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Review of S100A9 Biology and its Role in Cancer

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Abstract

S100A9 is a calcium binding protein with multiple ligands and post-translation modifications that is involved in inflammatory events and the initial development of the cancer cell through to the development of metastatic disease. This review has a threefold purpose: 1) describe S100A9 structural elements important for its biological activity, 2**)** describe S100A9 biology in the context of the immune system, and 3) illustrate the role of S100A9 in the development of malignancy via interactions with the immune system and other cellular processes.

S100A9 structure

S100A9 is a calcium binding protein. Each S100A9 monomer contains a high affinity calcium binding site at the C-terminus and a low-affinity calcium binding site at the N-terminus. The canonical high affinity calcium binding site consists of the typical 12 amino acids of helix 3 (E), loop 2, and helix 4 (F) that has the shape of a human hand (EF-hand). The non-canonical low affinity calcium binding EF-hand is defined by 14 amino acids of helix 1 (E), loop 1, and helix 2 (F) (Figure 1). Helices 2 and 3 are connected by the hinge region. Upon binding to calcium there is a conformational change whereby helix 3 rotates, thus exposing a hydrophobic cleft that is postulated to serve as an anchoring point for macromolecular interactions (Figure 1) [1].

S100A9 may exist as a homodimer, heterodimer with an S100A8 partner $(S100A8/A9)_2$, or as a heterotetramer with an S100A8 partner $(S100A8/A9)_4$. The three dimensional structures of the calcium bound S100A9 homodimer, S00A8/A9 heterodimer, and heterotetramer of S100A8/A9 are known [2-4]. The natural state of the protein is dependent on the environment in which it resides, but from the above studies, and others, it appears that the S100A8/A9 heterodimer is found in most biological interactions; however, in many of these studies, the presence of the heterotetramer was not specifically evaluated. S100A8/A9 is highly protease resistant in a fashion similar to prion proteins [5]. In the heterodimer, the C-terminus of S100A9 and the N-terminus of S100A8 are aligned in an anti-parallel fashion similar to other homodimeric S100 proteins. The heterodimer is recognized by the E210 antibody [6, 7]. S100A8/A9 heterodimerization is not dependent on calcium but formation

of heterotetramers is calcium dependent. Zinc also induces tetramer formation [8]. There is a truncated form of murine S100A9 (amino acids 1-102) that is the result of protease activity *in vitro* and exhibits reduced zinc binding, but this truncated peptide still retains the native disulfide bond formation between cysteine-79 and cysteine-90 [9]. Based on this data and structural data listed above, the zinc binding site is proposed to be located on the C-terminal region near a series of histidine residues but a Zn^{2+}-S100A9 structure has not been determined to date. The structure of S100A9 has been conserved through evolution as evidenced by the fact that murine S100A9 heterodimerizes with human S100A8. This suggests biochemical functional equivalence of the human and the murine proteins despite a relatively low degree of sequence homology (59%) [10]. S100A9 appears to be specific in its dimerization partners as S100A12, another S100 protein involved in inflammation, does not dimerize with S100A9 [11].

S100A9 was first identified in the context of multiple inflammatory reactions which has led to confusing nomenclature in the literature (Table 1). In 1987, it was found in infiltrating macrophages of rheumatoid arthritis patients and named MRP-14 (myeloid related protein of molecular weight 14 kD) [12]. Other investigators have called it migration inhibitory factor related protein (MRP) of molecular weight 14 kD due to its ability to translocate to keratin intermediate filaments in response to calcium stimulation [13]. The abundance of p14 (synonym for S100A9) in neutrophils and monocytes was confirmed in 1991 by Edgeworth, *et al.* and this was followed by the first large scale purification of the protein for structure determination [14]. S100 proteins obtained their name due to the fact that they are soluble in 100% ammonium sulfate [15]. S100A9 is now considered to be a member of the S100 family of calcium binding proteins [16]. There are more than 20 members of the S100 family each with unique roles in signal transduction. Given the numerous contexts in which S100A9 was discovered, a guide to the nomenclature was published in 2006 (Table 1) [16].

Calcium bound S100A9 binds to arachidonic acid, cytoskeletal elements (e.g. keratin intermediate filaments), Receptor for Advanced Glycation Endproducts (RAGE), Toll-Like Receptor 4 (TLR4), the major fatty acid transporter CD36, matrix metallo-proteinases (MMPs), fibronectin, and heparin sulfate glycosaminoglycans. The nature of S100A9 binding to these targets will be discussed later in this review.

S100A9 localization

The physical location of S100A9 varies according to the cell type and disease state. S100A9 is located in myeloid cells, cancer cells, and in tumor stroma. S100A9 is an abundant cytoplasmic protein in normal myeloid cells such as polymorphonuclear cells and monocytes. S100A8/A9 expressing macrophages are recruited to inflammatory sites in many cancers including pancreas adenocarcinoma, gastric adenocarcinoma, small cell lung carcinoma, pancreatic cystadenoma, lung adenocarcinoma, breast adenocarconoma, B-cell lymphoma, esophageal squamous cell carcinoma, and lung squamous cell carcinoma [17]. Supporting the concept of increasing levels of S100A9 with increasing inflammation is the finding of increased S100A9 protein levels both in gliobastoma multiforme treated with radiation [18] and in radiation-induced mammary carcinomas possibly due to increased infiltration of the tumor by inflammatory cells [19]. S100A9 is also up-regulated in other inflammatory diseases such as psoriasis. In this case, S100A9 is located both in the cytoplasm and plasma membrane in differentiated keratinocytes [20]. In the context of normal human gastrointestinal physiology, S100A9 is located in the cytoplasm and plasma membrane of pancreatic cell lines, whereas in the esophageal mucosa, S100A9 is located within the nuclei [21, 22]. S100A9 localization is important for its physiologic activities. S100A8 and S100A9 are minimally expressed in normal esophageal epithelium, but S100A9 is expressed across the spectrum of Barrett's esophagus through adenocarcinoma [23]. In the

normal esophagus, S100A9 is located in the basal aspect of the cells. With increasing dysplasia in Barrett's esophagous S100A8/A9 is found diffusely within the cytoplasm rather than isolated to the basal aspect of the cells [24]. S100A9 protein expression is increased in poorly differentiated tumors including undifferentiated (anaplastic) thyroid carcinomas [25, 26] and invasive adenocarcinoma of the breast associated with poor tumor differentiation [27]. It has become clear that S100A9 localizes with its partner S100A8 in many biological processes but may act as a sole player in other cancers. One study in particular examined a variety of cell lines and found S100A9, S100A8, or S100A8/A9 located within multiple cancer cell types. This information is reviewed in Table 2 [28]. The common theme that has emerged is that localization of S100A9 correlates to centers of inflammation in cancer or other pathological processes.

S100A9 signal transduction is important for inflammatory signal cascades and the oxidative potential of the NADPH complex

S100A9 transports arachidonic acid between the cytosol and the NADPH oxidase complex at the plasma membrane in neutrophils as part of an inflammatory signal cascade. Arachidonic acid is a polyunsaturated omega-6 fatty acid that is involved as a second messenger in cellular signaling. S100A9 transfers arachidonic acid to $gp91^{phox}$ of the NADPH complex while S100A8 binds to $p67^{phox}$ and rac-2 of the NADPH oxidase complex leading to the oxidative burst important in inflammatory cells (Figure 2) [29]. Thus is it is possible that a S100A8/A9 heterodimer could have multiple effects on the NADPH complex. The C-terminus of S100A9 (residues 103-105) in either the homodimeric or heterodimeric state with S100A8 facilitates arachidonic acid transport [30, 31]. S100A9 that has been phosphorylated at threonine-113 of the S100A8/A9 complex enhances activation of NADPH oxidase whereas zinc blocks arachidonic acid binding to S100A8/A9 [32]. The NADPH oxidative burst is decreased in neutrophils from S100A9 knockout mice. Two mutations, (H103A,H104A,H105A,K106A) S100A9 mutant and truncation of S100A9 to residues 1-100 eliminated the ability of S100A9 to activate the NADPH oxidase complex presumably due to lack of arachidonic acid migration to the plasma membrane [29]. Studies in HaCaT keratinocytes that over-express S100A9 demonstrate increased NADPH oxidase and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity whereas HeLa cells bearing the (H103A,H104A,H105A,K106A) S100A9 mutation are unable to bind arachidonic acid and fail to promote PMA (phorbol myristate acetate)-induced NADPH oxidase activity [33]. In general, S100A9 appears to contribute to the control of the oxidative potential of the NADPH complex.

Post-translation modifications of S100A9 also control its activities in inflammatory pathways

Both S100A8 and S100A9 have the capability of being nitrosylated (R-NO) which in general results in decreased inflammatory activity. However, S100A9 is nitrosylated in a calcium dependent manner. Translocation of cytosolic NADPH oxidase components such as $p47^{phox}$ and $p67^{phox}$ to the plasma membrane, and subsequent superoxide generation are inhibited by nitrosylated residues on the S100A8 subunit of the S100A8/A9 complex [29, 34]. Once outside the plasma membrane, the calcium-independent formation of S100A8 nitrosylation products function as a nitric oxide shuttle [34]. S-glutathionylation of S100A9 reduces its capacity to heterodimerize with S100A8 and bind fibronectin, but the S100A9 arachidonic acid binding capacity remains the same [35]. S-glutathionylation of S100A9 may therefore serve as a mechanism to limit the inflammatory response in the extracelullar matrix. Oxidation of methionine 63 and 83 abolishes S100A9's chemo-repulsive (fugetactic) effect on peripheral neutrophils [36]. Therefore, S100A8/A9 nitrosylation, glutathionylation,

and oxidation serve as post-translational mechanisms controlling both the magnitude and extent of the immune response.

S100A9 signaling is important in mediating inflammatory cascades in the vicinity of the plasma membrane via interaction with RAGE and TLR4

Current evidence supports myeloid secretion of S100A8/A9 which in turn binds to carboxylated glycans on RAGE or RAGE itself *in vitro* and in many cell types. The S100A8/A9-RAGE complex can activate signaling pathways including mitogen-activated protein kinase (MAPK) and NF-κB in colon tumor cells and NFκB in tissues from a murine model of skin carcinogenesis induced by DMBA/TPA [37-39]. The S100A9 subunit may be responsible for the association of S100A8/A9 to RAGE as *in vitro* binding data supports a thermodynamically favorable homo-dimeric S100A9-RAGE complex in the presence of zinc [40]. Controversy exists whether it is RAGE or TLR4-MD2 actions that are responsible for downstream S100A9 signaling in inflammatory processes. In actuality, these molecules appear to be important in different biological processes. For example, in a S100A9 null murine system, it was determined that TLR4-MD2 and not RAGE was responsible for S100A8/A9 mediated effects. In this model, S100A9 is upstream of TNF-α induction and the S100A8 subunit of the S100A8/A9 complex induced NF-κB via TLR4-MD2. When direct binding of S100A9 to RAGE and TLR4-MD2 is measured *in vitro*, the S100A9 homodimer has a greater affinity for RAGE or TLR4-MD2 than its heterodimeric counterpart, S100A8/A9 [40]. Therefore, it appears that S100A9 may be important for the structural interaction of the heterodimer with RAGE or TLR4-MD2, and S100A8 is important for regulation of the heterodimer's ability to complex with RAGE or TLR4-MD2. In another example, extra-cellular S100A8/A9 enhances LPS signaling at the plasma membrane leading to increased LPS-induced mortality in mice. Interestingly, this is accomplished by S100A8 and not S100A9 of the S100A8/A9 heterodimer binding directly to the TLR4-MD2 complex [41]. S100A8 and S100A9 are likely both important for mediating inflammatory cascades through their interactions with RAGE and TLR4, but the structural and mechanistic reasons why each subunit is important for specific inflammatory reactions requires further investigation.

S100A9 activity within the intracellular cytoskeleton and extra-cellular matrix is dependent on calcium and zinc

Calcium dependent tetramer formation of S100A8/A9 is essential for the formation of microtubules and has been measured in vitro [42]. In the polymerization of microtubules, S100A8 interacts with tubulin and S100A9 serves as the regulatory subunit [42]. In epithelial cells, calcium serves as a secondary messenger and binds to S100A8 and S100A9 and induces their translocation to keratin intermediate filaments thought to mediate cellular migration [13]. S100A9 localizes to the cytoplasm in monocyte cell culture systems and an increase in calcium levels promotes translocation of S100A9 to the membrane probably by protein kinase C (PKC) dependent mechanisms [43]. It is unclear whether S100A9 directly transports arachidonic acid out of the cell, but S00A8/A9 is likely secreted from neutrophil-like HL-60 cells via a calcium dependent protein kinase C pathway. Once in the extracellular space, calcium bound S100A9 is found complexed to arachidonic acid (Figure 3) [44]. In the extra-cellular space, S100A8/A9 not only binds to arachidonic acid in a calcium dependent manner, but has interactions with the major fatty acid transporter of endothelial cells (CD36) to promote fatty acid uptake and heparin sulfate proteoglycans via the S100A9 subunit thereby promoting myeloid cell migration [45, 46]. MMP2 and MMP9 need zinc to cleave S100A9, thereby limiting its activity in inflammatory pathways (Figure 3) [47]. S100A8/A9 is itself able to block the metalloproteinase (MMP) degradation of the

extra-cellular matrix by sequestration of zinc, thus forming a negative feedback loop [48]. The zinc binding motif on S100A9 (H92-E97) is responsible for inhibiting the spread and phagocytic activity of adherent peritoneal cells presumable by this mechanism [49]. By its interaction with cytoskeletal components and extracellular matrix factors, S100A8/A9 can promote inflammation and also create the molecular environment responsible for termination of biological activity in the presence of divalent ions such as calcium and zinc.

S100A9 expression mediates the inflammatory and migratory potential of myeloid cells

It is the goal of this section to outline general mechanisms of S100A9 induced inflammation and the roles of the S100A8 and S100A9 sub-units in influencing the migratory potential of myeloid cells that will be important in the discussion of S100A9 in different cancers. In general, levels of S100A9 are increased at sites of inflammation [50-53]. As described in previous sections, S100A9 activates NF-κB signaling pathways, although the precise mechanism of this initiation is still unclear. The ability of S100A9 to influence the inflammatory cascade and migration in multiple types of myeloid cells is dependent on divalent ions such as calcium and zinc. S100A9 is recruited to sites of inflammation by its interaction with glycans. In a rheumatoid arthritis model, S100A8 and S100A9 induced the expression and secretion of pro-inflammatory cytokines by monocytes such as IL-6, CXCL8, IL-1β, and TNF-α [54]. Mice treated with an anti-glycan antibody in a LPS-based colitis model exhibited reduced S100A8/A9 expression in colon tissues and had reduced levels of TNF-α, nitric oxide, IL-23 mRNA, and mucosal addressin cell adhesion molecule 1 (MAdCAM-1) [55]. S100A9 null mice and S100A9 null granulocytes *in vitro* also demonstrate reduced recruitment of granulocytes. There was also a reduction in granulocyte infiltration when S100A9 phosphorylation was blocked by p38 MAPK inhibition in human monocytes. Mechanistically, it appears that the S100A8 subunit interacts with tubulin and the S100A9 subunit is the regulatory element that promotes movement such as kinesis of cytoskeletal elements [56]. The conclusion that S100A9 is important for myeloid migration is supported by the evidence that S100A9 null neutrophils demonstrated defective chemoattractant induced calcium signaling as mediated by phospholipase C pathways [57]. S100A9 phosphorylation at Threonine-113 by protein kinase C modulates S100A9 translocation to the cell membrane of human neutrophils [58, 59]. This phosphorylation event appears to control cytoskeletal rearrangements important for myeloid cell migration. Another way in which S100A9 promotes cell movement is in monocytes where it localizes to the type III intermediate filament vimentin in a calcium dependent manner [60]. S100A9 is therefore involved in both the recruitment, and containment of inflammatory cells during the inflammatory response.

S100A9 regulates the maturation of myeloid cells

In the inflammatory response, monocytes differentiate into mature macrophages, initially expressing both S100A8 and S100A9. Later, macrophages at inflammatory sites will lose S100A8 expression [61]. Even though S100A9 is not important until later in the development of different cell types, knock-out of its binding partner S100A8 in mice causes early resorption of the embryo [62]. Up-regulation of S100A9 correlates with differentiation of the promyelocyte to a myelocyte/granulocyte and correlates with the expression of CD11b in neutrophilic cells already expressing CD15. S100A9 is up-regulated prior to the expression of CD15 in monocytes. In both monocytes and macrophages, S100A9 production correlates with the expression of CD11b on the cell surface [63]. Kruppel-related zinc finger protein and the transcriptional intermediary factor 1 beta (TIF1) appear to be involved in a myeloid protein regulatory element (MRE) binding complex regulating S100A9 gene expression and promotion of differentiation [63]. However, differentiation of HL-60 cells in

response to ATRA was reduced by 40% when treated with S100A9-siRNA so elements other than S100A9 may be important for maturation. S100A9 levels also correlate with the trans-differentiation of neutrophilic granulocytes to macrophages when stimulated with CSF-1 [64]. The role of S100A9 in the maturation of myeloid cells is not completely known, but it is interesting to note that early myeloid cells called myeloid derived suppressor cells (MDSC) may be induced by S100A9 and are able to suppress the immune response to cancer cells.

S100A9 recruits myeloid derived suppressor cells (MDSCs) promoting cancer growth via inflammatory pathways

The relationship between S100A9 and MDSC has been carefully studied [53, 65-67]. Murine MDSCs express both CD11b and Gr1. Human MDSCs are a heterogeneous population of early myeloid cells that exhibit a multitude of cell surface markers including: CD11b, $HLADR^{low/-}$, CD33, CD15, CD14, and IL4Rα [68]. MDSCs are also characterized by their ability to inhibit T cell function and the immune reaction to cancer cells by the release of reactive oxygen species (e.g. nitric oxide), cytokines, and arginase. Lymphoma tumors in S100A9 null mice grow less rapidly than in wild type mice and these results are dependent on reduced recruitment of MDSCs. Over-expression of S100A9 increases MDSC recruitment and inhibits differentiation of dendritic cells [69]. As a second example, S100A9 knockout mice are better able to reject EL4 lymphomas compared to wild type mice [70]. This appears to be due to lack of MDSC recruitment to tumor sites. On the biochemical level, it may be possible that S100A8/A9 on MDSCs binds to carboxylated glycans on endothelial surfaces or RAGE on the tumor cell to promote migration of MDSCs. S100A8/A9 is also expressed by tumor cells and may provide a mechanism for recruiting additional MDSC into the tumor microenvironment by binding to RAGE on MDSCs and promoting NFκB inflammatory pathway signal transduction [38, 71]. S100A9 appears to be important in the recruitment of MDSC to tumor sites and inhibition of the immune responses to cancers.

S100A9 is differentially expressed in various cancers

S100A9 is up-regulated in numerous cancer types including breast cancer (invasive ductal carcinoma), colitis-associated colon cancer, hepatocellular carcinoma, gastric cancer, pulmonary adenocarcinoma, colorectal cancer, breast apocrine carcinomas, non-small cell lung cancer, and squamous cervical cancer [27, 28, 38, 72-80]. S100A9 levels are also increased in the stroma of nasopharyngeal carcinoma, and in transitional cell carcinomas of the bladder [81, 82]. Given the number of cancers that appear to over-express S100A9, the biochemistry and molecular biology of this protein will be reviewed in the context of cancer biology.

In non-small cell lung adenocarcinoma, S100A9 is associated with a poor prognosis. Patients with early stage lung cancer who had over-expression of S100A9 within the cancer cells exhibited a significantly worse overall five year survival [83]. In a second small study, forty patients were divided into two groups consisting of poor survival (median 7.7 months) and extended survival (median 92.7 months). S100A8/A9 levels were increased in the poor survival group in the non-macrophage component of the stroma, but in the extended survival group S100A8/A9 was increased in the non-macrophage component of tumor cell islets [79]. This apparent controversy can be explained by the fact that M1 macrophages were increased in the extended survival group at the tumor site. S100A8/A9 may also function in M1 macrophages in conjunction with reactive oxygen species to suppress cancer. In a murine Lewis lung carcinoma model, it has been shown that S100A9 promotes both the development of metastatic disease and myeloid cell recruitment [84]. In this model,

expression of S100A9 was decreased in the presence of an antibody to TGF-β. Addition of exogenous TGF-β to mice increased S100A9 levels in the presence of VEGF-A and TNF-α [84]. Therefore, S100A9 is likely linked to the TGF-β pathway that promotes metastasis. S100A8/A9 may function within M1 macrophages to suppress cancer, but may function with TGF-β, VEGF-A and TNF-α to promote cancer. Depending on the molecular environment S100A9 can promote or inhibit tumor growth in lung cancer.

In pancreatic adenocarcinoma, S100A9 co-localizes with CD14 on monocytes and macrophages in the stroma. In pancreatic cancer S100A9 may interact with the TGF-β signaling pathway to influence cell growth and migration. Over half of pancreatic cancers are proposed to have a mutated component of the Smad4-mediated TGF-β signaling pathway [85]. With the lack of Smad4, S100A9 may be preferentially expressed compared to S100A8 in pancreatic adenocarcinoma [86]. Over 50% of pancreatic cancers lose expression of Smad4 late in the disease course and knockdown of Smad4 in pancreatic cancer cells abolishes TGF-β-mediated cell cycle arrest. The abolition of cell cycle arrest permits cancer cell growth, but the TGF-β protein is still present. Smad4 knockdown does not inhibit the TGF-β induced epithelial mesenchymal transition that is responsible for the evolution of pancreatic cancer cell type that has the ability to migrate and metastasize [87]. As TGF-β promotes the development of metastatic disease in lung cancer, it is very likely that S100A9 may promote these activities in pancreatic adenocarcinoma through interactions with the TGF-β pathway, although the specifics have not yet been elucidated. One possibility is that Smad4 mutations may accentuate the S100A9-TGF-β pathway in pancreatic adenocarcinoma. Therefore, in lung, pancreas and other cancers, S100A9 likely contributes to tumor cell migration through the activity of TGF-β, and S100A9 also leads to the recruitment of myeloid cells such as inhibitory MDSCs.

In hepatocellular carcinoma, NFκB binds to the S100A9 promoter and activates transcription. S100A9 proceeds to activate reactive oxygen species dependent signaling pathways protecting hepatocellular carcinoma cells from apoptotic cell death [88]. In this system S100A9 appears to be co-expressed with S100A8. Nemeth et al. were able to validate increased S100A8 and S100A9 levels in hepatocellular carcinoma in murine and human tissue [88].

S100A9 is up-regulated in colon adenocarcinomas. Murine model exists where colon adenocarcinomas can be induced by the pro-inflammatory agent 1,2-dimethylhydrazine (DMH) or the genotoxic agent azoxymethane. Both S100A8 and S100A9 exhibit increased expression in the resultant tumor cells [89, 90]. In a series of colorectal cancers obtained from fresh surgical specimens, S100A9 levels were increased and a protein signature was identified that inversely correlated with levels of S100A9. This signature included liver fatty acid binding protein, actin-binding protein/smooth muscle protein 22-alpha and cyclooxygenase 2 [91]. Also, S100A9 containing macrophages and polymorphoneutrophils (PMNs) accumulate along the invasive margins of human colorectal carcinomas [92]. These data suggest that S100A9 is involved in the invasive phenotype and development of colorectal tumors.

S100A9 may have both tumor supportive and tumor suppressive roles in breast cancer. In invasive ductal carcinoma of the breast, immunopositivity for S100A9 correlated with mitotic activity, the MIB-1 proliferation index (monoclonal antibody to recombinant Ki-67), HER2 over-expression, poor tumor differentiation, vessel invasion, nodal metastasis and poor pathological stage [93]. S100A9 is associated with high grade, negative ER and PR status, high Ki67 and p53 expression, and ERBB2 and EGFR expression. S100A9 expression is also closely correlated to a 10 protein basal signature CK5/6, CD10, EGFR, CAV1, CD44, ETS1, MET, Moesin, GATA3, and CK19. The presence of S100A9 in node

negative breast cancer patients has prognostic value [94]. However, other studies reveal a more complex relationship. For example, S100A8/A9 gene over-expression in the presence of calcium conferred an invasive/migratory phenotype to the breast cancer line MCF10A presumably via the H-Ras signaling pathway [95]. An increase in S100A8/A9 protein levels may also result in S100A9 zinc mediated sequestration, inability of MMP9 to cleave S100A9, and activation of RAGE signal transduction pathways all of which are known to be pro-tumorigenic. In contrast, S100A9 induced growth repression in infiltrating ductal carcinoma of the breast in MCF-7 cells. Smith *et al.* have shown that oncostatin (OM) inhibits the growth of infiltrating ductal carcinoma of the breast through the binding of oncostatin-activated STAT3 to the S100A9 promoter in MCF-7 breast cancer cells [96]. Activation of the oncostatin receptor can also activate the MEK/ERK pathway. In this system, blocking the ERK pathway inhibited S100A8 expression more efficiently and blocking the P38 MAPK pathway inhibited S100A9 expression more efficiently [95]. The dichotomy of S100A9 function needs to be reconciled with the fact that S00A8/A9 over-expression has been associated with adverse pathological features in infiltrating ductal carcinoma of the breast. Other evidence suggests that S100A9 appears to promote growth at low concentrations and inhibit growth at high concentrations. In a murine model, exogenous administration of S100A8/A9 was found to inhibit MM46 mammary carcinoma cells with a minimum inhibitory concentration between 50-100 g/mL. When zinc was added to this same system, it abrogated the ability of S100A8/A9 to inhibit MM46 apoptosis by at least 80% but was unaffected even by millimolar concentrations of either exogenous calcium or magnesium [97, 98]. Just like another S100 protein, S100B, p53 binds to the promoter of S100A9 [99, 100]. Also, increasing levels of S100A9 promotes apoptosis via p53-dependent and p53-independent pathways [99]. These activities of S100A9 are not only dependent on the native protein, but the relative local concentration gradients, its ligands and presumably its post-translational modifications. Thus, S100A9 appears to be inhibitory to breast cancers at higher concentrations and may be promoting tumor growth at lower concentrations, but additional research is needed to distinguish between dosage effects and model differences.

Esophageal adenocarcinoma is another cancer in which controversy exists over how S100A9 expression is involved in the pathogenesis of the disease. On the one hand, S100A9 is down-regulated in Barrett's esophagus progression via increased expression of microRNA-196a [101] and is reduced in esophageal adenocarcinoma cancer [22] [102, 103]. There are reports which show increased expression of S100A9 in esophageal cancers while other groups document reduced expression of S100A9. Notably, RAGE expression correlates with S100A9 over-expression in esophageal preneoplasia in a rat model, supporting a role for S100A9-mediated inflammatory pathways in esophageal cancer [104]. Clearly additional work needs to be done to elucidate the role of S100A9 in esophageal adenocarcinoma.

Clinically, prostate tumors with less differentiation tend to express more S100A9. For example, tissue specimens were obtained from 75 patients and higher Gleason score prostate cancer patients tended to have higher levels of S100A9 and RAGE along with expression of S100A8 in prostatic tissue [105]. In prostate cancer cell lines, S100A8/A9 co-localizes with RAGE and likely activates inflammatory signaling pathways such as NF-κB [106]. However, circulating S100A8/A9 in blood is not associated with prostate cancer risk [107]. Other studies are underway to validate the relationship of S100A9 levels to prognosticate outcomes and response to therapy.

Pharmaceutical targeting of S100A9

Multiple therapeutic strategies for blocking S100A9 and/or its activity are currently under development in either inflammatory diseases or in the setting of cancer. Recently, a series of compounds have been shown to limit the inflammatory activities of S100A9. Quinoline-3-

carboxamides bind to the S100A9 homodimer and limit the zinc and calcium dependent interactions of S100A9 with RAGE, TLR4-MD2 and arachidonic acid. [40]. More recently, these compounds were tested in patients with metastatic prostate cancer in a phase II trial for patients post-radiation and were found to be useful based on their anti-angiogenic properties. The progression free survival increased from an average of 3.3 to 7.6 months [108, 109]. Second, in PARP-1 knock-out mice and in mice treated with a Poly(ADP-ribose) polymerase (PARP) inhibitor S100A9 levels were reduced by approximately 66% [110]. These manipulations led to decreased 7,12-dimethylbenz(a)anthracene plus 12-O-tetradecanoylphorbol-13-acetate-induced skin carcinogenesis in a mouse model. S100A9 inhibitors may therefore be able to slow specific tumors.

Current S100A9 research may result in future pharmaceutical targets

In the cardiovascular system, the S100A8/A9 heterodimer enhanced secretion of the pro-inflammatory factors IL-6, ICAM-1, VCAM-1 and MCP1 in HUVEC cells in a dose dependent manner. The effects of S100A8/A9 were reduced by inhibition of ERK1/2 and p38 in the MAP kinase pathways. It has been postulated that blocking S100A8/A9 may represent a modality to treat atherosclerosis via the down-regulation of inflammatory pathways [111]. McCormick et al. found that arteries lacking atherosclerosis lacked expression of S100A8 or S100A9. S100A9 may associate with lipid structures and may promote dystrophic calcification by altering the ability of phospholipid to bind calcium [112]. These strategies all have the common theme of S100A9 involvement in aberrant regulation of the immune system and will likely also have therapeutic implications in cancer.

Summary

S100A9 is a calcium binding protein important in the pathogenesis of different cancer types. S100A9 structure was reviewed (Figure 1) including the nature of EF hands, the binding of divalent cations, and the structural basis for ligand binding. S100A9 is expressed in normal cell types such as myeloid cells, but its levels are generally increased in inflammation and cancer. The S100A9 protein is important for mediating inflammatory processes in myeloid cells and other cell types involved in inflammation (Figure 2). S100A9 activity is dependent on the concentration gradients of both calcium and zinc in the extra-cellular space (Figure 3). The review concluded with a discussion on development of targeted therapeutics. S100A9 levels may prove useful for prognosticating patient outcomes. Lastly, targeting S100A9 will likely prove useful in a number of inflammatory diseases and cancer states.

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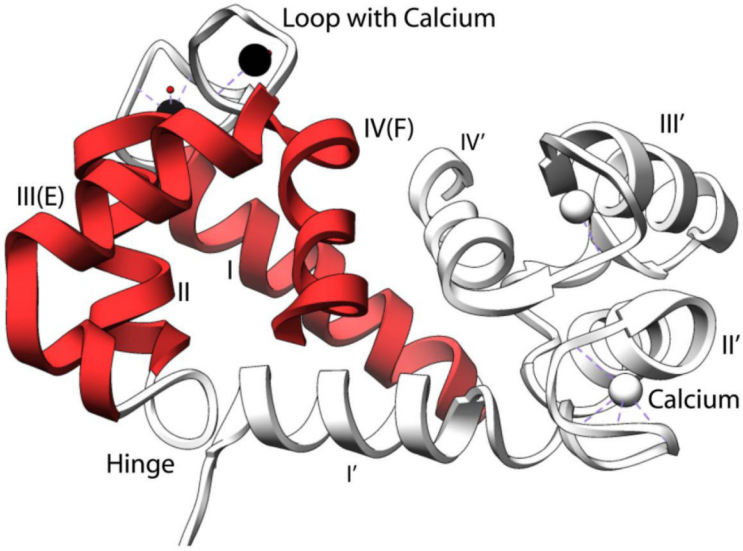

Figure 1.

Ribbon diagram of homodimeric calcium bound S100A9 from protein data bank file 1IRJ using the program Chimera.[113] Depicted in red is one subunit with the EF hand labeled. Secondary structure elements of homodimeric S100A9 include: helix one Q7-S23; helix two Q34-D44; hinge region L45-N55, calcium binding loop one: V24-N33, helix 3 E56-L66 (helix E of canonical EF Hand), calcium binding loop two D67-S75, and helix 4 F76-M94 (helix F of canonical EF hand). The C-terminal tail is disordered in the crystal structure and is therefore not shown in the figure. In this region, H92-E97 is the zinc binding motif. In the C-terminal disordered tail residues 103 to 105 represent the arachidonic acid binding region. There is also a truncated form of S100A9 of 12.7 kDa that is missing residues 1-4 found but has unclear biological function[114].

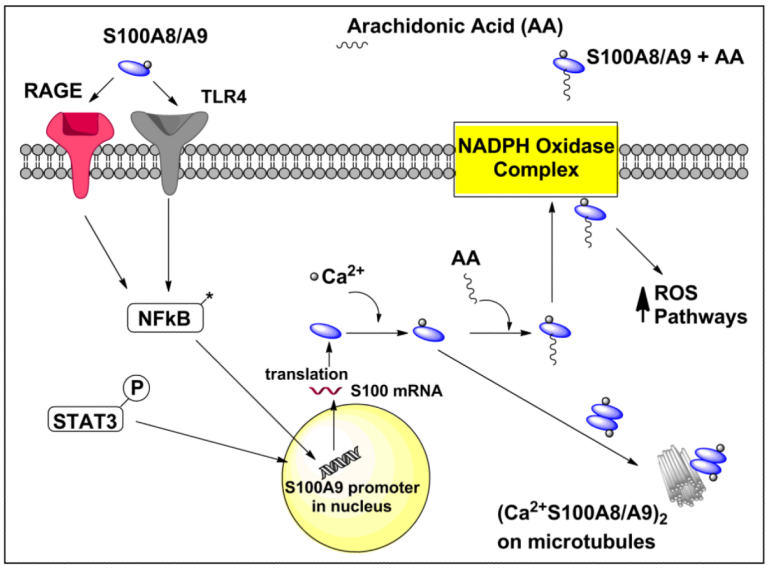

Figure 2.

Intracellular S100A9 activity: S100A8/A9 binds to cell receptors initiating signal transduction through NFκB pathways promoting increased S100A9 transcription. Ca^{2+}-S100A9 complexes with microtubules increasing cell motility. In addition, Ca^{2+}-S100A9 increases the oxidative potential of cells by migrating to the NADPH complex via PKC dependent mechanisms and activating reactive oxygen species pathways. S100A9 is then released into the extra-cellular space.

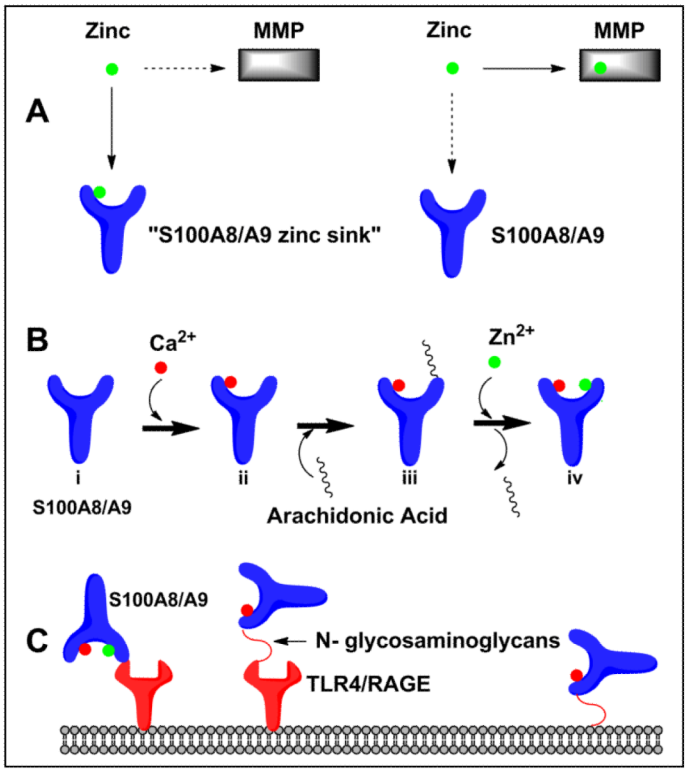

Figure 3.

Extra-cellular S100A9 activity A) S100A8/A9 sequesters zinc inhibiting the extracellular matrix degradation capacity of MMPs

B) S100A8/A9 or S100A9 serves to transport arachidonic acid (AA). Calcium binding to Apo-S100A9 (i) forms Ca^{2+}-S100A9 (ii). The Ca^{2+}-S100A9 (ii) protein binds to arachidonic acid forming the Ca^{2+}-S100A9 –AA complex (iii). Zinc binding to Ca^{2+}-S100A9 (ii) forms the $Zn^{2+}Ca^{2+}$-S100A9 complex (iv) and can no longer bind arachidonic acid.

C) S100A9 or S100A8/A9 binds to cell surface receptors initiating signal transduction cascades.

Table 1

Synonyms for S100A9 and S100A8.[16] Calprotectin = S100A8/A9

	Synonym
S100A8	**-Calgranulin A (CAGA) -CGLA -P8 -Myeloid related protein of molecular weight 8 kDa -Migration inhibitory factor related protein of molecular weight 8 kDa (MRP8), -CFAG, -LiAg, -60B8AG**
S100A9	**-Calgranulin B (CAGB) - -CGLB -p14 -Myeloid related protein of molecular weight 14 kDa -Migration inhibitory factor-related protein of molecular weight 14 kDa (MRP14) -CFAG -LiAg -60B8AG**

Table 2

Examples of cell lines determined by micro-array to express S100A8 or S100A9 [28].

Cell Line	Tissue of Origin	S100A8	S100A9
Hs683	Glioma	+	+
T47D	Breast	+	+
SNU484	Gastric	+	+
HeLa	Cervical	+	+
SK-Hep-1	Hepatocellular	−	+
MCF7	Breast	−	+
LNCaP	Prostate	+	−