /Mg^{2+} buffer, over night at 4 °C. Assays with oxyclozanide were made in presence of 20 μM Zn^{2+} as previously described [16]. After blocking, 30 nM of human-RAGE-Fc was added to each well, always in the presence of the correspondent concentration of the inhibitor. After 48 h of incubation, plates were washed and the specific binding was detected as described above.

2.3.4. Anti-S100A4 antibodies

Two specific mouse mAbs anti-human S100A4, 5C3 (neutralizing activity) and 5H4 (non-functional), developed in our laboratory, were used to test their blocking ability. After coating (1 μM S100A4) and blocking, antibodies at 10 and 30 nM in 1% BSA/PBS-Ca^{2+}/Zn^{2+}/Mg^{2+} buffer were added and incubated for 2 h at room temperature. Then, plates were washed 3 times with PBS and human RAGE-Fc at 30 nM was added. Incubation continued during 48 h and the specific binding was determined as described above. A replica plate was used to study the amount of anti-S100A4 antibody present at the end of the experiment. After washing the antibody, 1% BSA/PBS-Ca^{2+}/Zn^{2+}/Mg^{2+} was added without receptor, and the incubation continued during 48 h. Then, the plate was revealed as described above using a goat anti-mouse HRP conjugate (Jackson; at a 1:50,000 dilution).

2.4. Statistical analysis

In all experiments, values are expressed as mean ± standard error of the mean (SEM) as indicated. Statistical analyses were performed by the two-tailed nonparametric Mann Whitney-*U*-test, using the GraphPad Prism software, version 5.04 for Windows. Differences were considered statistically significant at $p < 0.05$. For

Table 1
Primers pairs for amplification of the S100 proteins.

Gene	Forward primer	Reverse primer
Human S100A4	5′-act cac ata tgg cgt gcc ctc tgg aga agg ccc tgg atg tg-3′	5′-act cat gag ctc atc att tct tcc tgg gct gct tat ctg gga a-3′
Mouse S100A4	5′-act cac ata tgg caa gac cct tgg agg agg ccc tgg atg ta-3′	5′-act cat gag ctc atc act tct tcc ggg gtt cct tat ctg ggc a-3′
Human S100A7	5′-act cac ata tga gca aca ctc aag ctg aga ggt cca taa tag-3′	5′-act cat gag ctc atc act ggc tgc ccc cgg aac agg gcg ctg c-3′
Human S100P	5′-act cac ata tga cgg aac tag aga cag cca tgg gca tga tc-3′	5′-act cat gag ctc atc att tga gtc ctg cct tct caa agt act t-3′

saturation experiments, the equation for one site specific binding was used (nonlinear regression) to determine the binding constants, where $Y = B_{max} * X/(K_d + X)$, and the Schatcard analysis was used to visualize the saturation binding data.

3. Reslts

3.1. RAGE binding to S100 proteins

To assess the interaction between RAGE and S100 proteins, different concentrations of each protein were immobilized and several concentrations of human RAGE-Fc were added for different periods of time. As showed in the Fig. 1A, the total binding was dependent on RAGE concentration (from 10 to 300 nM) using a fixed concentration of each S100 protein as a coating (2 μM). The specific binding was calculated by subtracting the OD (450 nm) in the absence of S100 proteins (PBS) considered as the non-specific binding (Fig. 1B). As showed in the table below the Fig. 1C, the K_d values ± std. Error for the interaction of the human RAGE-Fc with S100A4, S100P and S100A7, were 49.59 ± 2.69, 45.90 ± 1.48 and 59.15 ± 4.31 nM, respectively. RAGE binding was also dependent on the S100 concentration, from 250 to 2000 nM (Fig. 1D). The specific binding of the receptor for each concentration of coated protein was calculated as described above (Fig. 1E). Finally, we observed an increase in the interaction between RAGE-Fc and S100 proteins in a time-dependent manner, when added the receptor at 30 nM (Fig. 1F).

3.2. Interspecific interaction between RAGE and S100 proteins

To assess the potential of the inhibitors against human S100 proteins as therapeutic agents, human-to-mouse xenograft tumor models are usually needed. In this system, the S100 protein produced by human tumor cells may activate mouse stromal cells through RAGE receptor, and vice versa.

Here, we wanted to study, if it is possible the interspecific interaction between RAGE and S100 proteins and we demonstrated that the interaction between both the human and the mouse RAGE was possible and almost identical with all S100 proteins tested (Fig. 2). In addition, we used EGFR-Fc chimera to be sure of the specificity of the assay, and we observed no interaction between this receptor and the three S100 proteins.

3.3. S100A4 competes with S100A7 and S100P to interact with RAGE

To determine if S100A4, S100A7 and S100P interact with the same region of the receptor, we analyzed how the presence of S100A4 could block the specific interaction of the other S100 proteins. Fig. 3 shows the binding of RAGE to each S100 protein, after being preincubated with several amounts of human S100A4 for 4 h at 37 °C. In these conditions, the presence of S100A4 impedes the interaction of human RAGE with the coated S100 proteins in a dose-dependent manner. Similar effects were observed for S100A4, S100A7 and S100P proteins, suggesting that they all interact with the same, or very close, region of the receptor.

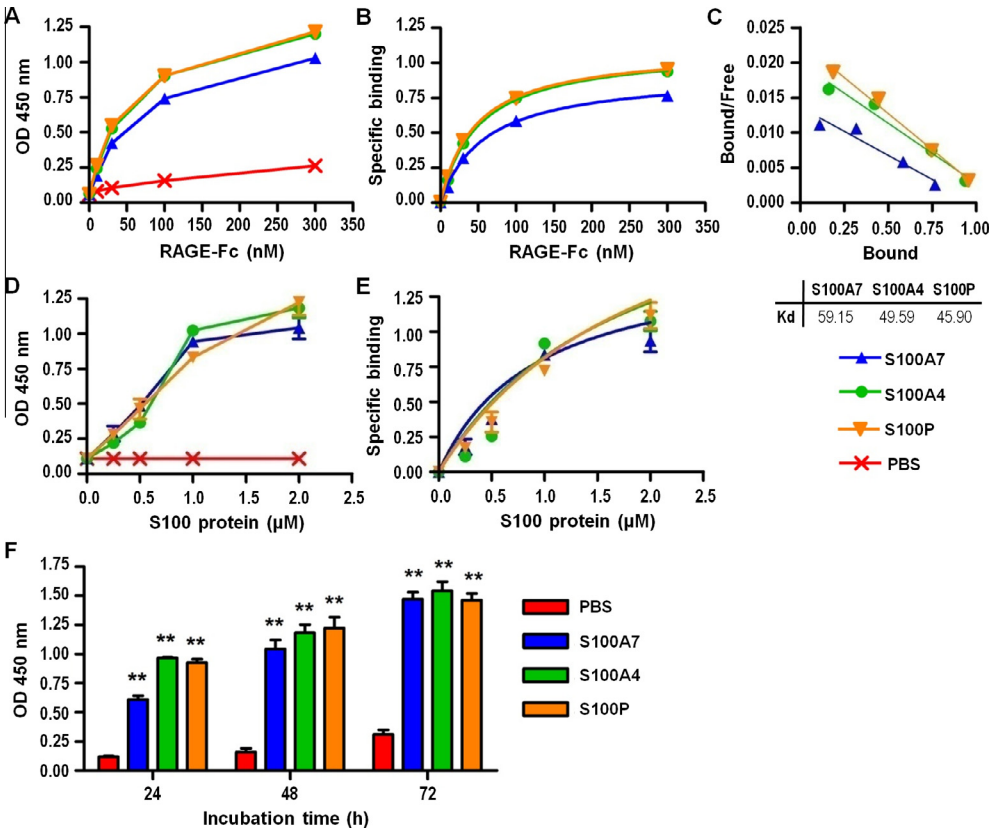

Fig. 1. Interaction between RAGE and S100 proteins. (A) Different concentrations of RAGE-Fc were incubated with a fixed concentration of S100 proteins (2 μM) for 48 h at 4 °C. Wells coated with PBS were considered as the unspecific binding of the receptor. (B) Specific binding of RAGE-Fc after subtracting the unspecific binding. (C) Scatchard plot of the saturation curve. The K_d values ± std. Error of RAGE-Fc for S100 binding were estimated to be 59.15 ± 4.31 nM (S100A7), 49.59 ± 2.69 nM (S100A4) and 45.90 ± 1.48 nM (S100P) using the one site binding equation. (D) Different concentrations of each S100 protein were coated and incubated with 30 nM of RAGE-Fc for 48 h at 4 °C. (E) Specific binding of RAGE-Fc after subtracting the unspecific binding from the total binding at each concentration of coating. (F) Time-dependent interaction between RAGE-Fc (30 nM) and S100 proteins (2 μM). Graphs show mean ± SEM of 3 independent experiments. *Mann–Whitney U-test, **p < 0.01.*

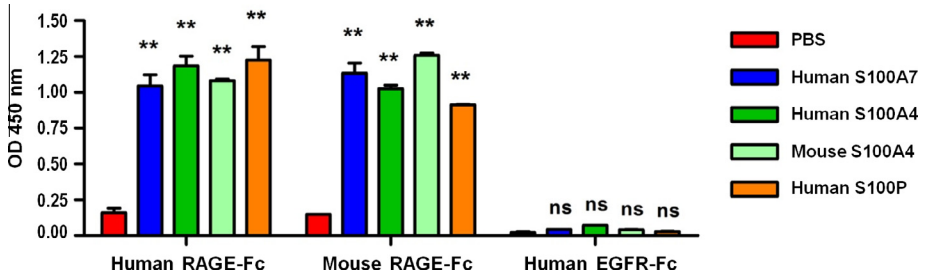

Fig. 2. Interspecific interaction between RAGE and S100 proteins. Each S100 protein was coated at 2 μM and the indicated receptors were added at 30 nM for 48 h. EGFR-Fc was used as a negative control. Wells coated with PBS were considered as the unspecific binding of the receptor. Graph shows mean ± SEM of 3 independent experiments. *Mann–Whitney U-test ns $p > 0.05$, $^{**}p < 0.01$.*

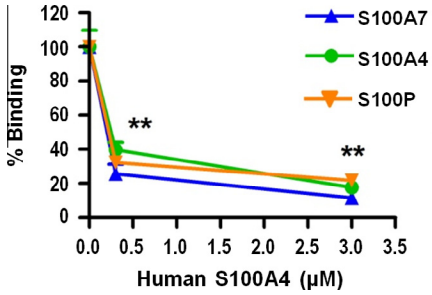

Fig. 3. Competition assay for the S100A4, S100A7 and S100P interaction to RAGE. Human RAGE-Fc (30 nM) was incubated with several amounts of human S100A4 prior to the addition to S100A7 and S100P coated wells (2 μM), for 48 h. Wells coated with S100A4 were considered as the positive control (100% of competition). Percentage of binding was calculated by normalizing the specific binding for each S100 to the receptor, compared with the values in absence of S100A4 protein (100% of interaction). Graph shows mean ± SEM of 3 independent experiments. *Mann Whitney U-test $^{**}p < 0.01$.*

3.4. Anti-human RAGE antibody blocks the RAGE–S100 interaction

To further confirm the specificity of the interaction, we used a goat polyclonal antibody anti-human RAGE widely referenced [19].

Based on the presented results, the chosen conditions to test the inhibitors were: 1 μM of human S100 protein, 30 nM of human RAGE-Fc and 48 h of interaction. Fig. 4A shows the specific binding of RAGE to each S100 protein after being preincubated the receptor with several amounts of anti-RAGE antibody for 4 h at 37 °C. The OD value was normalized to the interaction in the absence of the antibody, considered as the 100% of interaction. The polyclonal antibody anti-human RAGE was able to block the interaction in a dose-dependent manner with a maximum of inhibition of 70% when 300 nM of antibody was used. Similar effects were observed for S100A4, S100A7 and S100P proteins, although at 10 nM of antibody the decrease was not statistically significant for S100A7 protein.

3.5. Inhibition of the S100A4–RAGE interaction using specific chemical compounds

It has been described, using SPR analysis, that oxyclozanide is able to block the interaction of S100A4 and S100A9 to RAGE [16], however there are no references regarding the ability of another molecule, cromolyn (neutralizing agent of the S100P–RAGE interaction [17]), to block the interaction between S100A4 and RAGE. To assess whether the ELISA assay was suitable to study the capacity of chemical compounds to block this interaction, oxyclozanide and cromolyn were used. Fig. 4B shows the specific binding of RAGE (30 nM) to S100A4 (1 μM) in the presence of the indicated concentrations of oxyclozanide and cromolyn. After 48 h of interaction, the receptor bound to S100A4 was significantly reduced when incubated with oxyclozanide in a statistically significant dose-dependent manner. In contrast, cromolyn had no effect on the interaction.

3.6. Inhibition of the interaction using specific mAbs against S100A4 protein

We previously published that S100A4 increased synergistically the migration of endothelial cells in combination with VEGF. The mAb anti-S100A4, 5C3, neutralized this S100A4-induced activity whereas the antibody 5H4 had no effect. Fig. 4C shows the results of the migration assays performed as described in the previous publication [6].

To evaluate whether the ELISA assay was also appropriate to screen inhibitory biological compounds such as mAbs, these two S100A4-specific molecules were used. Antibodies were preincubated with the coated S100A4 (1 μM) for 2 h at room temperature. Then, the antibody was washed and the receptor was allowed to bind to the protein during 48 h. Fig. 4D shows the percentage of binding of RAGE-Fc to S100A4 protein in the presence of 10 or 30 nM of each antibody, normalized to the binding without antibody that was considered as the 100% of interaction. Antibody 5C3 blocked the interaction between S100A4 and RAGE in a statistically significant dose-dependent manner, whereas the 5H4 mAb was not able to block the interaction.

Additionally, we used a replica plate to analyze the amount of each antibody present at the end of the assay. Here, we observed the same quantity of both antibodies (data not shown), demonstrating that the mere presence of the antibody did not interfere the possible interaction.

4. Discussion

Communication between all cell components of the tumor is crucial for cancer progression [20]. S100 proteins, play an important role in this communication [21] and have been shown to interact with the receptor RAGE to regulate a large number of cellular functions such as cell migration and invasion, survival, tumor growth, angiogenesis and metastasis [22]. Several strategies have been directed to block this interaction, using either small molecules or specific mAbs. Therefore, there is a great need of an easy assay to perform an initial screening of these compounds to select the best inhibitors.

In the present study we set up an ELISA-based assay to study the interaction between three members of the S100 protein family and RAGE, optimizing the conditions to screen neutralizing agents.

It is well known the importance of Ca^{2+} and Zn^{2+} on the interaction between S100 proteins and RAGE [23]. For this reason, we

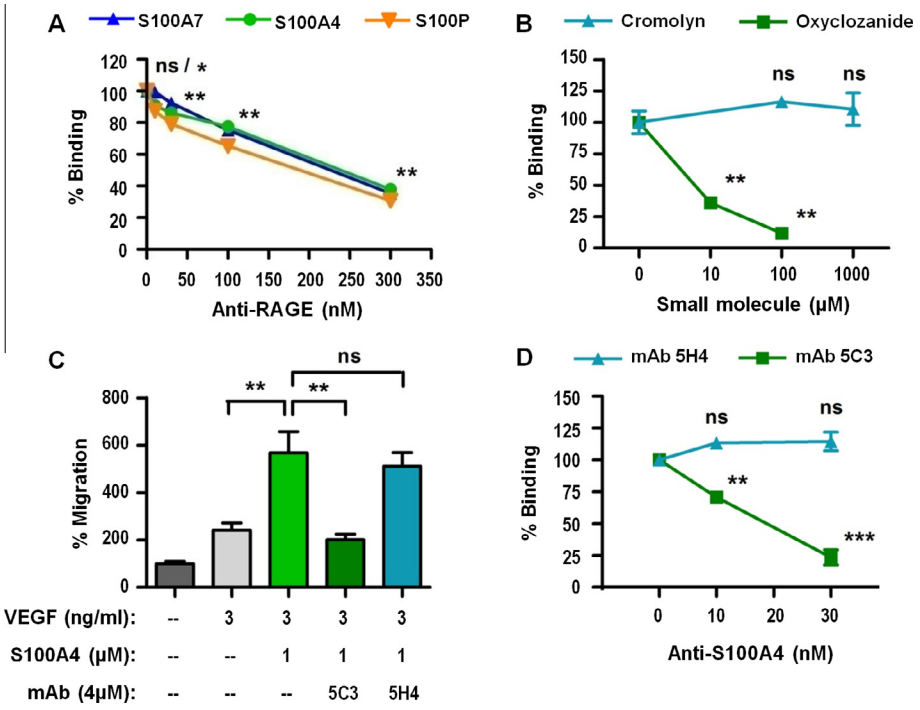

Fig. 4. Validation of the method by using RAGE and S100 inhibitors. (A) Dose-dependent effect of the polyclonal antibody anti-human RAGE on the specific interaction. Human RAGE-Fc (30 nM) was incubated with several amounts of the polyclonal antibody prior to the addition to the S100 coated wells (2 μM), for 48 h. (B) Dose-dependent effect of the oxyclozanide on the specific interaction. RAGE-Fc (30 nM) was added and incubated to the S100A4 coated wells (2 μM), for 48 h, in the presence of the correspondent concentration of each molecule. (C) Transwell migration assay using HUVEC cells. 5C3 mAb was used as a neutralizing antibody and 5H4 mAb was used as a non-functional antibody. (D) Dose-dependent effect of the anti-S100A4 antibodies on the specific interaction. S100A4 protein (1 μM) was incubated with either 10 or 30 nM of each antibody, for 2 h. Then, plates were washed and RAGE-Fc was added (30 nM) and incubated for 48 h. In all experiments, percentage of binding was calculated by normalizing the specific binding of each S100 to the receptor, compared to the values in absence of the inhibitor in each case (considered the 100% of interaction). Graphs shows mean ± SEM of 3 independent experiments. *Mann Whitney U-test ns* $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

used in all the assays a referenced concentration of salts ($CaCl_2$, $ZnCl_2$ and $MgCl_2$) diluted in the binding buffer.

Our main results demonstrated a dose and a time-dependent binding between all three S100 proteins and RAGE. Apparently, as determined by the Scatchard analysis, the K_d value did not differ significantly between each S100 protein (around 50 nM), suggesting that they have similar affinity to the receptor. Although this interaction has been previously described by other approaches, this is the first time that these three proteins are compared together in terms of affinity to its receptor using a new ELISA-based approach, optimizing previous described protocols [18].

Taken into consideration the real application of this method in searching inhibitors able to work in human-to-mouse xenograft tumor models, we wanted to determine if both human and murine S100 proteins interact with the human and murine forms of RAGE. Our results demonstrated that the interaction between human S100 proteins and mouse RAGE was possible and almost identical to that of the human form of the receptor, although both forms of the receptor show only a 78% of homology. In the case of human S100A4 and human S100P, the interaction was slightly lower for mouse RAGE-Fc than for the human form, whereas in the case of mouse S100A4 the interaction was slightly higher for the mouse receptor.

It was described that S100A4 competes with S100A12 to interact with RAGE [24] and that S100A12 binds to the V-domain of the receptor [23]. In addition, we previously demonstrated that S100A4 binds to the same region [6]. To determine if S100A4, S100A7 and S100P interact with the same domain of the receptor a competition assay was made. We observed that RAGE was unable to interact with both S100A7 and S100P in presence of S100A4 protein supporting this hypothesis.

To further confirm the specificity of the interaction and to assess the effectiveness of the protocol, we used a goat polyclonal antibody anti-human RAGE widely referenced as a biological inhibitor [19]. We observed a statistically significant decrease in the interaction of RAGE to the human S100s in the presence of the antibody, even at low concentrations (10 nM). Since the effect of the antibody was almost identical for all three S100 proteins, we could suppose that they all bind to the same region of the receptor.

Having established the optimal conditions of the assay, we wanted to study its utility to screen blocking agents for the interaction. For this, we selected the human S100A4 protein, the referenced inhibitory small molecule oxyclozanide [16], and the neutralizing mAb 5C3 [6].

Using these conditions we demonstrated that oxyclozanide blocks the interaction in a dose-dependent manner, whereas cromolyn was not able to inhibit the interaction even at a concentration 10 times higher than oxyclozanide.

Furthermore, we confirmed previous results obtained with the 5C3 mAb using SPR studies on blocking the interaction with RAGE [6], whereas the 5H4, a non-neutralizing anti-S100A4 antibody did not. Additionally, we determined the amount of 5C3 and 5H4 antibodies present at the end point and we did not observe differences between both antibodies, indicating that the 5H4 antibody was present as 5C3 but did not block the interaction due probably to its different epitope.

In conclusion, we have developed a highly sensitive ELISA-based assay to study the interaction of S100 proteins with RAGE,

suitable to be adapted to study other protein–protein interactions. Furthermore, this assay has proven to be a good way to perform a preliminary screening of inhibitory molecules, both chemical compounds and mAbs, before using more sophisticated and expensive techniques such as SPR.

Acknowledgments

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