

The Role of Calcium in Calprotectin Dimerization as a Cancer Biomarker

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Background: S100A8 and S100A9 as two subunits of heterodimeric calprotectin are identified mainly in leukocytes and are involved in inflammatory processes and several cancerous pathogens. This study was performed in order to evaluate the interaction of recombinant calprotectin subunits and to estimate calprotectin's tertiary and secondary structures.

Objectives: The aim of this study was to investigate the effects of calcium in calprotectin dimerization as a cancer biomarker.

Materials and Methods: Heterodimeric calprotectin was formed with incubation of recombinant S100A8 and S100A9 subunits in the presence of Ca (1 mM), at 25 °C for 15 minutes. Tertiary and secondary structures of S100A8, S100A9 and their complex were investigated, using fluorescence and circular dichroism (CD) spectroscopy, respectively.

Results: Interaction of S100A8 and S100A9 in the presence of Ca²⁺ were revealed by decreasing the emission intensity of intrinsic fluorescence and increasing of the external fluorescence and also changes in the CD spectra of subunits after Ca²⁺ interactions.

Conclusions: The expression of recombinant calprotectin, as an effective protein, can help in diagnosis or treatment of inflammatory and cancer processes in the future. Furthermore, Ca²⁺ induced a partial change in secondary and tertiary structure of calprotectin subunits and this change is probably necessary for protein dimerization.

Keywords: Calcium; Calprotectin; Biomarkers; Cancer; Fluorescence Spectroscopy

1. Background

Calprotectin is a member of the S100 family of proteins, and is a marker of inflammation and a calcium and zinc-binding protein. Expression of calprotectin has been reported mainly in neutrophils (30-60% in the cytosol), followed by monocytes and macrophages (mainly associated with membranes), and to a lesser extent in other cells. Expression of S100A8 and S100A9 and hence calprotectin are induced following recruitment of macrophages to inflammatory sites; calprotectin is not stored in tissue macrophages (1). The calprotectin structure is comprised of a hetero-dimer with two calcium-binding chains and two calcium-binding sites per chain. The heavy chain is a 14 KD protein, also known as MRP14/S100A9/P14/LIH and the light chain is an 8 KD protein, also known as MRP8/S100A8/L1L/P8 (1-3). The chains bind non-covalently in the presence of calcium. Other compounds namely, hetero or homo-dimer of the two chains, tetrameric or more monomers per polymer chain have also been identified. Twenty-one S100 genes, including those for calprotectin, are clustered on human chromosome 1q21. Until now homo-dimer of S100 proteins including S100A8 and S100A9 have been reported; the primer functional form was reported to be heterodimeric consisting of antiparallel arrangement of S100A8/S100A9 known as calprotectin,

which is induced in the presence of calcium. S100A8 and S100A9 are produced primarily in myeloid cells and cells triggered by inflammation of myeloid lineage with the exception of lymphocytes. S100A9 gene deletion leads to the loss of S100A8. Expression of S100A9 and S100A8 proteins in phagocytes are associated with a set of actions in the innate immune system. The expression of these proteins occur during differentiation of macrophages and dendritic cells; both proteins can be simultaneously expressed in monocytes, endothelial cells, keratinocytes and epithelial cells by several mediators such as interleukin (IL1)-alpha, IL1-beta, IL10, IL22, tumor necrosis factor (TNF) alpha and lipoteichoic acid (LPS) (4). Different roles have been reported for calprotectin, including; antimicrobial, cytotoxicity, cytokine-like activity, anti-proliferation, induction of apoptosis, chemotactic effects, leukocyte-endothelium interaction, cell adhesion, immune regulation, inflammation and coagulation responses. Levels of calprotectin were found increase following infections and inflammatory disease states (1, 3). Normally, calprotectin has been reported to be at a concentration of about 5 mg in plasma and 2 mg in stool, with a maximum of 10 mg per liter. S100A8 and S100A9 are soluble mediators, which are involved in cancer processes; they are

damage associated molecular patterns (DAMP), involved in tumor progression and malignancy. As DAMP ligands for cell surface receptors, they trigger signaling cascades mediating cellular responses to cytokine and chemokines (4). Calprotectin secretion occurs as a result of a pathological attack when the leukocytes increase in tissues and this occurrence can be traced in the plasma, cerebrospinal fluid, urine or feces (5). Calprotectin is produced as an early inflammatory response protein and has reported to be increased in many different human cancers (3). S100A9 is reported to be involved in de-differentiation of cells by making changes in cytoskeleton in a calcium-dependent manner, and by signaling to normal cells, they can lead changes in neoplasms (6). Genomic changes in S100A8/S100A9 loci in tumors with portions removed, double displacement and condensation have also been reported which may be associated with malignancy. The question is whether calprotectin or its subunits as a diagnostic marker can predict disease progression or metastasis in some cancers? The aim of the present study was to investigate the interactions of calprotectin's subunits with calcium. There are many evidences that indicate calprotectin is increased in inflammatory diseases as well as many cancers including skin, breast, stomach, prostate and colon (2, 7).

2. Objectives

The present study aimed to evaluate the effects of calcium on calprotectin subunits dimerization.

3. Materials and Methods

3.1. Materials

The rS100A8 and rS100A9 were obtained from a previous research. Anilino-naphthalene-8-sulfonic acid (ANS), was purchased from Sigma (Sigma-Aldrich, Germany). Calcium chloride (CaCl_2), was purchased from Merck (Germany).

3.2. Complex Formation of r-S100A8/S100A9

For the preparation of r-S100A8/A9 complex, equal volumes of r-S100A8 and r-S100A9 ($1 \mu\text{M}$), were incubated with calcium chloride (1 mM) in PBS dialysis buffer for at least 15 minutes at 25°C . Complex formation was investigated by fluorescence and circular dichroism (CD) spectroscopy.

3.3. Circular Dichroism Spectroscopy

The content of regular secondary structures of r-S100A8, r-S100A9 and r-S100A8/A9 complex were examined in the far ultraviolet (UV) region (190-260), which correspond to peptide bond absorption, using an AVIV model J810 spectropolarimeter (JASCO) to give the content of regular secondary structure of proteins. Far UV-CD spectra of 0.04 mg/mL solution of proteins in PBS buffer (pH = 6.5) were obtained with 1 mm path length quartz cell. The background was corrected against the buffer blank. The data were calculated as molar ellipticity ($\text{deg}\cdot\text{cm}^2/\text{dmol}$)

assuming a mean residue number of 10^7 and average molecular weight of 25 kDa for S100A8/A9 complex using the CD deconvolution software. The molar ellipticity was determined as $[\theta] = 100 \times (\text{MRW}) \times \theta_{\text{obs}} / (c \cdot l)$, where θ_{obs} is the observed ellipticity in degrees at a given wavelength and c is the light path length in cm.

3.4. Intrinsic Fluorescence Spectroscopy

Intrinsic fluorescence of r-S100A8, r-S100A9 and r-S100A8/A9 complex, after treatment with calcium chloride (1 mM), were studied using the Cary eclipse model 100 bio spectrofluorometer equipped with a 150 W xenon lamp and a DR-3 data recorder. The excitation and emission slits were set at 5 and 5 nm, respectively. The intrinsic fluorescence was measured by exciting the protein solution with 1 cm path length cell at 280 nm in PBS dialysis buffer at pH = 6.5 and 25°C and emission spectra were recorded at the wavelength range of 300-450 nm.

3.5. 8-Anilino-1-Naphthalene Sulfonate Fluorescence Spectroscopy

External fluorescence spectroscopy of r-S100A8/A9 complex was performed with stock solution of 8-anilino-1-naphthalene sulfonate (ANS) (10 mM). The ANS fluorescence of r-S100A8/A9 complex was treated with calcium chloride. Excitation and emission slits were set at 5 and 5 nm, respectively. Emission spectra were recorded from 400 to 650 nm with excitation at 380 nm in increments of 1 nm.

4. Results

4.1. Fluorescence Spectroscopy of r-S100A8/A9 Complex

Intrinsic fluorescence spectra showed changes in the tertiary structure of r-S100A8 and r-S100A9 subunits after complex formation (Figure 1). Calcium connection to the binding sites of S100A8 and S100A9, lead to the displacement of the aromatic residue from hydrophobic environment to surface of proteins. Increasing emission spectra of ANS fluorescence showed a more hydrophobic structure for r-S100A8/A9 after treatment with Ca in comparison with only r-S100A8/A9 (Figure 2).

4.2. Circular Dichroism Assessments of r-S100A8/A9 Complex

Circular dichroism is an ideal technique for monitoring the transitional switch between regular secondary structures in proteins, which can occur as a result of changes in experimental parameters such as treatment with Ca^{2+} . The far UV-CD spectra characterize the secondary structures of proteins due to peptide bond absorption, thus changes in these spectra usually reflect major backbone changes in proteins. The far UV-CD spectra of r-S100A8/A9 complex indicate significant changes in the secondary structures, compared with rS100A8 and rS100A9 (Figure 3).

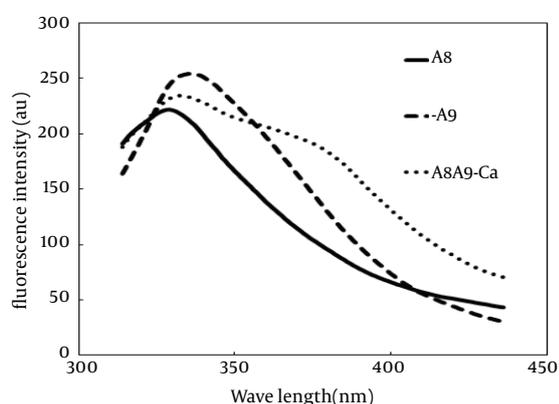


Figure 1. Intrinsic Fluorescence Emission Spectra of r-S100A8, r-S100A9 and r-S100A8/A9 (1 μ M) After Treatment With Ca^{2+} (1 mM)

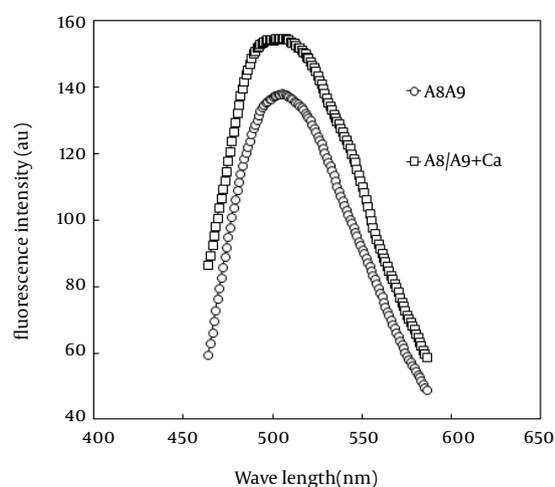


Figure 2. 8-Anilino-1-Naphthalene Sulfonate External Fluorescence Emission Spectra of r-S100A8/A9 (1 μ M) in the Absence and Presence of Ca^{2+} (1 mM)

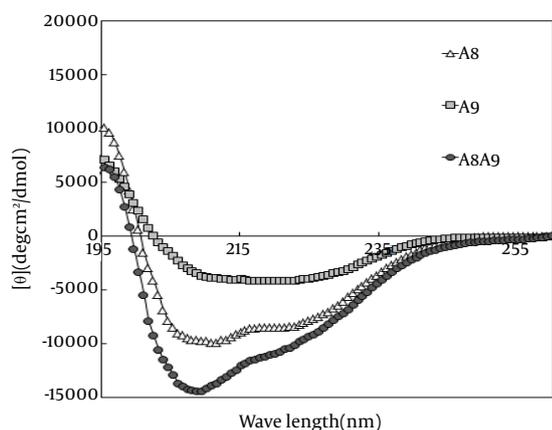


Figure 3. Far Ultra Violet-Circular Dichroism Spectra (Molar Ellipticity $[\theta]$) of r-S100A8, r-S100A9 and r-S100A8/A9 Complex (1 μ M) After Treatment With Ca^{2+} (1 mM)

5. Discussion

In this study, the interaction of calprotectin monomers in the presence of Ca^{2+} was investigated. The existence of the heterodimeric calprotectin was confirmed by decreasing intrinsic fluorescence, increasing ANS external fluorescence and change in CD spectra of rS100A8 and A9 after their interaction with Ca^{2+} . The role of calcium has been demonstrated in calprotectin function (8). The same structural changes have been reported in the presence of excess Ca^{2+} , which in turn increases the propensity of calprotectin to form protein aggregates (9). Spectroscopic techniques were used to verify protein-protein interaction, this required the induction of change in the spectroscopic parameters following complex formation, i.e., change in fluorescence intensity, wavelength maximum or polarization, fluorescence resonance energy transfer efficiency, circular dichroism or nuclear magnetic resonance (NMR) chemical shift or intensity (10). High levels of S100A8 and S100A9 occur during inflammatory processes. Also, there is a close relationship between inflammation and carcinogenesis, while chronic inflammation can increase the risk of tumorigenesis. Even in the absence of inflammation as a causative factor, tumor formation can be due to genetic changes associated with immune cells triggering inflammation. S100A8/S100A9 secretion can also be provoked by tumor cell necrosis followed by hypoxia-induced tumor growth. They can induce tumor formation as a result of the inflammatory process or elicit inflammatory response. They can mediate or prompt tumor formation and/or anti-tumor responses. As an anti-tumor, S100A8/S100A9, act to induce cytotoxicity and apoptosis in tumor cells. Promotion of growth signals, blocking growth-inhibitors, apoptosis inhibition, potential uncontrolled proliferation, initiation of angiogenesis, tumor invasion and metastasis, are the essentials for malignant tumor (4). However, in spite of the anti-tumor properties of S100A8/S100A9 and the possibility of their use as tools for cancer therapy (still not proven in vivo), this complex triggers a number of responses leading to tumor formation. The effective dose of S100A8/S100A9 for tumor cell apoptosis is 20-25 micrograms per milliliter, whereas lower concentrations of S100A8/S100A9 cause proliferation of tumor cells. Pro-apoptotic effects of S100A8/S100A9 ensue receptors for advanced glycation end products (RAGE), also, effects on growth promotion follow RAGE-induced signaling pathways by phosphorylation of mitogen-activated protein kinase (MAPK) and the activity of NF-KP (involved in cell signaling pathways) (11-14). Toll-like receptors (TLR) are membrane receptors associated with innate immune inflammatory response against pathogens. S100A8/S100A9 boost inflammatory responses by TLR4 and recently, the important role of TLR in carcinogenesis has been identified (4). The molecular pathways mediated by S100A8/S100A9 are potential targets for development of new

cancer treatments and detection of cancer biomarkers for early diagnosis and treatment processes (Table 1). In colon cancer, the abnormal increase in stool calprotectin, which is stable against enzymatic degradation, may be used as a biomarker for screening patients with acute colorectal cancer from healthy individuals (15). However, calprotectin lacks the required efficiency for screening patients with inflammatory bowel disease or polyps, and neoplasms. Furthermore, since calprotectin levels are increased to some extent in these subjects, stool calprotectin testing, could help isolate patients suspected of having colon cancer to be subjected to colonoscopy for determination of their health or disease progression (16). Also, levels of S100A9 expression in the stool can be a marker for diagnosis of metastasis and follow-up treatment in colorectal cancer. Using a combination of stool blood and calprotectin can yield more accurate diagnosis of colorectal cancer (17). Overexpression of S100A8 and S100A9 occur in breast cancer, which are associated with poor cell differentiation and mitotic activity of the tumor cells respectively, and therefore, may be used as a marker for diagnosis, monitoring therapy as well as detecting metastasis (18). Also, they may be used as drug targets (19). S100A9 expression increases in prostate cancer, which may be used to differentiate patients with benign tumors from those with malignant tumors. Moreover, it can be used as a marker for the onset of metastasis (20, 21). Overexpression of calprotectin, S100A8

and S100A9 in ovarian cancer, may be used as a diagnostic marker to detect malignant tumors and in endometrial cancer this overexpression can act as a diagnostic marker, and monitor therapy or disease progression (22, 23). Overexpression of calprotectin and S100A8 have been reported in endometrial cancer, which can be used as a diagnostic marker and for monitoring therapy or disease progression (7). High levels of S100A8/S100A9 expression may be used as a marker for the diagnosis of severe inflammation. S100A9 increases in thyroid cancer cells, and thus may be used as a marker for predicting the disease process and perhaps can be one of the drug targets in the future (6). S100A8 and S100A9 may be used for the diagnosis of esophageal cancer (24) and gastric cancer and metastasis (25). Increased expression of S100A9 in lung cancer macrophages is a risk factor for the onset of metastasis and predicts weak recovery for patients; this increased expression can also act as a diagnostic marker for liver and larynx cancer (26). Therefore, calprotectin or its subunits may be used as a cheap, non-invasive, readily available and easy marker. The structure and function of this protein and its subunits showed a narrow dependency to Ca^{2+} . In this study the structural change of calprotectin and its subunits were confirmed by fluorescence and CD techniques. It seemed that Ca^{2+} induced a partial change in secondary and tertiary structure of subunits and was probably necessary for protein dimerization.

Table 1. S100A8/A9 and Calprotectin as Biomarkers in Different Cancers

Kinds of Cancer	S100A8	S100A9	Calprotectin
Bladder	in cancer cells	-	-
Blood	in cancer cells	-	-
Breast	in cancer cells	in cancer cells	-
Cervical	in cancer cells	-	-
Colorectal	-	-	in fecal
Endometrial	in plasma	-	in plasma
Gastric	in serum	in serum	-
Hepatocellular	-	in cancer cells	-
Laryngeal	-	in cancer cells	-
Lung	-	in cancer cells	-
Oral	in cancer cells	-	-
Ovarian	in fluid ovarian cystic and serum	in fluid ovarian cystic and serum	in serum
Prostate	in serum	in serum	-
Skin	in cancer cells	in cancer cells	-
Thyroid	-	in cancer cells	-

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References

- Kurata A, Terado Y, Schulz A, Fujioka Y, Franke FE. Inflammatory cells in the formation of tumor-related sarcoid reactions. *Hum Pathol.* 2005;**36**(5):546-54.
- Kostakis ID, Cholidou KG, Kallianidis K, Perrea D, Antsaklis A. The role of calprotectin in obstetrics and gynecology. *Eur J Obstet Gynecol Reprod Biol.* 2010;**151**(1):3-9.
- Meucci G, D'Inca R, Maieron R, Orzes N, Vecchi M, Visentini D, et al. Diagnostic value of faecal calprotectin in unselected outpatients referred for colonoscopy: A multicenter prospective study. *Dig Liver Dis.* 2010;**42**(3):191-5.
- Srikrishna G. S100A8 and S100A9: new insights into their roles in malignancy. *J Innate Immun.* 2012;**4**(1):31-40.
- Gibson RJ, Bowen JM. Biomarkers of regimen-related mucosal injury. *Cancer Treat Rev.* 2011;**37**(6):487-93.
- Ito Y, Arai K, Yoshida H, Tomoda C, Ryushi., et al. S100A9 expression is significantly linked to dedifferentiation of thyroid carcinoma. *Pathol Res Pract.* 2005;**201**(8-9):551-6.
- Ni Bhriain H, Trovik J, Wik E, Stefansson IM, Akslen LA, Salvesen HB, et al. Plasma calprotectin concentrations in women with endometrial carcinoma. *Gynecol Oncol.* 2009;**114**(3):491-5.
- Sohnle PG, Hunter MJ, Hahn B, Chazin WJ. Zinc-reversible antimicrobial activity of recombinant calprotectin (migration inhibitory factor-related proteins 8 and 14). *J Infect Dis.* 2000;**182**(4):1272-5.
- Yousefi R, Imani M, Ardestani SK, Saboury AA, Gheibi N, Ranjbar B. Human calprotectin: effect of calcium and zinc on its secondary and tertiary structures, and role of pH in its thermal stability. *Acta Biochim Biophys Sin (Shanghai).* 2007;**39**(10):795-802.
- Rao VS, Srinivas K, Sujini GN, Kumar GN. Protein-protein interaction detection: methods and analysis. *Int J Proteomics.* 2014;**2014**:147648.
- Hermani A, Hess J, De Servi B, Medunjanin S, Grobholz R, Trojan L, et al. Calcium-binding proteins S100A8 and S100A9 as novel diagnostic markers in human prostate cancer. *Clin Cancer Res.* 2005;**11**(14):5146-52.
- Moon A, Yong HY, Song JI, Cukovic D, Salagrama S, Kaplan D, et al. Global gene expression profiling unveils S100A8/A9 as candidate markers in H-ras-mediated human breast epithelial cell invasion. *Mol Cancer Res.* 2008;**6**(10):1544-53.
- Turovskaya O, Foell D, Sinha P, Vogl T, Newlin R, Nayak J, et al. RAGE, carboxylated glycans and S100A8/A9 play essential roles in colitis-associated carcinogenesis. *Carcinogenesis.* 2008;**29**(10):2035-43.
- Ohkubo Y, Iwakawa M, Seino K, Nakawatari M, Wada H, Kamijuku H, et al. Combining carbon ion radiotherapy and local injection of alpha-galactosylceramide-pulsed dendritic cells inhibits lung metastases in an in vivo murine model. *Int J Radiat Oncol Biol Phys.* 2010;**78**(5):1524-31.
- Karl J, Wild N, Tacke M, Andres H, Garczarek U, Rollinger W, et al. Improved diagnosis of colorectal cancer using a combination of fecal occult blood and novel fecal protein markers. *Clin Gastroenterol Hepatol.* 2008;**6**(10):1122-8.
- Zhao L, Wang H, Sun X, Ding Y. Comparative proteomic analysis identifies proteins associated with the development and progression of colorectal carcinoma. *FEBS J.* 2010;**277**(20):4195-204.
- Ayling RM. New faecal tests in gastroenterology. *Ann Clin Biochem.* 2012;**49**(Pt 1):44-54.
- Davidson B, Stavnes HT, Forsund M, Berner A, Staff AC. CD105 (Endoglin) expression in breast carcinoma effusions is a marker of poor survival. *Breast.* 2010;**19**(6):493-8.
- Yang WS, Moon HG, Kim HS, Choi EJ, Yu MH, Noh DY, et al. Proteomic approach reveals FKBP4 and S100A9 as potential prediction markers of therapeutic response to neoadjuvant chemotherapy in patients with breast cancer. *J Proteome Res.* 2012;**11**(2):1078-88.
- Hille A, Rave-Frank M, Christiansen H, Herrmann MK, Kertesz T, Hermann RM, et al. Faecal calprotectin and lactoferrin values during irradiation of prostate cancer correlate with chronic radiation proctitis: results of a prospective study. *Scand J Gastroenterol.* 2009;**44**(8):939-46.
- Muller H, Haug U, Rothenbacher D, Stegmaier C, Brenner H. Evaluation of serum and urinary myeloid related protein-14 as a marker for early detection of prostate cancer. *J Urol.* 2008;**180**(4):1309-12.
- Shield-Artin KL, Bailey MJ, Oliva K, Liovic AK, Barker G, Dellios NL, et al. Identification of ovarian cancer-associated proteins in symptomatic women: A novel method for semi-quantitative plasma proteomics. *Proteomics Clin Appl.* 2012;**6**(3-4):170-81.
- Mielczarek-Palacz A, Sikora J, Kondera-Anasz Z, Nocon M. [Changes in calprotectin concentration-inflammation marker in serum of women with gynecological cancer]. *Ginekol Pol.* 2011;**82**(11):822-6.
- Taccioli C, Chen H, Jiang Y, Liu XP, Huang K, Smalley KJ, et al. Dietary zinc deficiency fuels esophageal cancer development by inducing a distinct inflammatory signature. *Oncogene.* 2012;**31**(42):4550-8.
- Wu W, Juan WC, Liang CR, Yeoh KG, So J, Chung MC. S100A9, GIF and AAT as potential combinatorial biomarkers in gastric cancer diagnosis and prognosis. *Proteomics Clin Appl.* 2012;**6**(3-4):152-62.
- Kawai H, Minamiya Y, Takahashi N. Prognostic impact of S100A9 overexpression in non-small cell lung cancer. *Tumour Biol.* 2011;**32**(4):641-6.