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RESEARCH COMMUNICATION

Regulation of S100A8/A9 (Calprotectin) Binding to Tumor Cells by Zinc Ion and Its Implication for Apoptosis-Inducing Activity

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S100A8/A9 (calprotectin), which is released by neutrophils under inflammatory conditions, has the capacity to induce apoptosis in various cells. We previously reported that S100A8/A9 induces apoptosis of EL-4 lymphoma cells via the uptake of extracellular zinc in a manner similar to DTPA, a membrane-impermeable zinc chelator. In this study, S100A8/A9-induced apoptosis was examined in several cell lines that are weakly sensitive to DTPA, suggesting S100A8/A9 is directly responsible for apoptosis in these cells. Since zinc inhibits apoptosis of MM46, one of these cells, the regulation by zinc of the capacity of S100A8/A9 to bind MM46 cells was studied. When MM46 cells were incubated with S100A8/A9 in standard or zinc-depleted medium, the amounts of S100A8/A9 bound to cells was markedly lower at 3 h than at 1 h. In contrast, when MM46 cells were incubated with S100A8/A9 in the presence of high levels of zinc, binding to cells was the same at 1 and 3 h. When the cells were permeabilized with saponin prior to analysis, a larger amount of cell-associated S100A8/A9 was detected at 3 h. The amount was further increased in cells treated with chloroquine, suggesting that S100A8/A9 was internalized and degraded in lysosomes. Although it has been reported that S100A8/A9 binds to heparan sulfate on cell membranes, the amount of S100A8/A9 bound to MM46 cells was not reduced by heparinase treatment, but was reduced by trypsin treatment. These results suggest that S100A8/A9 induces apoptosis by direct binding to MM46 cells, and that this activity is regulated by zinc.

INTRODUCTION

S100A8 and S100A9 are calcium- and zinc-binding proteins from the S100 protein family that are abundant in the cytosol of neutrophils [1, 2]. These proteins are released from neutrophils as a heterodimeric complex, S100A8/A9, under inflammatory conditions. The complex is also known as calprotectin, since it showed antifungal activity [3]. The smaller 8 kd component, S100A8, has also been termed migration inhibitory factor-related protein (MRP)-8 and calgranulin A, while the larger 14 kd component, S100A9, has been termed MRP14 and calgranulin B [4, 5]. It has been reported that amounts of S100A8/A9 in blood or extracellular body fluid are increased under many pathological conditions, for instance, rheumatoid arthritis [6], inflammatory bowel diseases [7], viral or microbial infections [8, 9], tumors [10], and many inflammatory conditions [11]. Notably, abscess fluids contain more than 1 mg/mL of the protein complex [12].

Although S100A8/A9 is abundant under many inflammatory conditions, the physiological role of this factor is not fully understood. Proposed extracellular activities of the protein complex include antifungal activity [13, 14] and chemotactic activity against neutrophils [15]. On the other hand, we reported that S100A8/A9 induced apoptosis in tumor cells [16, 17] and normal fibroblasts [18]. Therefore, it is proposed that S100A8/A9 is a neutrophil-derived effector acting against tumor cells and is a factor regulating inflammatory processes via apoptosis-inducing activity [19].

Zinc ions are a regulator for the antifungal activity of S100A8/A9: chelation of zinc by the protein complex is speculated to be the mechanism of the fungistatic activity [14, 20, 21]. We found that the apoptosis-inducing activity of S100A8/A9 is inhibited in the presence of zinc ions

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[17]. By contrast, the activity was augmented in medium deprived of divalent cations (other than Ca^{++} and Mg^{++}) [18]. We also reported that the protein complex induces apoptosis of EL-4 mouse lymphoma cells via exclusion of extracellular zinc in a manner similar to a membrane-impermeable zinc chelator, DTPA [22]. However, there are several reports dealing with the binding capacity of S100A8 and/or S100A9 and the binding molecules on different types of cells: S100A9 and S100A8/A9 reportedly bind heparan sulfate on the surface of endothelial cells [23], and S100A8/A9 binds carboxylated glycans on endothelial cells [24]. It was also proposed that a scavenger receptor, CD36, binds the complex of S100A8/A9 and arachidonic acid [25]. Moreover, the receptor for advanced glycated end product (RAGE) reportedly binds S100B, S100A1, and S100A12 proteins [26, 27], although it is unknown whether RAGE is a general receptor for the members of the S100 protein family.

Recently, it was proposed that S100A8/A9 induced apoptosis in colon cancer cell lines via the exclusion of zinc from the target cells or some unknown mechanism [28]. Since S100A8/A9 has a very broad target specificity (we have not encountered any cell lines that are resistant to the apoptosis-inducing activity of S100A8/A9), S100A8/A9 may induce apoptosis by a mechanism that requires the binding of the factor to target cells, in addition to the mechanism of extracellular zinc exclusion.

In this paper, we examined the cell death-inducing activities of S100A8/A9 and the zinc chelator DTPA in five tumor cell lines, and observed that S100A8/A9 induced cell death of DTPA-sensitive cell lines as well as three cell lines including MM46 mouse mammary carcinoma cells, which are resistant to DTPA. Further studies showed that the action of S100A8/A9 against MM46 cells was inhibited by zinc. The effect of zinc on the binding of S100A8/A9 to MM46 cells suggest a novel mechanism in which zinc-regulated S100A8/A9 association with target cells induces apoptosis.

MATERIALS AND METHODS

Reagents

Diethylenetriaminepentaacetic acid (DTPA), *NNN′-N′*-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN), chloroquine, heparinase I, and sodium heparin were purchased from Sigma (St Louis, Mo). FlouZin-3,AM was from Molecular Probes (Eugene, Ore). Trypsin (1:250) was from GIBCO (Grand Island, NY). Mouse monoclonal anti-human MRP8 (S100A8) and anti-human MRP14 (S100A9) antibodies were from BMA (Augst, Switzerland). Goat FITC-conjugated anti-mouse IgG antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). $ZnSO_4 \cdot 7H_2O$, Saponin, NaN_3, and other chemicals were from Wako Pure Chemical Industries (Osaka, Japan).

Recombinant human MRP8 and MRP14

Recombinant human S100A8 and S100A9 were prepared as described previously [22]. Briefly, competent *Escherichia coli* strain BL21 (DE3) cells (Novagen, Madison, Wiss) were transformed using the vector pET1120-MRP8(S100A8)wt or pET1120-MRP14(S100A9)wt [29]. The transformed cells were grown in (2× YT) medium supplemented with 100 μg/mL ampicillin for 24 h, and produced the proteins as inclusion bodies. The harvested cells were solubilized with B-PER Bacterial Protein Extraction Reagent (PIERCE, Rockford, Ill). The inclusion bodies were solubilized with Inclusion Body Solubilization Reagent (PIERCE), and the proteins were refolded according to the manufacturer's directions. The proteins were purified by reverse-phase column chromatography (Resource RPC, Amersham Pharmacia, Buckinghamshire, UK) furnished in a BioLogic HR system (BIO-RAD, Hercules, Calif), followed by UNO-Q anion exchange chromatography (BIO-RAD).

Cell lines

EL-4 mouse thymoma, MOLT-4 human leukemia, and MH134 mouse hepatoma cell lines were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS). MM46 spontaneous mammary carcinoma cells and Ehrlich carcinoma cells were passaged weekly in the peritoneal cavity of male C3H/He mice and ddY mice (Japan SLC Inc, Shizuoka, Japan), respectively.

MTT assay

The MTT assay was used to evaluate cell death. Each cell line (1×10^4 cells/well) was cultured with test samples in 96-well microtest plates (Iwaki, Chiba, Japan) with 200 μL of RPMI1640 medium supplemented with 100 U of penicillin, 60 μg/mL kanamycin, and 5% FCS (hereafter referred to as the standard medium) at 37°C in a humidified atmosphere of 5% CO_2 in air. After the appropriate periods, 25 μL of 3-(4,5-dimetyl-2-thiazolyl)-2,5-diphenyl -2H-tetrazolium bromide (MTT, 5 mg/mL) was added to the cultures and the plates were incubated for an additional 3 h. Then, 150 μL of the supernatant was discarded, 100 μL of acidified isopropanol (0.04 N HCl in 2-propanol) was added to each well, and the optical density (595 nm) was measured with a microplate reader (Multiscan MS-UV, Labsystems, Basingstoke, UK). Data are represented as the mean ± SD of triplicate estimations.

Divalent cation-depleted medium

To prepare divalent cation (other than Ca^{++} and Mg^{++})-depleted medium, 100 mL of RPMI-1640 medium containing 5% FCS that had been extensively dialyzed against phosphate-buffered saline (PBS) was applied to a Chelex 100 column (Bio-Rad, 1.0 × 20 cm). Then, 500 μM $CaCl_2$ and 400 μM $MgCl_2$ were added to supplement the calcium and magnesium. This medium is referred to hereafter as the divalent cation-depleted medium.

Intracellular zinc concentration

EL-4 or MM46 cells (1×10^5 cells/well) were incubated in 24-well plates with 1 mL of the standard medium with samples at 37°C for 6 h. After 3 washes with PBS, the cells were treated with 1 mM FlouZin-3,AM for 30 min at 37°C. The cells were washed 3 times with PBS and analyzed using a FACSscan flow cytometer (Beckman Coulter, Philadelphia, Pa).

S100A8/A9 binding assay

EL-4 or MM46 cells (1×10^5 cells/well) were incubated in 24-well plates with a specific concentration of S100A8/A9 sample for 1 or 3 h at 37°C in 5% CO_2 in air, unless otherwise indicated. The cells were washed 3 times with PBS containing 2% FCS, and were treated with anti-S100A9 antibody at a dilution of 1:100 for 30 min on ice. The cells were washed 3 times with PBS (2% FCS), and treated with the secondary FITC-conjugated anti-mouse IgG antibody at a dilution of 1:100 for an additional 30 min on ice. The fluorescence intensity associated with cells was analyzed with a FACSscan flow cytometer. The morphology of the cells was observed with a fluorescence microscope (Nikon, Eclipse, TE2000U, Tokyo, Japan), after being fixed with 4% formaldehyde and treated with anti-S100A9 antibody and the secondary FITC-conjugated antibody as above.

To estimate the cell-associated amount of S100A8/A9, which includes S100A8/A9 in intracellular compartments, the treated cells were fixed with 4% formaldehyde after three washes with PBS, and treated with PBS containing 0.5% saponin and 1% bovine serum albumin for 10 min at room temperature. After that, the cells were treated with anti-S100A9 antibody and the FITC-conjugated secondary antibody as described above.

Trypsin and heparinase treatments of MM46 cells

MM46 cells were treated with 0.25% trypsin or 4 U/mL heparinase I at 37°C for 5 or 60 min, respectively. After the digestion, the cells were washed three times with PBS, and used as targets for the binding of S100A8/A9.

RESULTS

Cytotoxicity of S100A8/A9 and DTPA against several cell lines

To examine whether the cytotoxicity of the S100A8/A9 heterodimer is solely due to exclusion of extracellular zinc by chelation, the cytotoxic activity of recombinant human S100A8/A9 against several tumor cell lines was compared with that of a membrane-impermeable zinc chelator, DTPA, and a membrane-permeable zinc chelator, TPEN. Consistent with our previous report, S100A8/A9, as well as DTPA alone, induced the death of EL-4 cells at 40 h of culture (Figure 1a). S100A8/A9 and TPEN efficiently induced loss of viability of MOLT-4 cells, while the effect of DTPA on the cells was weak at 40 h. On the other hand, S100A8/A9 and TPEN at 5 μM showed cytotoxicity against MM46 cells, MH134 cells, and Ehrlich carcinoma cells at 24 h, whereas DTPA did not show any cytotoxicity. Even at 100 μM, DTPA did not reduce the viability of MM46 cells at 24 h (data not shown).

Since we previously reported that the MM46 cell death induced by S100A8/A9 is due to apoptosis and the time course of the apoptotic induction of this cell type was well characterized [30], we used MM46 cells in the subsequent experiments. We also found that S100A8/A9 induced activation of caspase-3 and mitochondrial permeability transition in MM46 cells (unpublished observations). Figure 1b shows the time-course of the cytotoxic activities of S100A8/A9 and DTPA against MM46 cells. S100A8/A9 exerted full cytotoxicity in MM46 cells from 24 h. In contrast, DTPA did not show any cytotoxicity at 24 h. It took 3 days for DTPA to fully induce cell death, suggesting that S100A8/A9 induces MM46 cell death via a fundamentally different mechanism than that of DTPA.

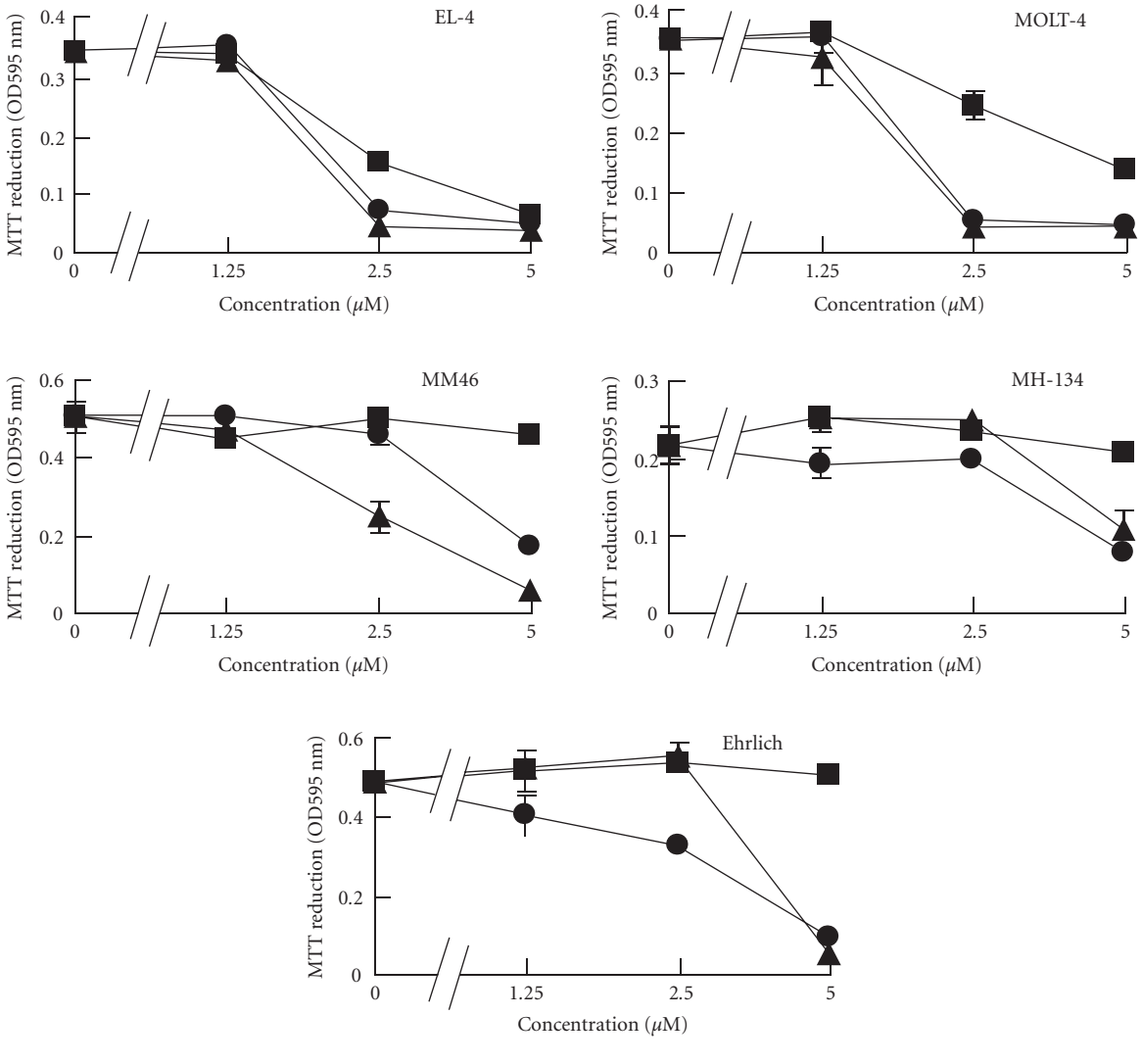
We previously reported that the S100A9 homodimer induced EL-4 cell death at 10 μM, while the activity of the S100A8 homodimer is very weak: it showed marginal cytotoxicity even at 40 μM. On the other hand, the S100A8/A9 complex has more potent activity against EL-4 cells: the onset of cytotoxicity occurred at 5 μM (Figure 1a and [22]). Because S100A8/A9-induced apoptosis of MM46 cells may involve a different mechanism from that in EL-4 cells, the effects of the individual subunits on MM46 cells were also investigated. Figure 3a shows that neither S100A8 nor S100A9 on their own show cytotoxicity against MM46 cells.

To examine whether MM46 cells are less sensitive than EL-4 cells to zinc, the intracellular zinc concentrations of the two cell lines after treatment with S100A8/A9, DTPA, or TPEN were compared in a FACS analysis using FlouZin-3,AM as a probe. As shown in Figure 2, membrane-impermeable DTPA induced a reduction in the intracellular zinc concentration of MM46 cells at 6 h. The degree of reduction was similar to that observed for the membrane-permeable TPEN, and the patterns of reduction were indistinguishable from those of EL-4 cells. The effect of S100A8/A9 on the intracellular zinc was comparable to that of DTPA and TPEN, in both EL-4 cells and MM46 cells.

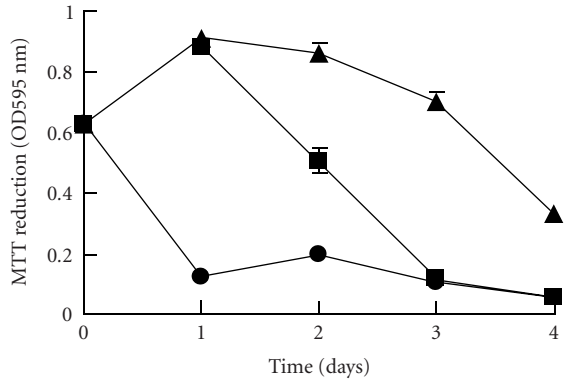
The data in Figure 1 indicates that MM46 cells are more resistant to the shortage of intracellular zinc than EL-4 cells. This suggests that S100A8/A9 can induce cell death via a mechanism other than zinc exclusion, and that direct binding of S100A8/A9 to MM46 cells is involved.

Binding of S100A8/A9 to MM46 cells

We next analyzed the binding of S100A8/A9 and the homodimers to MM46 cells using flow cytometry. Monoclonal antibodies against S100A8 or S100A9 were used as the first antibodies, followed by secondary FITC-conjugated anti-mouse IgG antibody. After demonstrating that the binding of S100A8/A9 to MM46 cells could be detected by either anti-S100A8 or anti-S100A9 antibody



(a)



(b)

Figure 1. Cell death-inducing activity of recombinant human S100A8/A9 complex, DTPA and TPEN against various cell lines. (a) The indicated cell lines were cultured with S100A8/A9 (●), DTPA (■), or TPEN (▲) for 24 h (MM46 cells, MH-134 cells, and Ehrlich cells) or 40 h (EL-4 cells and MOLT-4 cells). (b) Kinetics of induction of MM46 cell death by S100A8/A9 and DTPA. MM46 cells were cultured without (▲) or with 10 μM S100A8/A9 (●) or 10 μM DTPA (■) for the indicated periods. The viability of each cell line was evaluated by MTT assay. Bars represent standard deviation.

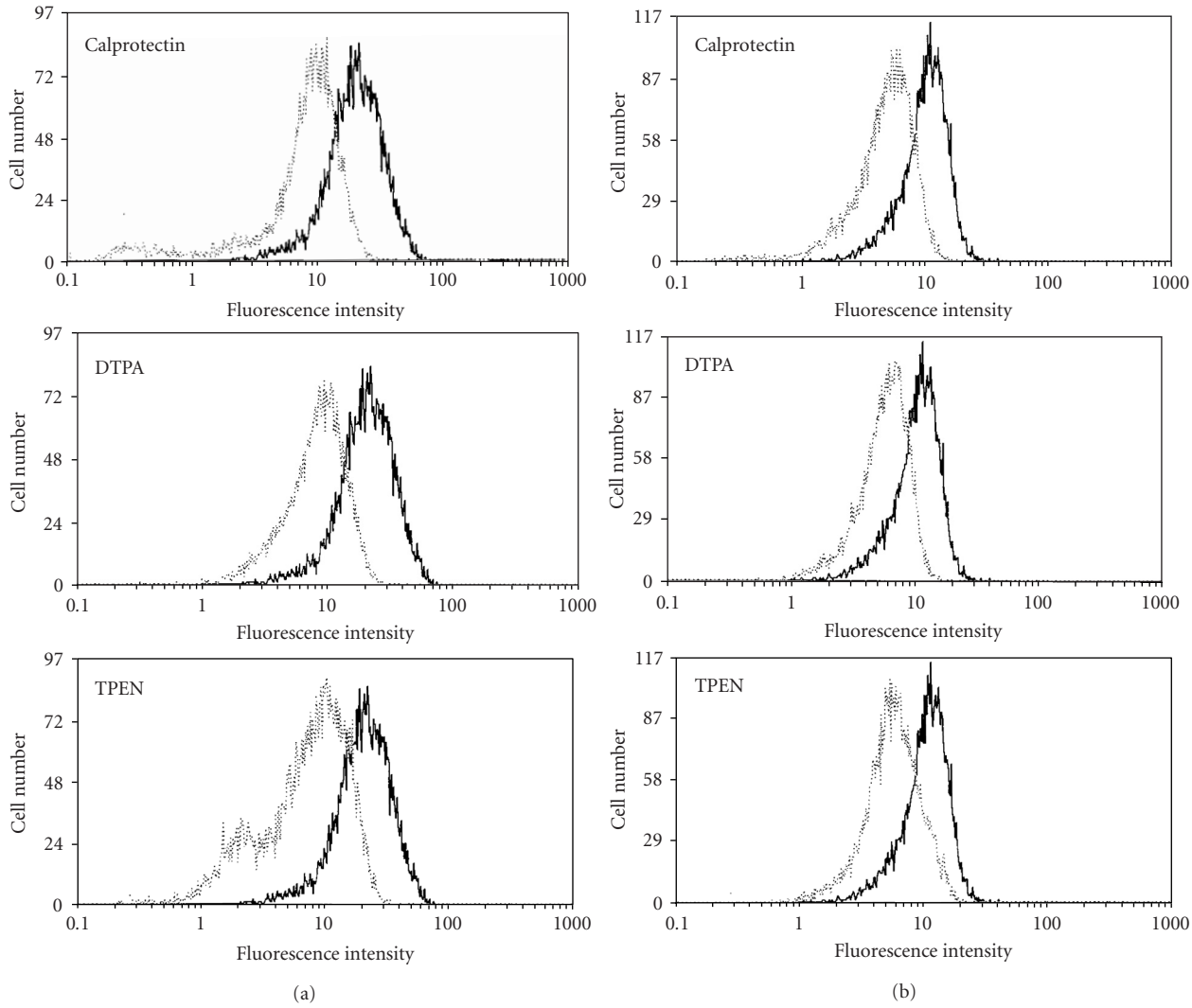


Figure 2. Reduction of the intracellular zinc concentration of (a) MM46 cells and (b) EL-4 cells treated with S100A8/A9 complex, DTPA, or TPEN. The cells were cultured without (solid line) or with (dotted line) 5 μM S100A8/A9, 5 μM DTPA, or 5 μM TPEN for 6 hours, respectively. The intercellular zinc concentration was measured using Zn-specific fluorescence probe, FluoZin3,AM as described in "materials and methods."

(Figure 3b), it was decided to use the anti-S100A9 antibody for the detection of the complex in all subsequent assays. The binding assays were performed at 37°C; a control experiment at 4°C gave quite similar histograms at 1 h (data not shown). Remarkably, the S100A9 homodimer showed a similar degree of binding in the assay as the S100A8/A9 complex (Figure 3b), in spite of its lack of cytotoxic effect on MM46 cells. The S100A8 homodimer showed a lesser degree of binding. We note that the lower activity of S100A8 may be caused by its relative instability compared to S100A9 and the S100A8/A9 complex.

A dose-response curve was determined for S100A8/A9 binding at 1 h, and was depicted in Figure 3c. The amount of S100A8/A9 increased in a dose-dependent manner up to 5 μM, but then markedly decreased at 10 μM and beyond.

Zinc regulation of the binding of S100A8/A9 to tumor cells

We previously reported that the cytotoxic activity of S100A8/A9 against EL-4 cells is inhibited by zinc, and that S100A8/A9-induced cell death occurs at lower concentrations in divalent cation-depleted (other than Ca^{++} and Mg^{++}) medium [16, 17, 18, 22]. Although the cytotoxic effect of S100A8/A9 on MM46 cells observed at 24 h appears to not be directly induced by exclusion of zinc in the medium (Figure 1), S100A8/A9 cytotoxicity was moderately and completely inhibited by the presence of 2.5 μM and 5 μM zinc, respectively (Figure 4a). As observed for EL-4 cells, the dose-response curve shifted to lower concentration ranges in the divalent cation-depleted medium (Figure 4b). These data suggest that zinc modulates the cytotoxic activity of S100A8/A9.

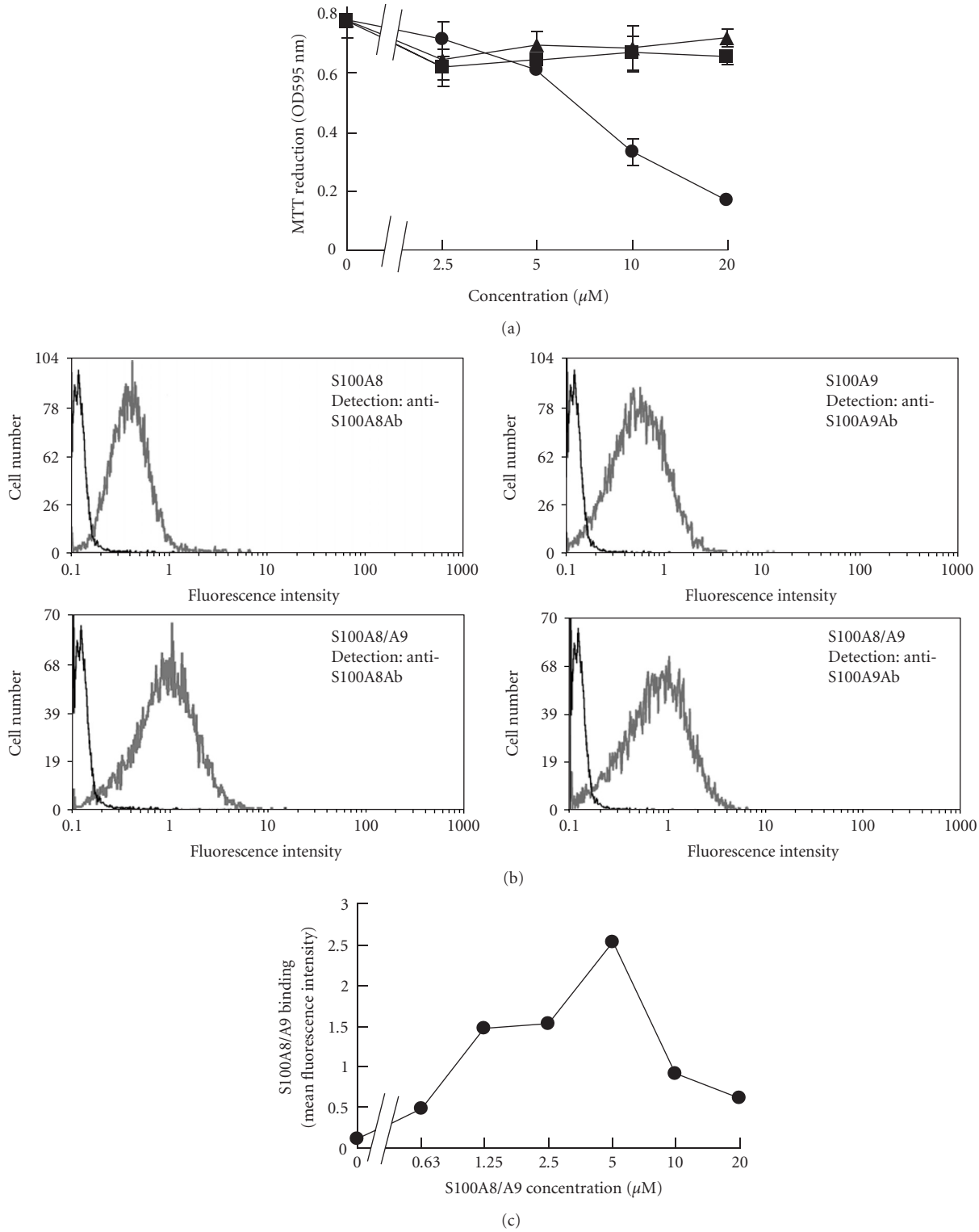


Figure 3. (a) Apoptosis-inducing activity of S100A8, S100A9, and S100A8/A9 against MM46 cells. Cells were cultured with different concentrations of S100A8/A9 complex (●), S100A8 (■), or S100A9 (▲) for 24 hours. Cell viability was estimated by MTT assay. Bars represent standard deviation. (b) Binding profile of S100A8/A9 complex, S100A8, or S100A9 to MM46 cells. The cells were incubated with (gray line) or without (black line) 5 μM S100A8, 5 μM S100A9, or 5 μM S100A8/A9 for 1 h, respectively. After the incubation, the cells were washed and treated with anti-S100A8 antibody or anti-S100A9 antibody, followed by FITC-conjugated anti-mouse IgG antibody, and analyzed as described in "materials and methods." (c) Dose-response relationship of the binding of S100A8/A9. MM46 cells were incubated with the indicated concentrations of S100A8/A9 for 1 h and the binding is expressed as the geometric mean fluorescence intensity of 10 000 cells.

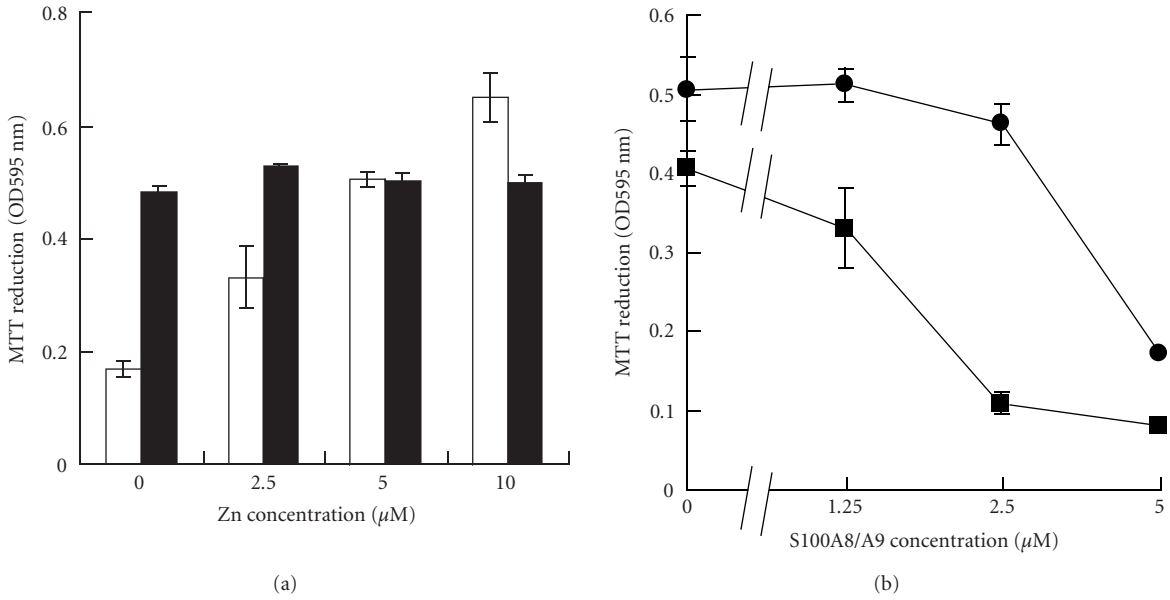


FIGURE 4. Zinc inhibits apoptosis-inducing activity of S100A8/A9 complex against MM46 cells. (a) MM46 cells were cultured without (open column) or with (filled column) 5 μM S100A8/A9 in the presence of the indicated concentrations of zinc sulfate for 24 h. (b) Augmentation of the apoptosis-inducing activity of S100A8/A9 in divalent cation-depleted medium. MM46 cells were cultured with 10 μM S100A8/A9 in the standard medium (●) or divalent cation-depleted medium (■) for 24 h and the viability was evaluated by MTT assay. Bars represent standard deviation.

To check the effect of zinc on the association of S100A8/A9 with MM46 cells, we measured binding in the presence of various concentrations of zinc. At 10 μM S100A8/A9, the amount of protein that binds to MM46 cells at 1 h was very small in the divalent cation-depleted medium, but binding was augmented in a dose-dependent manner in the presence of zinc (Figure 5a).

We next examined the kinetic changes of the binding of S100A8/A9 to MM46 cells. A S100A8/A9 concentration of 10 μM was used because this concentration has been shown to induce apoptosis in standard medium. As shown in Figure 5b, the amounts of S100A8/A9 in the divalent cation-depleted medium at 30 min and 1 h were much smaller than the amounts in the standard medium, and decreased markedly at 3 h. In the standard medium, binding of the protein complex was observed at 30 min, and was slightly increased at 1 h, but the binding was much decreased at 3 h. Interestingly, the binding of the S100A9 homodimer detected at 1 h was not decreased at 3 h (Figure 5c).

To assess whether zinc affects the kinetic changes in the binding of S100A8/A9 in standard medium, the binding of the protein complex was analyzed in the presence of 20 μM zinc sulfate, conditions under which the cytotoxicity of S100A8/A9 is completely inhibited. Unexpectedly, binding at 3 h in the presence of excess zinc was not decreased, but rather, the amount in the MM46 cells at 3 h was increased (Figure 5b). Morphologically, several dots of fluorescence were observed on the cells at 1 h in the absence and the presence (stoichiometric or excess) of zinc, suggesting that S100A8/A9 forms aggregated structures on the cell membrane.

In EL-4 cells, in which apoptosis appears to be induced via the exclusion of zinc by S100A8/A9, the initial binding of protein complex to cells was markedly decreased at 3 h (Figure 5d). The binding of S100A8/A9 to EL-4 cells was increased in the presence of 20 μM zinc sulfate. Thus, as in MM46 cells, the addition of zinc enables S100A8/A9 to remain bound to EL-4 cells at 3 h.

Internalization of S100A8/A9 in MM46 cells

We have noted that the binding of S100A8/A9 to cells is reduced during the incubation in the standard medium or in the divalent cation-depleted medium. To elucidate the mechanism for this phenomenon, we examined whether the protein complex is internalized by MM46 cells. For this, MM46 cells treated with S100A8/A9 were fixed and permeabilized with saponin, and the cell-associated S100A8/A9 was detected using the anti-S100A9 antibody. At 1 h of incubation, the amount of cell-associated S100A8/A9 in the permeabilized cells was almost the same as that in the unpermeabilized cells. The amount detected without saponin treatment was markedly reduced at 3 h. However, the difference in the amount in the permeabilized cells between 1 h and 3 h was very slight (Figure 6a), suggesting that S100A8/A9 was internalized within the cells during 1–3 h.

Next, to check the possibility that the internalized S100A8/A9 molecules were degraded in lysosomes, MM46 cells were treated with S100A8/A9 in the presence

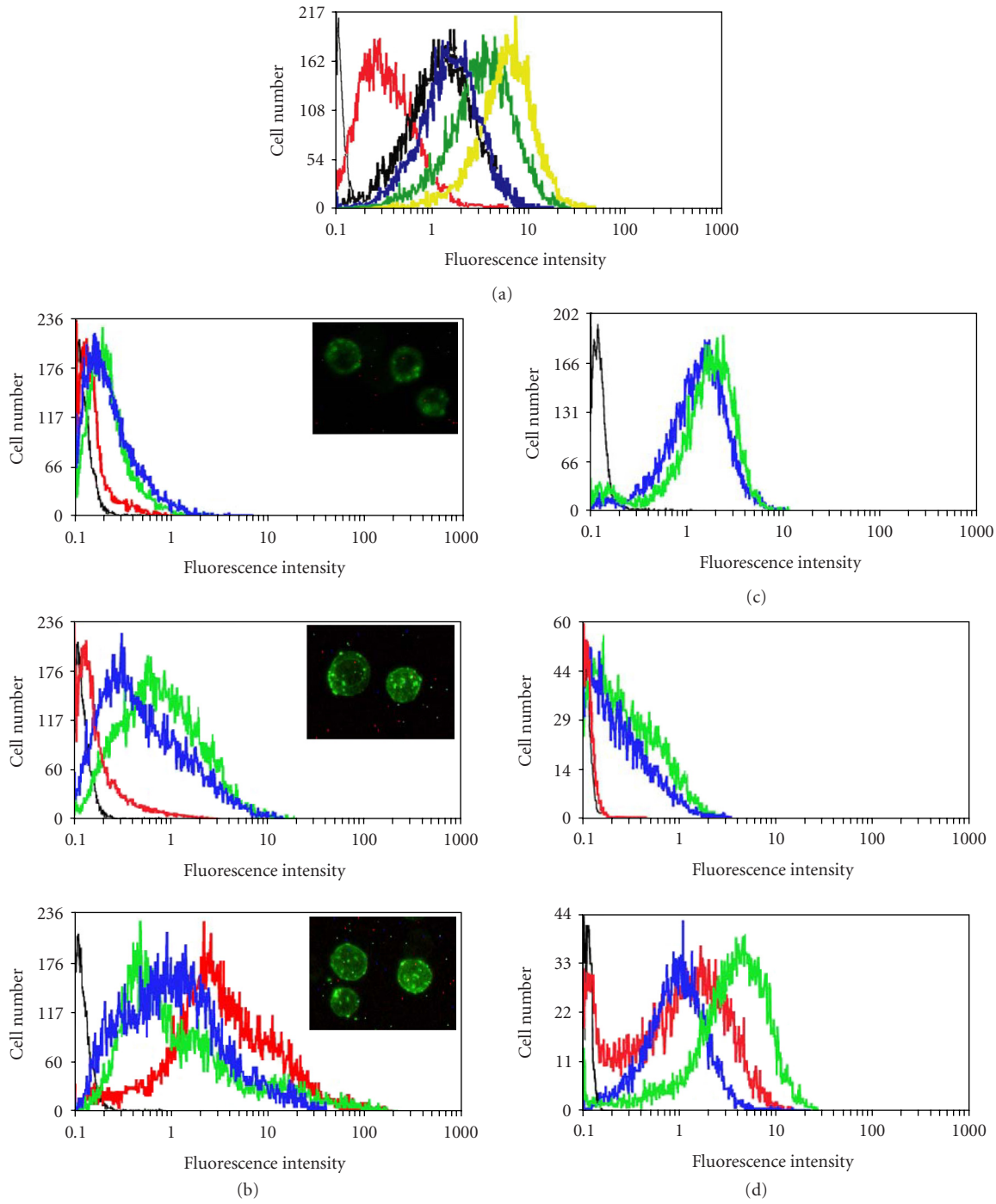


Figure 5. Profile of the binding of S100A8/A9 to MM46 cells in divalent cation-depleted medium, in standard medium or the presence of excess zinc. (a) Enhancement of the binding of S100A8/A9 to MM46 cells by zinc. The cells were incubated with 5 μM S100A8/A9 in the divalent cation-depleted media supplemented with 1.25 μM (red), 2.5 μM (black), 5 μM (blue), 10 μM (green), or 20 μM (yellow) zinc sulfate at 37°C for 1 h. (b) Kinetic changes in the binding profile of S100A8/A9. MM46 cells were incubated with 10 μM S100A8/A9 for 30 min (blue), 1 h (green), or 3 h (red) at 37°C in the defined divalent cation medium (upper panel), in the standard medium (middle panel), or in the standard medium with 20 μM zinc sulfate (lower panel). Insets: morphological appearance of MM46 cells incubated with 10 μM S100A8/A9 for 1 h in the defined divalent cation medium (upper), in the standard medium (middle), or in the standard medium with 20 μM zinc sulfate (lower). The binding of S100A8/A9 on the fixed cells was visualized as described in "materials and methods." (c) Binding of S100A9 to MM46 cells. The cells were incubated at 37°C with 10 μM S100A9 for 1 h (green) or 3 h (blue). (d) Changes in the profile of binding of S100A8/A9 to EL-4 cells. EL-4 cells were incubated in the standard medium (upper panel) or 20 μM zinc sulfate (lower panel) with 10 μM S100A8/A9 at 37°C for 30 min (blue), 1 h (green), or 3 h (red). (a), (b), (c), and (d) black line: histograms of the control cells which were incubated without any sample for 1 h in the respective medium.

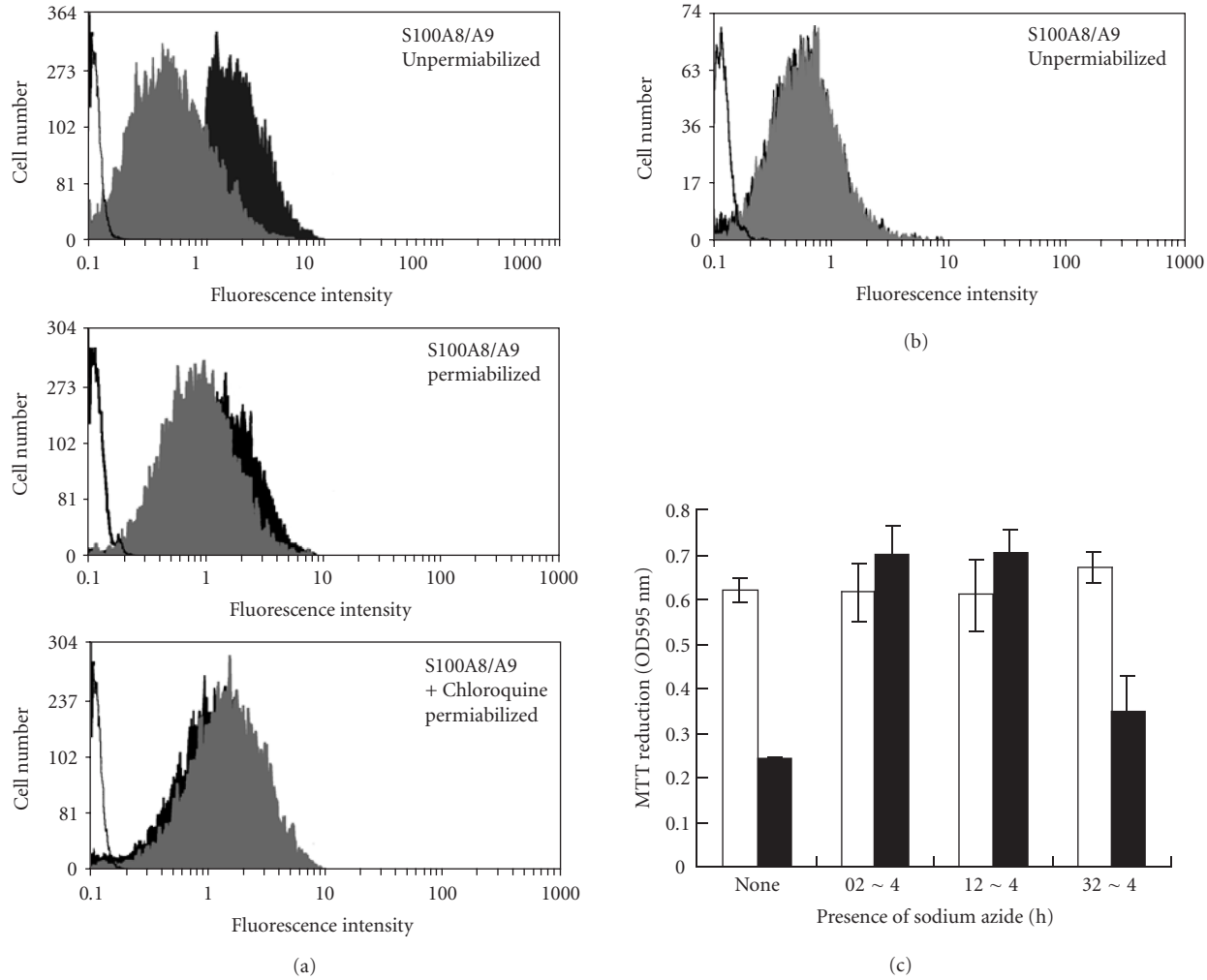


Figure 6. Detection of intracellular S100A8/A9 complex in MM46 cells. (a) MM46 cells were treated at 37°C without (black line), or with 10 μM S100A8/A9 for 1 h (black histogram) or 3 h (gray histogram). In the upper panel, the treated cells were washed and S100A8/A9 binding was immediately analyzed with anti-S100A9 antibody. In the middle panel, the treated cells were washed, fixed, and permeabilized with saponin, and the cell-associated S100A8/A9 was detected with anti-S100A9 antibody. In the lower panel, the cells were incubated with S100A8/A9 in the presence of 100 μM chloroquine, and after that, the cells were washed, fixed, and permeabilized with saponin, and the cell-associated S100A8/A9 was detected with anti-S100A9 antibody. (b) Effects of sodium azide on the changes in the binding profile of S100A8/A9. The cells were incubated at 37°C without (black line) or with S100A8/A9 plus 50 mM sodium azide for 1 h (black histogram) or 3 h (gray histogram). After that, the cells were washed and S100A8/A9 binding was immediately analyzed with anti-S100A9 antibody. (c) Effect of sodium azide on the apoptosis-inducing activity of S100A8/A9. MM46 cells were cultured with (filled column) or without (open column) 10 μM S100A8/A9 for 24 h. Sodium azide (50 mM) was present in the culture during the indicated periods. Bars represent SD.

of chloroquine, a lysosome enzyme inhibitor, and the cells were fixed, permeabilized, and analyzed. The cell-associated S100A8/A9 at 1 h incubation was little affected by chloroquine treatment, and the amount of S100A8/A9 at 3 h was slightly larger. Importantly, the amount at 3 h was much greater than that observed for permeabilized MM46 cells not treated with chloroquine (Figure 6a). These results suggest that some of the protein complex is degradated in lysosomes after being internalized by the cells.

S100A8/A9 binding was further characterized by examining the effect of sodium azide, an inhibitor of energy metabolism, on the change in the amount of bound S100A8/A9 during the incubation. Figure 6b shows that the amount of S100A8/A9 bound at 1 h was not decreased at 3 h in the presence of sodium azide, suggesting that an energy-dependent process is responsible for the internalization of S100A8/A9. Since sodium azide inhibited the reduction in the binding of S100A8/A9 to the cells, we checked whether the inhibitor inhibits the apoptosis-inducing activity of S100A8/A9. Figure 6c shows that sodium azide did indeed inhibit apoptosis. The addition of sodium azide at 1 h brought about a similar degree of inhibition, whereas at 3 h apoptosis was not inhibited.

These results raise the possibility that extensive ATP synthesis after 3 h is not necessary to induce apoptosis, and that internalization of S100A8/A9 in the cells up to 3 h is adequate for the apoptosis.

Heparan sulfate is not involved in induction of apoptosis by S100A8/A9

It was reported that S100A8/A9 binds heparan sulfate on endothelial cells in a zinc-dependent manner [23]. Since heparan sulfate is the universal component on cell membranes of various kinds of cells [31], it is important to determine if binding of S100A8/A9 to heparan sulfate is necessary for the induction of apoptosis. For this, we first measured the binding to MM46 cells treated with heparinase I or trypsin. The binding of 10 μM S100A8/A9 to the cells in the divalent cation-depleted medium or the standard medium (conditions under which S100A8/A9 induces apoptosis) was not attenuated by heparinase I treatment, but markedly attenuated by trypsin treatment (Figure 7a). On the other hand, the binding of S100A8/A9 in the presence of 20 μM zinc sulfate was moderately attenuated in the heparinase I-treated cells, although it was more extensively attenuated by trypsin treatment.

The effect of heparan sulfate on S100A8/A9-induced apoptosis was studied. Heparin reportedly binds S100A8/A9 in the presence of zinc and inhibits the binding of the protein complex to heparan sulfate on endothelial cells [23]. Sodium heparin (40–200 μM) did not inhibit apoptosis induced by 10 μM S100A8/A9 either in the divalent cation-depleted medium or in the standard medium (data not shown). These results suggest that the binding of S100A8/A9 to heparan sulfate is not involved in the induction of apoptosis.

DISCUSSION

We previously reported that S100A8/A9 induced apoptosis of EL-4 cells through the exclusion of extracellular zinc [22]. Recent studies have described that S100A8/A9 is able to kill not only cells that are sensitive to zinc exclusion by a membrane impermeable zinc chelator, DTPA, but also cells resistant to zinc deprivation by DTPA, suggesting that S100A8/A9 induces apoptosis via dual mechanisms: a zinc exclusion-dependent mechanism and possibly a receptor-dependent mechanism [28]. S100A8/A9-induced death of DTPA-resistant cell lines such as MM46 mammary tumor cells provides evidence in support of the latter mechanism.

We showed that DTPA reduced the intracellular zinc concentration in both EL-4 and MM46 cells to the same extent as a membrane-permeable zinc chelator, TPEN. However, most MM46 cells survived in the presence of DTPA on day 1 and half were still alive on day 2. Since almost all of the EL-4 cells undergo apoptosis on day 2 with DTPA [22], MM46 cells are clearly more resistant to deprivation of intracellular zinc. This implies that S100A8/A9 induced apoptosis of MM46 cells via a mechanism other than zinc exclusion. This hypothesis is supported by the observation that S100A8 and S100A9 alone, despite having significant zinc-binding capacity, showed weaker cell death-inducing activity against EL-4 cells [22], but they were entirely ineffective against MM46 cells. This prompted the exploration of the capacity of S100A8/A9 to bind to MM46 cells.

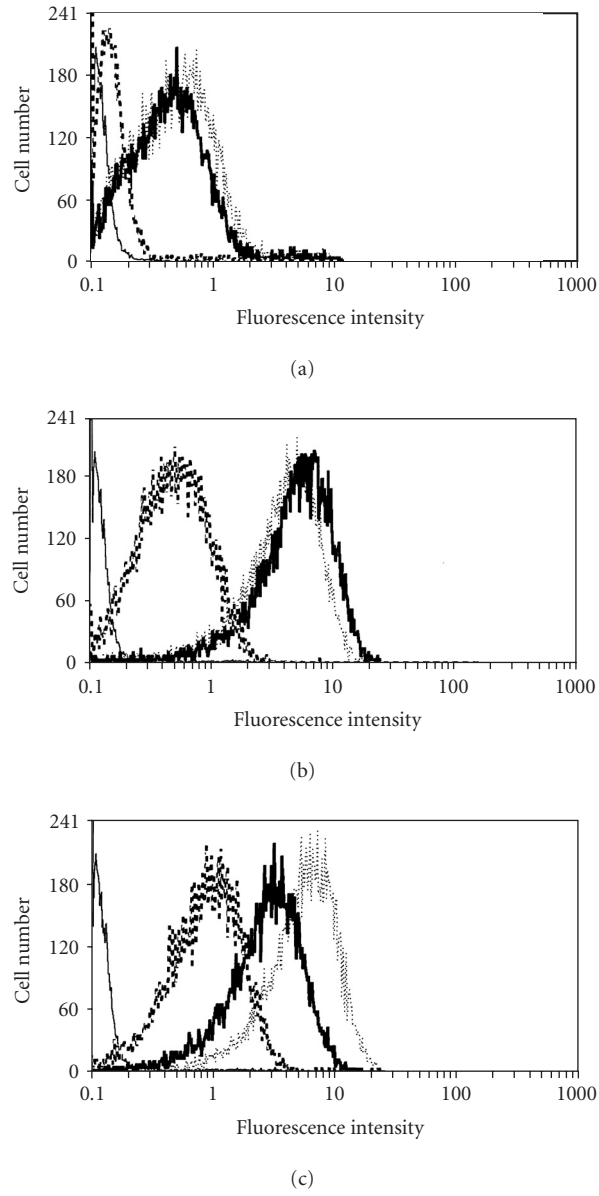


Figure 7. Binding of S100A8/A9 complex to MM46 cells which were treated with trypsin or heparinase. Untreated MM46 cells (thin dotted line) or cells treated with trypsin (bold dotted line), or heparinase (bold line) were incubated with 5 μM S100A8/A9 in the defined divalent cation medium (a), in the standard medium (b), or in the standard medium with 20 μM zinc sulfate (c) for 1 h, respectively. The histograms of the control cells which were incubated without any sample for 1 h in the respective medium are represented by a narrow solid line (solid line). The amount of S100A8/A9 bound to the cells was measured as described in "materials and methods."

S100A8 and S100A9 homodimers as well as the S100A8/A9 heterodimer were found to effectively bind to MM46 cells. However, the site of binding on MM46 cells is not known. We note that there are several reports dealing with the binding molecules for S100A8/A9 on cell membrane: S100A8/A9 reportedly binds to heparan sulfate proteoglycan [23] or carboxylated glycans [24] on endothelial cells. S100A8/A9 and arachidonic acid complex reportedly interact with CD36 [25]. Furthermore, it was also proposed that a receptor for advanced glycation end products (RAGE) on macrophages and other cells might be a universal receptor for the S100 protein family [26].

Among these molecules, heparan sulfate proteoglycan is widely distributed on various cell types [31], and it was reported that the binding of S100A8/A9 to heparan sulfate or heparin depends on the presence of zinc [23]. In this paper, we also found that the amount of cell-bound S100A8/A9 in the divalent cation-depleted medium was smaller than that in the standard medium, and that the former increased proportionately with the increase in the zinc concentration. Examination of the binding of S100A8/A9 to heparan sulfate on the cell membrane showed that the induction of apoptosis of MM46 cells by S100A8/A9 is not mediated by the binding to heparan sulfate, since binding was little affected by heparinase treatment of the target cells, and the apoptosis-inducing activity was not inhibited in the presence of a high concentration of heparin. In these conditions, the binding of S100A8/A9 was largely attenuated by the prior treatment of the cells with trypsin, suggesting that the binding molecule on the cells has a protein-like nature. The identification of the S100A8/A9 "receptor" on MM46, or other tumor cells, is the next important problem to be solved.

In our experiments, the binding of S100A8/A9 to MM46 cells in the presence of excess zinc, was diminished significantly by treatment with heparinase. Thus, heparan sulfate appears to play a part in the binding of S100A8/A9 in zinc-rich environments. S100A8, S100A9, and S100A8/A9 reportedly show some biological activities at low concentrations, conditions under which the zinc concentration surpasses the concentration of the proteins. For example, cellular activities of S100A8/A9 include chemotactic activity against inflammatory cells [15], and S100A9 has been shown to have growth-stimulating activity against fibroblasts [32]. In addition, S100A8/A9 purified from rat inflammatory neutrophils at low concentration has an enhancing effect on ^{3}H-thymidine incorporation into human dermal fibroblasts, but at higher concentrations, induces apoptosis [18]. These observations suggest that zinc alters the biological activities of S100A8/A9, presumably by modulating its affinity for binding molecules/receptors on target cells.

Under physiological conditions such as inflammation, it can be postulated that S100A8/A9 induces growth of fibroblasts to repair tissue damage at low concentrations. But as expression levels rise above the cellular levels of free zinc under conditions of severe inflammation, S100A8/A9 will suppress tissue repair by inducing apoptosis of the surrounding cells. Clearly, additional studies are needed to test and refine this hypothesis.

We found that a greater amount of S100A8/A9 was required for the induction of apoptosis in the standard medium than in the divalent cation-depleted medium. It is therefore possible that the zinc-free form of S100A8/A9 induces apoptosis by binding a molecule distinct from the zinc-bound S100A8/A9. However, it is difficult to prove whether the binding seen in the divalent cation-depleted medium represents the binding of the zinc-free form of S100A8/A9 because some S100A8/A9 molecules may be capable of utilizing zinc ions from other cells. Alternatively, the internalization of S100A8/A9 into an intracellular compartment might be important for the induction of apoptosis, for the following three reasons. (1) It was observed that the amount of S100A8/A9 bound to the cell surface at 1 h incubation was decreased at 3 h, but the decrease was inhibited by zinc or sodium azide. (2) S100A8/A9, at least in part, seems to be internalized and degraded by lysosomes, because chloroquine increased the amount of the cell-associated protein complex. (3) Sodium azide presents throughout the assay inhibited apoptosis, but when added at 3 h, no inhibitory effect on apoptosis was detected. We previously reported that the induction of apoptosis of MM46 cells by S100A8/A9 is divided into an induction phase and an execution phase [30]. In the later execution phase, the generation of reactive oxygen species from about 8 h after the start of incubation is essential. It is therefore probable that sodium azide does not interfere with the execution phase, but rather interferes with the internalization of S100A8/A9 detected up to 3 h during the induction phase. However, other possible explanations cannot be ruled out, since the effect of sodium azide as an energy metabolism inhibitor is very broad.

In summary, previous studies have shown that S100A8/A9 can induce apoptosis in certain cell lines via its high affinity binding of zinc, causing depletion of zinc in cells. However, S100A8/A9 is capable of inducing apoptosis in cell lines such as MM46, which are not sensitive to exclusion of intracellular zinc. We have obtained evidence supporting the proposal that in these cells, an alternate mechanism of S100A8/A9-induced apoptosis is operative, involving binding to cell surface "receptors." Moreover, we have shown that the binding of cells is in turn modulated by the level of zinc, suggesting that the binding to cells by Zn-bound S100A8/A9 is different from the Zn-free state. The elucidation of the target binding sites at the cell surface should greatly enhance our insight into the mechanism underlying S100A8/A9-induced apoptosis and its modulation by zinc.

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