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S100A8/A9 Induces Apoptosis and Inhibits Metastasis of CasKi Human Cervical Cancer Cells

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Abstract S100 proteins, a family of Ca^{2+}-binding proteins, have been linked to several human diseases in recent years. Deregulated expression of S100 proteins, including S100A9 and its partner S100A8, was reported to be associated with neoplastic disorders. In our previous study using serial analysis of gene expression, we identified decreased expressions of S100A9 and S100A8 in human cervical squamous cell carcinoma. To investigate the functions of S100A8 and S100A9 in cervical cancer, we purified recombinant S100A8 and S100A9 proteins and treated CaSki human cervical cancer cells with these proteins. We found that S100A8/A9 induced apoptosis and inhibited migration of CaSki cells; S100A8/A9 also reduced the expression of matrix metalloproteinase (MMP)-2 in CaSki cells. In summary, this study suggests that S100A8 and S100A9 have inhibitory effects on the proliferation of CaSki carcinoma cells by inducing cell apoptosis and on the invasiveness of CaSki cells.

Keywords Apoptosis · Cervical cancer · Metastasis · MMP-2 · S100 proteins

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Introduction

Cervical cancer is the second most common malignant tumor in women [1]. Recently, a considerable number of epidemiological surveys and experimental studies indicate that infection of high risk human papilloma virus (HPV) is a necessary cause for the development of cervical cancer based on the fact that HPV is detected in 99.7% of all squamous cell carcinomas of the uterine cervix [2]. The HPV infection is very common in sexually active women. Although at least 90% of HPV infections disappear spontaneously within 1 to 2 years [3], a few cervical lesions with persistent HPV infection progress into cervical intraepithelial neoplasia (CIN) and even invasive cervical carcinoma. Thus, HPV appears to be a necessary but not sufficient cause for cervical cancer. Studies also suggest that the initiation of cancer requires participation of other factors involved in the activation of oncogenes, inactivation of tumor suppressor genes, and the interaction between tumor and its microenvironment. Cervical cancer is clearly associated with aberrance of tumor suppressor genes such as p53, p16, and Rb [4]; but it is not known whether the alteration is the cause of cancer, nor is it clear about the molecular mechanism underlining the changes.

Previous studies have demonstrated that changes of expression of one or several key genes in cervical epithelium lead to altered expression of a cascade of downstream genes and hence the changes of gene expression profiles of cervical tissue and eventually result in the cancerous cervical epithelium [5]. It is important to unravel the pathogenesis of cervical carcinoma by defining the causative gene(s) and studying its/their functions. We previously identified potential key genes for cervical cancer after comparing gene expression profiles of malignant vs. non-malignant cervical epithelium using the serial analysis

of gene expression (SAGE) method followed with verification by real-time quantitative RT-PCR. We also observed that Ca^{2+}-binding protein or Ca^{2+}-regulating protein, particularly S100A8 and S100A9, showed significant decrease of gene expression in cervical squamous epithelial cell carcinoma [6].

S100A8 and S100A9 are members of the S100 family of Ca^{2+}-binding proteins that consist of 21 members. S100 proteins do not have endogenous protein enzyme activity per se; but after binding with Ca^{2+}, their structures change to expose the hydrophobic area to bind with different target proteins to transmit Ca^{2+} signal and regulate Ca^{2+} concentration in cells [7]. Thus S100 proteins play important biological roles in muscular contraction, gene expression, hormone secretion, cell proliferation, and cell differentiation [8, 9].

S100A8 and S100A9 often function through the formation of heterodimer and are mainly expressed in neutrophilic granulocytes and mononuclear macrophages. They could resist microorganisms, induce apoptosis, and are the markers for various inflammatory diseases [10]. Recent studies demonstrate decreased expression of S100A8/A9 heterodimer in various squamous epithelial tumors (e.g., esophageal squamous carcinoma and head/neck squamous carcinoma) [11, 12], consistent with a role of the heterodimer in the pathogenesis of squamous carcinomas. However, the expression of S100A8/A9 increases in various gland cell tumors and is associated with low differentiation of tumors [13, 14]. Thus, the signaling cascade and biological role of S100A8/A9 during the pathogenesis of cervical carcinoma remain unresolved and await clarification.

The current study investigated the role of S100A8/A9 in the pathogenesis of cervical carcinoma. CaSki cells, originally collected from intestinal metastasis nidus of human cervical squamous carcinoma, were selected as a model for this study because of their strong invasiveness and the HPV16 virus positivity. CaSki cells were treated with purified S100A8 and S100A9 proteins followed by analyses of tumor biological behaviors including apoptosis, invasion, and metastasis.

Materials and Methods

Cells and Reagents

CaSki cells were purchased from American Type Culture Collection (ATCC, Mananssas, VA) and were cultured in DMEM supplemented with 10% FCS (Hyclone, Logan, UT, USA) at 37°C in a humid atmosphere containing 5% CO_2. The plasmids of pET28 expression vector containing human S100A8 and S100A9 (full-length) cDNAs were kindly provided by Dr. Philippe A. Tessier (University of Laval, Quebec, Canada). Antibodies directed to S100A8, S100A9, S100A8/A9, Caspase-3, and MMP-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Hochest33342 was purchased from Sigma (München, Germany). The Annexin-V kit was purchased from Biosea Biotechnology (Beijing, China).

Protocol

S100A8/A9 heterogeneous dimer was formed and verified. To study the effects of S100A8/A9 on the CaSki cells, we purified the S100A8 and S100A9 proteins first and used S100A8/A9 to treat the cells at 20 μg/mL of final concentration. Apoptosis were measured with caspase-3 and Hochest staining and FACS analysis; metastasis was evaluated by MMP-2 expression and cell migration.

Recombinant Protein Purification

Expression vectors pET28a-S100A8 and pET28a-S100A9 were transformed to E. coli BL21 (DE3). Single colony of bacteria was inoculated in 5 mL LB culture medium, and grown at 37°C overnight. The bacterial culture was then diluted 1:50 and grown at 37°C until A600 reached 0.6 followed by addition of 1 mM IPTG and subsequent growth at 16°C for 16 h. After the incubation, the bacteria were centrifuged at 5,000 g for 10 min; the pellet was resuspended in PBS/NaCl (0.5 M)/imidazole (1 mM) and lysed by sonication. The lysate was then centrifuged at 55,000 g for 30 min at 4°C, and the supernatant was collected. Recombinant His-Tag S100A8 and S100A9 were purified with nickel columns. S100A8 or S100A9 bound to the column were cleaved from their His-Tag by incubation with 10 U of biotinylated thrombin (Novagen, Germany) for 20 h at room temperature. Recombinant S100A8 and S100A9 were eluted with PBS. The digestion and elution processes were repeated once to cleave the remaining undigested recombinant proteins, and streptavidin-agarose (Novagen, Germany) was added to remove the contaminating thrombin. The proteins were kept at 80°C for up to 2 months until usage.

Formation of S100A8/A9 Heterodimers

In vitro formation of S100A8/A9 heterogeneous dimer was verified through SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. For SDS-PAGE, S100A8 and S100A9 were digested with Hanks-Hepes solution with 1.3 mM Ca^{2+}, quantified with BCA method, and mixed at equal amounts. The mixture was kept at room temperature for 30 min before reductive or non-reductive loading buffer was added. SDS-PAGE electrophoresis and Coomassie Brilliant Blue staining were then performed.

S100A8/A9 heterogeneous dimer was detected with an antibody in the Western blot analysis.

Cell Lysis and Western Blot Analysis

CaSki cells were washed with PBS at 4°C and solubilized in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 50 mM sodium fluoride, and 1 mM PMSF. Denatured protein samples were subjected to SDS-PAGE in electrophoresis buffer (25 mM Tris-Base, 250 mM glycine, and 0.1% SDS). Proteins were then transferred to Immobilon-P membranes in transfer buffer (50 mM Tris-base, 380 mM glycine, and 20% methanol) for 2 h at 400 mA at 4°C, and detected by overnight incubation with appropriate antibodies. Protein bands were visualized after incubation with peroxidase-linked anti-mouse or anti-goat secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using the Super Signal West Pico Chemiluminescent Substrate Kit (Thermo Fisher Scientific, Pierce, IL, U.S.A).

Fluorescence-Activated Cell Sorting (FACS) Analysis

CaSki cells were grown in six-well plates with S100A8, S100A9, or S100A8/A9 heterodimers for 24 h. The cells were trypsinized and washed twice with cold PBS and then resuspended in 200 μL binding buffer at a concentration of 1×10^6 cells/mL. The cells were incubated with 10 μL annexin V-FITC conjugate and 5 μL propidium iodide in the dark for 15 min. In total, 300 μL binding buffer was then added in each tube prior to being analyzed with FACS analyses (Becton Dickinson, San Jose, CA, USA).

Hochest 33342 Staining

CaSki cells were treated with S100A8, S100A9 or S100A8/A9 heterodimers for 24 h and washed with PBS for 3 times. Hochest 33342 (10 μg/mL) was added to the solution and incubated at 37°C for 30 min. Observations were made under an inverted fluorescence microscope.

Migration Assay

Wound scratch assay was utilized to analyze cell migration on a two-dimensional epithelial sheet. CaSki cells were grown to confluence in 3.5 cm culture dishes in DMEM with 10% FCS followed with in 1% FCS for 24 h. The monolayers were then scratched with a 200 μl-pipette tip, washed with PBS, and incubated with 20 μg/mL recombinant S100A8, S100A9, or S100A8/A9. Migration of the

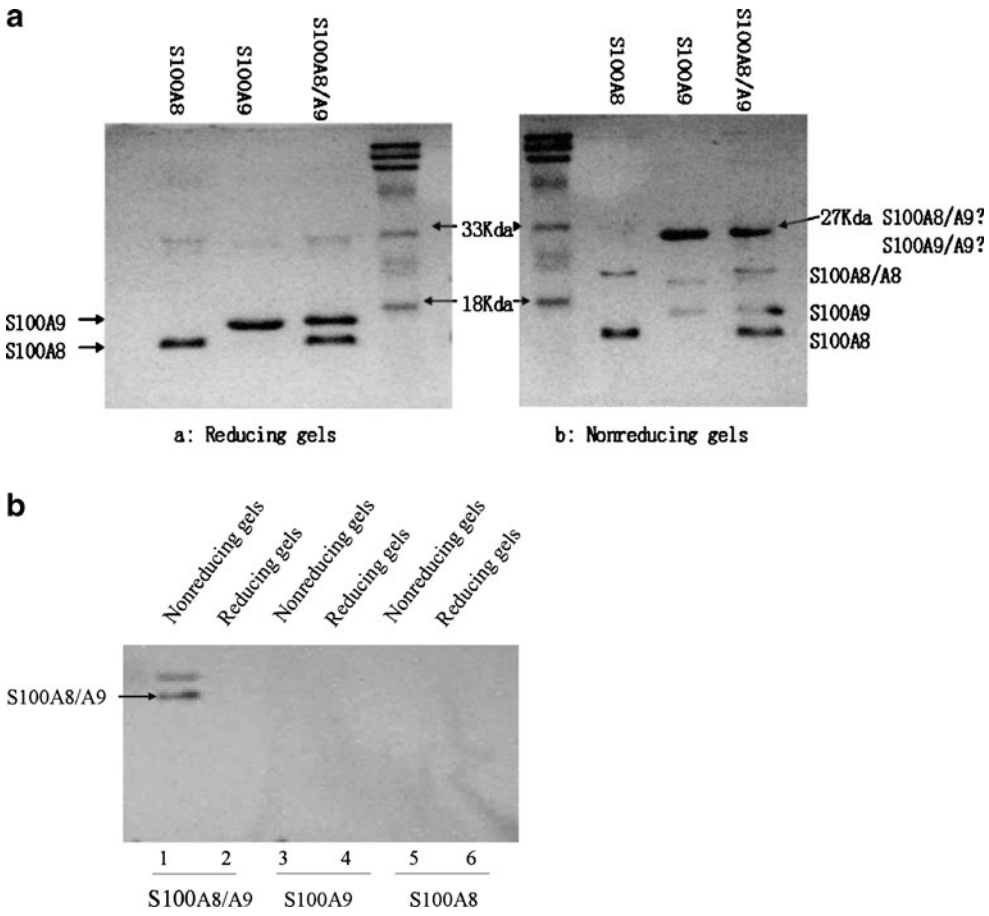

Fig. 1 Formation of S100A8/A9 heterogeneous dimer. **a** Recombinant S100A8 and S100A9 (3 μg/lane) were mixed together (in presence of Hanks-Hepes solution containing 1.3 mM $Ca2^+$) before being separated on SDS-PAGE under reducing (**a**) or nonreducing (**b**) conditions and stained with Coomassie brilliant blue. The presence of the monomeric, homodimeric, and heterodimeric forms of the proteins is indicated. **b** As shown by detection using antibody against S100A8/A9 heterogeneous dimer, there were bands of specific S100A8/A9 heterodimeric under non-reducing electrophoresis

cells was detected under a microscope; data were summarized from at least three independent scratch wounds, from which migration from at least three different positions were documented. The wound margin distances between the two edges of the migrating cell sheets were measured at 0, 4 h, 12 h, 24 h and 30 h after scratching. Differences between groups were statistically evaluated with single-factor variance analysis and were considered as statistically significant when $P<0.05$.

Results

Formation of S100A8/A9 Heterodimers

To verify the formation of the S100A8/A9 heterodimers, SDS-PAGE analyses using non-reductive gels were performed. Homogeneous dimers of S100A8 or S100A9 were detected. However, the molecular weight for S100A9/A9 (27-kDa) homogeneous dimer was almost identical to the heterogeneous dimer of S100A8/A9 (28-kDa, Fig. 1a), indicating that SDS-PAGE analysis can be used to demonstrate the formation of heterogeneous dimer. However, in our Western blot analysis, the antibody against S100A8/A9 heterogeneous dimer did not cross-react with S100A9/S100A9 homogeneous dimer. Thus the 27-kDa band detected in Western analysis represented the S100A8/A9 heterogeneous dimer (Fig. 1b), indicating that S100A8 and S100A9 could form heterogeneous dimer in vitro.

Effect of S100A8/A9 on CaSki Apoptosis

Detection of AnnexinV/PI-marked Cells Using FACS Analysis

To study effect of S100A8/A9 on CaSki apoptosis, cells were treated with S100 proteins followed by staining of AnnexinV/PI and FACS analysis. The apoptosis rate was 5.8±0.4%, 6.3±0.2%, and 6.3±0.7% for the negative control, S100A8 treatment, and S100A9 treatment, respectively. No significant difference ($P>0.05$) existed among these groups. However, the apoptosis rate was significantly higher for the cells treated with S100A8/A9 (13.3±0.6%) than any other groups ($P<0.05$) (Fig. 2), suggesting that S100A8/A9 heterogeneous dimer induces apoptosis of CaSki cells.

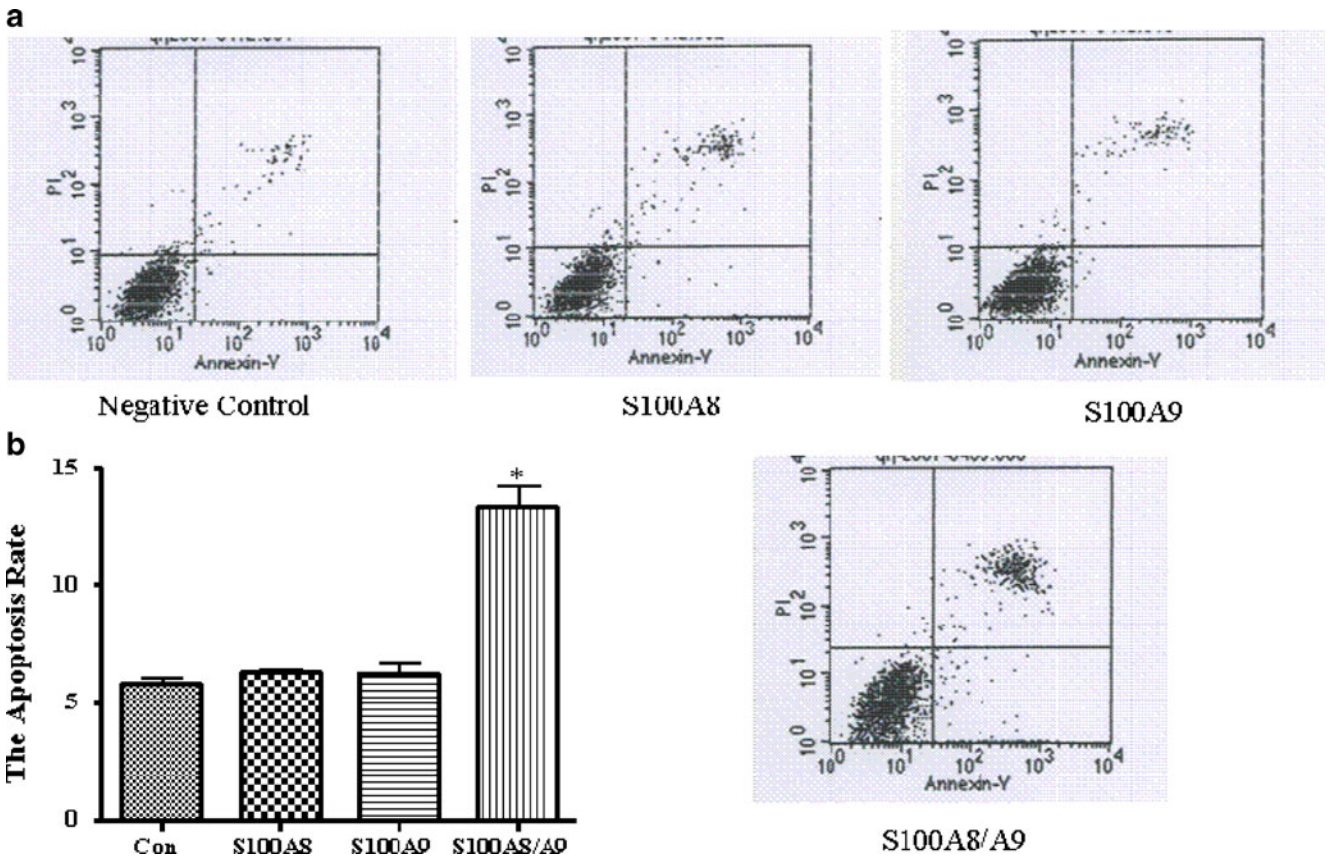

Fig. 2 Effect of S100A8/A9 on CaSki Apoptosis. Apoptosis rate was measured with FACS analysis of CaSki cells double stained with AnnexinV/PI 24 h after the treatment with 20 μg/ml S100A8/A9. The apoptosis rate was significantly higher in S100A8/A9 group (13.3±0.6%) than control groups ($P<0.05$, $n=3$)

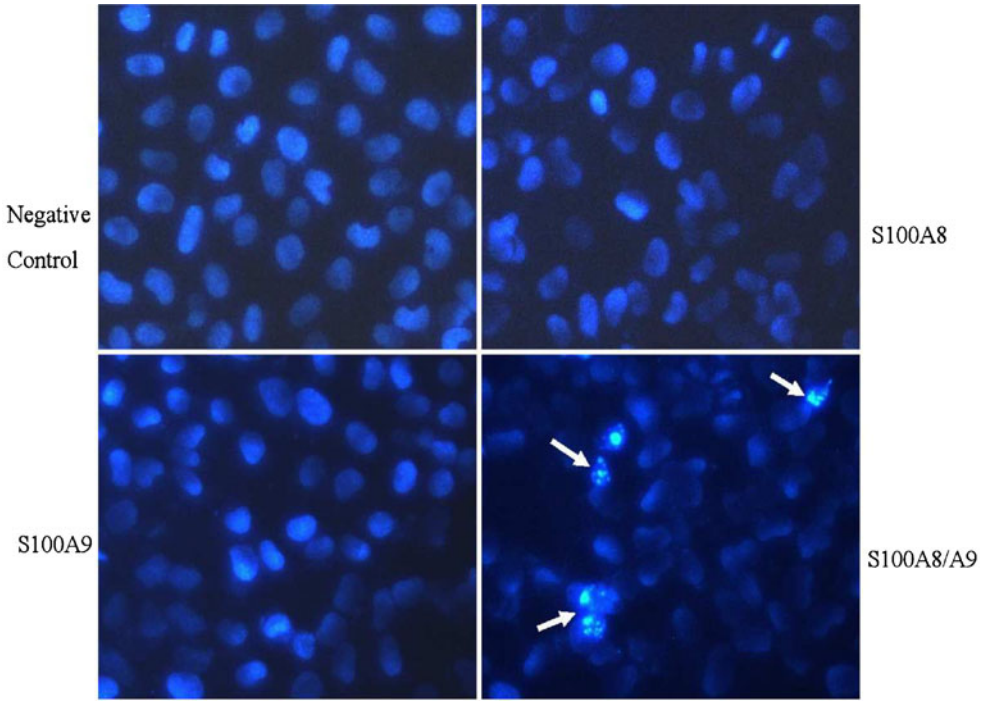

Fig. 3 Nucleus Hochest Staining 24 h after CaSki cells were treated with 20 μg/ml S100A8/A9

Morphology of Apoptotic Cell

Effects of S100A8/A9 on apoptosis were also determined by nuclear staining using Hochest 33342. Twenty-four h after treatment with 20 μg/ml S100A8/A9, nuclei in CaSki cells had significant pyknosis and cloudy fluorescence, whereas nuclei in control cells or cells treated 20 μg/ml S100A8 or 20 μg/ml S100A9 showed uniform blue fluorescence (Fig. 3). Thus, S100A8/A9 treated cells were more apoptotic than cells in other groups.

Caspase-3 Mediated S100A8/A9-induced Apoptosis

To determine the potential mechanism of S100A8/A9-induced apoptosis, Western blot analysis was performed. Twelve h after cells were treated with S100A8/A9, caspase-3 precursor was cleaved to 17 kDa and 20 kDa subunits (Fig. 4), suggesting that capsase-3 mediated S100A8/A9-induced apoptosis.

Effect of S100A8/A9 on In Vitro Invasiveness of CaSki Cells

Streak Repair

The ability of CaSki cells in streak repair was determined by measuring the residual streak width at 4 h, 12 h, 24 h, and 30 h after streaking. Treatment with S100A8/A9 significantly reduced the streak repair ability, compared with non treatment or treating with S100A8 or S100A9 alone ($P<0.01$) (Fig. 5), whereas no significant difference existed between the three control groups ($P>0.05$) (Table 1).

Effect of S100A8/A9 on MMP-2 Expression

To explore the molecular mechanism of S100A8/A9 induced invasiveness of CaSki cells, Western blot analysis of MMP-2 was performed. Starting from 12 h after treatment with S100A8/A9, MMP-2 expression began to decrease in a time-dependent manner (Fig. 6), suggesting a role of MMP-2 in S100A8/A9 mediated invasiveness of CaSki cells.

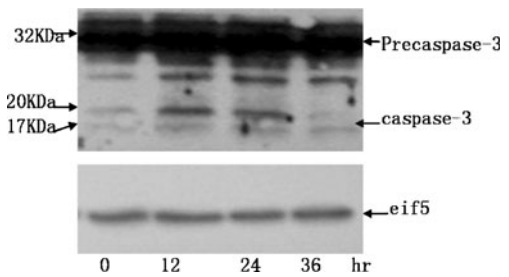

Fig. 4 Stronger caspase-3 activity in CaSki cells treated with S100A8/A9. Cells were treated with 20 μg/ml S100A8/A9 and collected at different time point. Western blot analysis was performed to detect caspase-3 and its active fragments. eif5, loading control

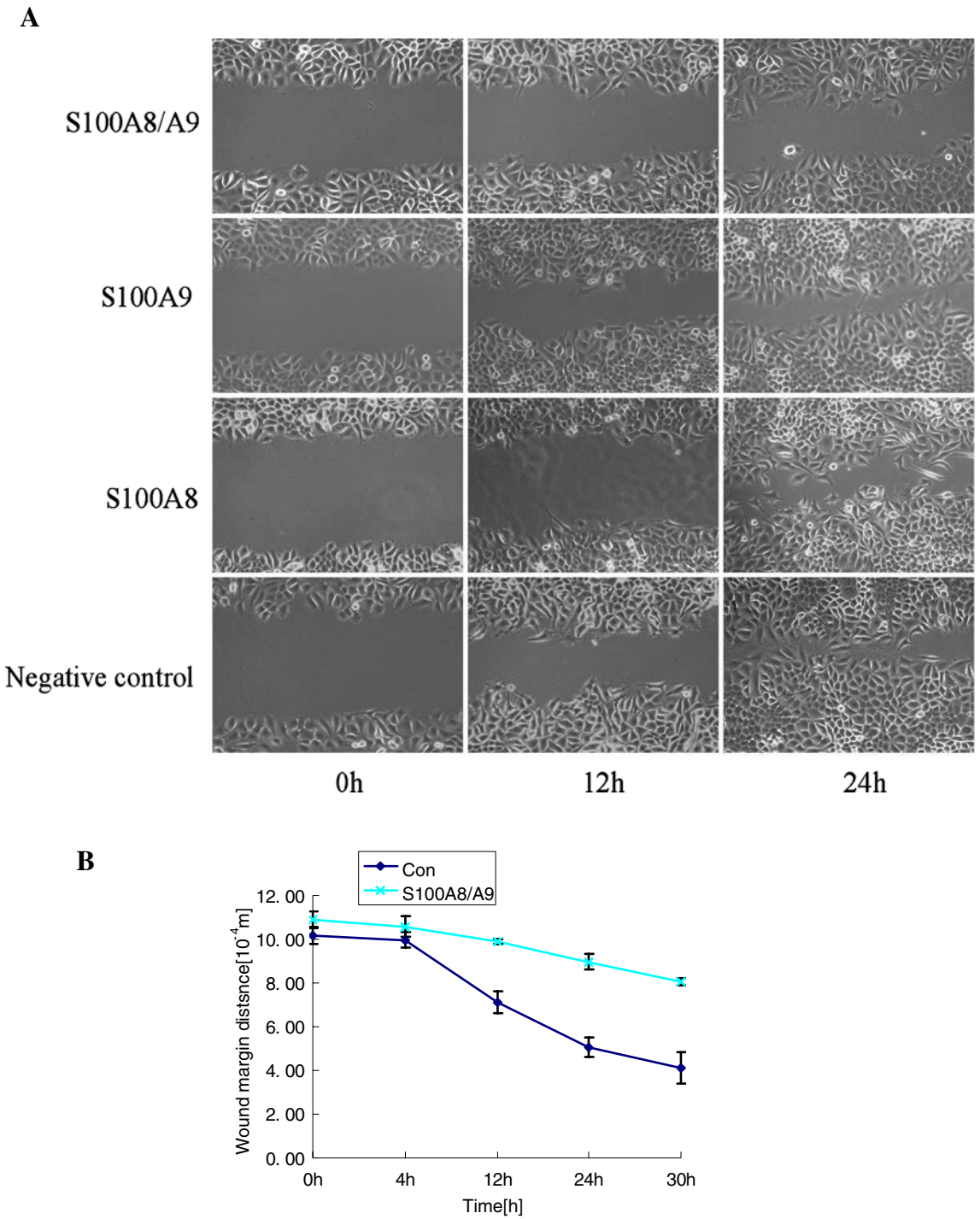

Fig. 5 Inhibitory effect of S100A8/A9 on the ability of streak repair of CaSki cell. **a** Representative microscopic pictures documenting inhibited closure of the wound gap over time. **b** Measurement of the distance of wound margin

Discussion

S100A8/A9 Heterogeneous Dimer

S100A8 and S100A9 are two members of S100 family proteins that mainly play their biological roles by forming heterogeneous dimer under certain Ca^{2+} concentration. According to previous study from other group that expressed and purified the S100A8/A9 dimer using E. coli expression system [15], we utilized the same system to obtain the heterogeneous dimer in vitro, which was verified by Western blot analysis.

Table 1 Effect of S100A8/A9 on the streak repair ability of CaSki cell

Time	Wound margin distance (10^{-4}m)				
	Control	S100A8	S100A9	S100A8/A9	*p*
0 h	10.17±0.4	10.81±0.47	10.6±0.14	10.89±0.4	
4 h	9.95±0.36	10.18±0.3	10.2±0.34	10.5±0.48	
12 h	7.13±0.5	8.29±1.07	8.38±0.1	9.89±0.13	
24 h	5.06±0.44	5.34±1.25	6.68±0.1	8.97±0.35	<0.05
30 h	4.11±0.7	4.41±0.28	4.83±0.26	8.05±0.18	<0.05

Apoptosis and Tumor

Initiation and progression of tumor is a comprehensive process that involves multiple factors, stages, and gene variations. In contrast to normal cells, malignant cells exhibit abnormality in proliferation, apoptosis, and differentiation. Many tumors are caused by the imbalance between the rates of cell proliferation and cell death. This study suggests that S100A8/A9 play a role as a tumor suppressor by inducing the apoptosis of tumor cells.

Apoptosis is a process that cells end their own lives under certain physiological or pathological conditions. Cells in apoptosis form colonies or split into several apoptotic bodies that eventually are engulfed by other cells. Organisms use apoptosis to remove injured or hyperplastic cells from the body and hence prevent the hyperplasia of injured cells and tumor formation. Thus, apoptosis disorder may lead to tumor formation.

It is well established that apoptosis causes many morphological and biochemical changes (e.g., nuclear pyknosis and breaking), which was verified by staining with Hochest 33342 in this study. One of the key features in apoptotic cells is the translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. Annexin-V has a strong affinity for PS and thus can measure apoptosis. In the current study, 24 h after treatment with S100A8/A9, there were significantly more Caski cells positive to Annexin V, indicating that S100A8/A9 could promote tumor cell apoptosis.

Our study suggests that S100A8/A9 induces apoptosis through caspase-3. Caspase family proteins play key roles in cell apoptosis and include regulating molecules (such as caspase-8, caspase-9, and caspase-2) and effect molecules (e.g., caspase-3, caspase-6, and caspase-7). Caspase-3 is a key functional molecule and plays an important role in the signal transduction pathway of apoptosis. Caspase-3 is present in cytoplasm as proenzyme (32-kDa) under normal state and is activated at early apoptosis. Activated caspase-3 is composed of two large subunits (17-kDa) and two small subunits (12-Kda) to cleave the corresponding substrates in cytoplasm and nucleus and finally cause cell apoptosis [16]. As verified by this study, S100A8/A9 could promote caspase-3 activation and thus induce cell apoptosis.

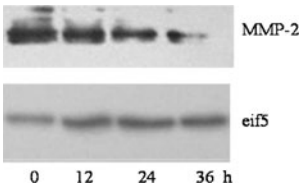

Fig. 6 Inhibitory effect of S100A8/A9 on MMP-2 expression. Cells were treated with 20 μg/ml S100A8/A9 and collected at different time point. MMP-2 expression was observed by Western blot. eif5, loading control

In Vitro Invasiveness of Tumor Cell

Metastasis is the last stage of tumor progress and the major cause for death of tumor patients. Tumor metastasis is a complex multi-stage process including decreased adhesion between tumor cells and between tumor cells and extracellular matrix, altered tumor cell framework, enhanced motility, increased capability to secrete various protein hydrolases, and activated angiogenesis. The enhanced motility is directly associated with the potential of tumor metastasis [17]. Migration of cell involves multiple steps including extending pseudopodium at cell head, establishing new adhesion, contracting cell body, and eliminating adhesion at cell tail; and it is mainly realized through mutual slide between fibril and myosin during streak test [18]. In this study, the streak repair ability decreased after the cells were treated with S100A8/A9, indicating that S100A8/A9 inhibits the invasiveness of tumor cells partially by inhibiting cell motility.

The degradation of basal membrane cellular matrix is the necessary step for tumor cells to infiltrate into nearby fibrous connective tissue and then metastasize to distant places; MMP and its TIMP are two of the main proteins involved in the process [19]. MMP-2 plays important role in the infiltration and metastasis of carcinoma cells. It promotes tumor invasion/metastasis by degrading basal membrane and tumor-wrapping substrate and by breaking through substrate barrier; it also enhances tumor growth/

spreading by inducing proliferation of capillary endothelial cells and hence formation of new blood vessels. MMP-2 expression increases in many tumor tissues, which is associated with breakage and fragmentation of the corresponding basal membrane [20–22]. After the cells were treated with S100A8/A9, the MMP-2 expression decreased. Thus, the invasion and metastasis of cells might be suppressed by inhibiting the degradation of extracellular substrate. However, the specific mechanism of this inhibition awaits further verification.

In summary, our current study indicates that S100A8/A9 might inhibit carcinoma by inducing the apoptosis of cervical carcinoma cell and inhibiting the metastasis.

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