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Implication of extracellular zinc exclusion by recombinant human calprotectin (MRP8 and MRP14) from target cells in its apoptosis-inducing activity

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***Background*: Calprotectin is a calcium-binding and zinc-binding protein complex that is abundant in the cytosol of neutrophils. This factor is composed of 8 and 14 kDa subunits, which have also been termed migration inhibitory factor-related proteins MRP8 and MRP14. We previously reported that rat calprotectin purified from inflammatory neutrophils induces apoptosis of various tumor cells or normal fibroblasts in a zinc-reversible manner.**

***Aim*: The present study was undertaken to elucidate which subunit is responsible for the apoptosis-inducing activity, and to explore the mechanism of zinc-reversible apoptosis induction.**

***Methods*: The apoptosis-inducing activity of recombinant human MRP8 (rhMRP8) and recombinant human MRP14 (rhMRP14) was examined against EL-4 lymphoma cells *in vitro*. To determine whether zinc deprivation by calprotectin was essential for the cytotoxicity, the activity of calprotectin was tested under conditions where physical contact between the factor and the cells was precluded by a low molecular weight cut-off dialysis membrane.**

***Results*: The cytotoxicity of rhMRP14 against EL-4 cells was first detected at 10 μM in a standard medium, whereas rhMRP8 caused only marginal cytotoxicity at 40 μM. A mixture of both proteins showed higher specific activity (onset of cytotoxicity at 5 μM). When the cells were cultured in divalent cation-depleted medium, each dose–response curve was shifted to about a four-fold lower concentration range. Calprotectin was found to induce cell death even when the complex and the target cells were separated by dialysis membrane. A membrane-impermeable zinc chelator, diethylenetriamine pentaacetic acid (DTPA), also induced target cell apoptosis in a similar time-course as calprotectin. Moreover, the activities of calprotectin and DTPA were completely inhibited by the presence of zinc ions.**

***Conclusion*: These data indicate that calprotectin has higher specific activity to induce apoptosis than the individual subunits, and that the mechanism is exclusion of zinc from target cells.**

Key words: S100A8/S100A9, Cytotoxicity, Neutrophils

Introduction

Neutrophils are known to be inflammatory cells that secrete many protein factors including enzymes, antibacterial proteins, and cytokines. They play a role in controlling inflammation, in addition to killing microorganisms.[1,2] While searching for the regulatory activity within the neutrophils, we found that rat neutrophils contain a factor having cytostatic activity against lymphocytes.[3] This factor is calprotectin.[4] We subsequently reported that rat calprotectin has apoptosis-inducing activity against various tumor cells[5] and normal cell types including fibroblasts.[6] The apoptosis-inducing activity was abrogated by zinc ions, but not by calcium ions.[4–6]

Calprotectin is a calcium-binding and zinc-binding protein complex composed of 8 and 14 kDa proteins.[7] These proteins, which belong to the S100 protein family,[8] have also been termed migration inhibitory factor-related proteins (MRP)-8 and MRP14,[9] calgranulin A and B,[10] or S100A8 and S100A9.[11] Neutrophils are believed to be a predominant producer of calprotectin since they contain a high amount of the proteins in cytosol.[12] The concentration of calprotectin in extracellular fluids increases under various inflammatory conditions,[13–17] suggesting that this factor has important functions influencing inflammatory processes. The concentration in normal human serum was reportedly less than 1 μg/ml, but the titers increase in many

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pathological conditions including cystic fibrosis, rheumatoid arthritis, or Crohn's disease (reviewed elsewhere[17]). Much intense increase in calprotectin concentration has been observed in body fluids of local inflammatory sites than in blood: the concentration in synovial fluid of patients with rheumatoid arthritis was reported to increase sometimes to more than 100 μg/ml.[13,15] Very high concentrations (1–20 mg/ml) of calprotectin reportedly existed in abscess fluid from human abdominal abscesses.[16]

Evidence unraveling the extracellular functions of calprotectin or its subunit have been accumulating recently. For example, antimicrobial activity[18–21] and regulatory activities towards migration or adherence of inflammatory cells have been proposed.[22,23] The exact physiological roles of this protein complex, however, await further investigation.

Our findings concerning the growth-inhibiting and cell-death-inducing activity suggest that calprotectin plays a regulatory role in inflammatory processes through its effect on the survival and/or growth of cells involved in the inflammation. It is also suggested that if a high concentration of calprotectin is present in the body fluid at locally inflamed sites, this might cause a delay in tissue repair and have a deleterious effect on the inflamed tissues. Therefore, discovery of the mechanism of the apoptosis induction by calprotectin might be important in controlling of inflammation. The present study was undertaken to determine whether human calprotectin has apoptosis-inducing activity, as observed previously for the rat protein complexes. The activities of the MRP8 and MRP14 alone were compared with that of the mixture using recombinant human MRP8 (rhMRP8) or recombinant human MRP14 (rhMRP14). We also explored the mechanism of induction of zinc-reversible apoptosis. The results show that an equimolar mixture of rhMRP8 and rhMRP14 has higher specific activity than the individual proteins, and suggests that apoptosis is induced by exclusion of extracellular zinc ions by calprotectin from target cells.

Materials and methods

Reagents

Diethylenetriamine pentaacetic acid (DTPA) and *N,N,N′,N′*-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) were purchased from Sigma (St Louis, MO, USA). $ZnSO_4 \cdot 7H_2O$ and other chemicals were from Wako Pure Chemical Industries (Osaka, Japan).

Recombinant human MRP8 and recombinant human MRP14

Recombinant human calprotectin (rhMRP8 and rhMRP14) was prepared as described elsewhere,[24] with some modifications. Briefly, competent *Escherichia coli* strain BL21(DE3) cells (Novagen, Madison, WI, USA) were transformed by pET1120-MRP8wt or pET1120MRP14wt vectors. The transformed cells were grown in (2 × YT) media supplemented with 100 μg/ml of ampicillin for 24 h, and the cells produced the proteins as inclusion bodies. The harvested cells were solubilized with B-PER™ Bacterial Protein Extraction Reagent (Pierce, Rockford, IL, USA). The inclusion bodies were solubilized with Inclusion Body Solubilization Reagent (Pierce), and the proteins were refolded according to the manufacturer's protocol. The proteins were purified by reverse-phase column chromatography (Resource™ RPC; Amersham Pharmacia, Buckinghamshire, England) furnished in a BioLogic HR system (Bio-Rad), followed by UNO-Q anion exchange chromatography (Bio-Rad, Hercules, CA, USA). The buffer systems used were the same as those described previously.[24]

Cell lines

Mouse EL-4 thymoma cells, human MOLT-4 leukemia and MCF-7 mammary carcinoma lines were maintained in RPMI 1640 medium supplemented with 5% fetal calf serum (FCS). MM46, a transplantable ascites tumor from a spontaneous mammary carcinoma in a C3H/He mouse, was passaged weekly in the peritoneal cavity of male C3H/He mice (Japan SLC Inc., Shizuoka, Japan).

3-(4,5-Dimetyl-2-thiazolyl)-2,5-diphenyl-2 *H*-tetrazolium bromide assay

To evaluate the target cell death, the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay was used.[25] EL-4 cells or MM46 cells (1×10^4 cells/well) were cultured in 96-well microtest plates (Iwaki, Chiba, Japan) with 200 μl of RPMI 1640 medium (100 U of penicillin, 60 μg of kanamycin/ml, supplemented with 5% FCS). The cultures were added with test samples, and cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After the indicated periods, 25 μl of MTT (5 mg/ml) was added to the cultures, and plates were incubated for an additional 3 h. Then, 150 μl of the supernatants 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).

Divalent cation (other than Ca^{2+} and Mg^{2+})-depleted medium

To prepare divalent cation-depleted medium, 100 ml of RPMI-1640 medium containing 5% FCS that had been extensively dialyzed against 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 calcium and magnesium.

Double-chamber culture

The requirement of direct contact between rhMRP8/rhMRP14 complex and EL-4 cells to exert cytotoxic effects was examined by the double-chamber culture system. For this, plastic cylinders (15 mm in height, 10 mm in inner diameter) were used with the edge on one side sealed with dialysis membrane (3500 cut-off; Spectrum Medical Ind., Los Angeles, CA, USA) as inner chambers. These inner chambers were set in the wells of 24-well culture plates (Iwaki) at a location approximately 3 mm distant from the dialysis membrane in the inner chamber and the bottom of the wells of the 24-well plates (the outer chambers). The total culture volumes of the inner and outer chambers were 425 μl and 1000 μl, respectively. Divalent cation-depleted medium without or with the rhMRP8/rhMRP14 complex (7 μM) was added to the chambers as indicated, and the double-chamber system was kept at 4°C for 3 days. Thereafter, EL-4 cells (2.5×10^4 cells and 5×10^4 cells) were added to the inner and outer chambers, respectively, and the cells were cultured at 37°C. After 48 h of culture, 200 μl aliquots of suspended culture was transferred to 96-well plates to measure the capacity of MTT reduction of EL-4 cells in each chamber, using the MTT assay already described.

DNA fragmentation

EL-4 cells were cultured in 12-well plates (Corning, Corning, NY, USA) at 3×10^6 cells with test samples for the indicated periods to analyze DNA fragmentation. After the cells were harvested and washed once with PBS, 1.5 μg DNA extracted with a Genomic DNA Purification Kit (Promega, Madison, WI, USA) was separated by electrophoresis on 1.5% agarose gel in TAE buffer (40 mM Tris–HCl, 19 mM acetic acid, and 1 mM ethylenediamine tetraacetic acid) containing 0.5 μg/ml of ethidium bromide.

Results

Cell-death-inducing activity of human recombinant calprotectin chains

We previously reported that calprotectin (MRP8/MRP14 complex) derived from rat inflammatory neutrophils induces apoptosis of various tumor cells[4,5,25] and normal fibroblasts,[6] and that the apoptosis-inducing activity is abrogated by the presence of zinc.[4–6] To determine whether human calprotectin shows this cytotoxic activity, and which subunit of the protein complex is responsible for the activity, we compared the cytotoxicity to EL-4 cells of rhMRP8, rhMRP14 and a mixture of the two, using the MTT assay.

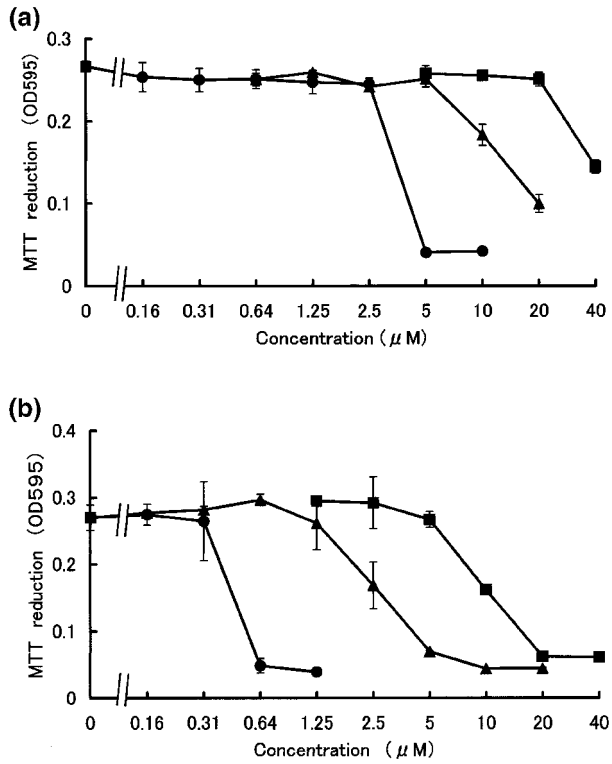
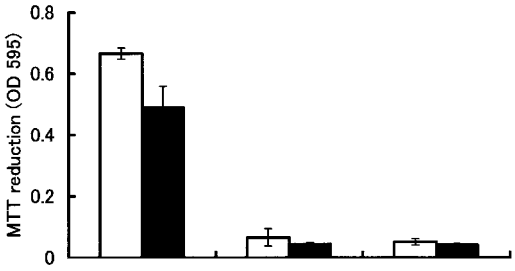


FIG. 1. Effect of rhMRP8, rhMRP14 or MRP8/MRP14 complex on the number of EL-4 cells. Cells were cultured with the indicated concentrations of (■) rhMRP8, (▲) rhMRP14, or (●) an equimolar mixture in (a) standard RPMI-1640 medium or (b) in divalent-cation-depleted medium for 40 h. Cell numbers were estimated by MTT reduction. Bars represent upper and lower values of duplicate estimations. When bars are not shown, they are smaller than the size of the symbols.

Figure 1a shows that rhMRP14 had more potent activity than rhMRP8. rhMRP14 inhibited MTT reduction in EL-4 cells, with an onset at a concentration of 10 μM and marked inhibition at 20 μM, whereas rhMRP8 showed no inhibition of MTT reduction until a concentration of 40 μM. On the contrary, equimolar solutions of MRP8 and MRP14 showed even more potent activity with almost complete inhibition at 5 μM. It was observed microscopically that the inhibition of MTT reduction paralleled the emergence of apoptotic bodies and apoptotic cells having an apoptotic appearance, such as condensation or segregation of nuclei.

We previously reported that the effects of calprotectin were shifted to lower concentration ranges in media depleted of divalent cations other than Ca^{2+} and Mg^{2+}.[6] We next examined whether the specific activities of rhMRP8, rhMRP14 or their mixture were also augmented in divalent cation-depleted medium. Figure 1b shows that, in fact, the dose–response curves are shifted to about a four-fold lower concentration. A similar observation was made in examining the effects of calprotectin on MM46 mammary carcinoma cells (data not shown).



Inner Chamber	–	–	+
Outer Chamber	–	+	+

FIG. 2. Test of the requirement for direct contact between rhMRP8/rhMRP14 and EL-4 cells to exert a cytotoxic effect. Divalent-cation-depleted medium without or with rhMRP8/rhMRP14 complex was added to the inner and outer chambers of a double-chamber culture system, in which the two chambers were separated by dialysis membrane (3500 cut-off) as described in Materials and methods. After being stored at 4°C for 3 days, EL-4 cells were added to the inner and outer chambers as indicated. After culturing for 48 h, MTT-reducing activity of EL-4 cells in the inner chamber (white bars) and the outer chamber (black bars) were measured. Bars represent standard deviations of triplicate estimations.

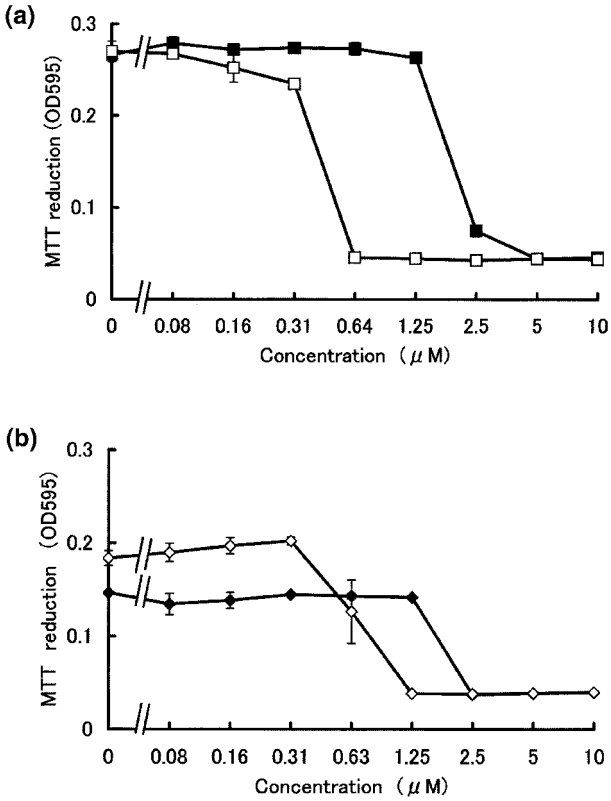


FIG. 3. EL-4 cell cytotoxicity of the cell-membrane-impermeable Zn-chelator DTPA or the cell-membrane-permeable Zn-chelator TPEN. The cells were cultured with (a) DTPA or (b) TPEN in standard medium (closed symbols) or in divalent-cation-depleted medium (open symbols). MTT assays were preformed after culturing for (a) 48 h or (b) 24 h. Bars represent standard deviations of triplicate measurements. When bars are not shown, they are smaller than the size of the symbols.

Lack of required calprotectin binding to the target cells in cell death induction

Sohnle and co-workers suggested that the candidastatic activity of calprotectin is caused by exclusion of zinc from medium.[26] Moreover, there is accumulating evidence that the exclusion of intracellular zinc by cell-membrane-permeable zinc chelators such as TPEN induces apoptosis of target cells.[27–30] We were therefore motivated to investigate whether the exclusion of extracellular zinc by calprotectin causes target cell apoptosis. Thus, although no reduction in the number of EL-4 cells grown is found in divalent cation-depleted medium (Fig. 1), the presence of even a trace amount of zinc supports cell growth.

If extracellular zinc chelation is the mechanism of apoptosis induction, it is possible that direct interaction of calprotectin with cells is not required. To test this hypothesis, we prepared a double-chamber culture in which inner and outer chambers were separated by a dialysis membrane (3500 cut-off), thereby precluding direct interactions between calprotectin and the EL-4 cells. To pre-equilibrate zinc concentrations before the addition of the cells, zinc-deprived medium with or without calprotectin was added in both chambers and allowed to stand at 4°C for 3 days to equilibrate zinc concentrations. As shown in Figure 2, pretreatment of inner and outer chambers with calprotectin resulted in the inhibition of MTT reduction of EL-4 cells in the both chambers. On the contrary, when only the outer chambers were pretreated, the inhibition was again observed not only in the outer chambers, but also in the inner chambers, showing that direct contact between calprotectin and the target cells was not necessary for induction of cell death. It was confirmed that calprotectin was not permeable to the opposite chambers: when only the outer chambers were pretreated with calprotectin as already described, the subunit proteins in the supernatants from the inner chambers were not detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with silver staining, while the proteins in 0.64 μM calprotectin solution, which was the minimal concentration for apoptosis induction in divalent-cation-depleted medium, were clearly detectable (data not shown).

Apoptosis-inducing effects of zinc chelators

To obtain further evidence that exclusion of extracellular zinc induces cell death, we next studied the toxicity of zinc chelators. As shown in Figure 3a, DTPA (which is a cell-membrane-impermeable chelator) causes almost complete loss of MTT

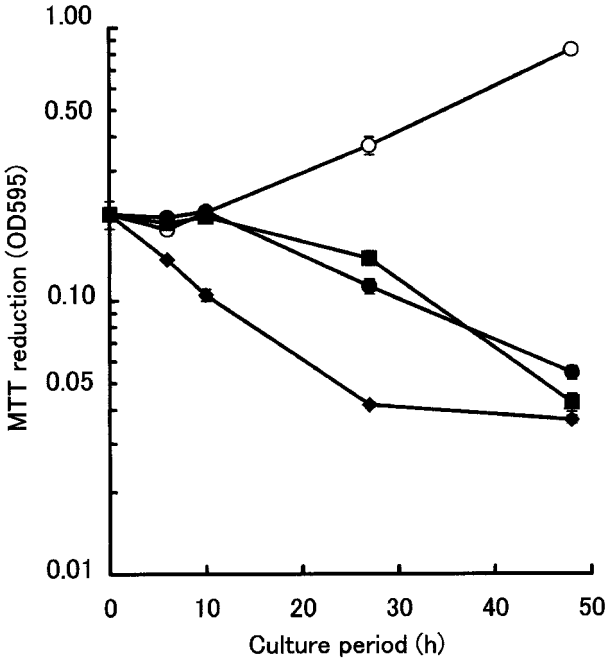


FIG. 4. Time-courses of the cell-death-inducing activity of rhMRP8/rhMRP14 complex, DTPA or TPEN. EL-4 cells were cultured (○) without, or (●) with rhMRP8/rhMRP14 complex (5 μM), (■) with DTPA (10 μM) or (♦) with TPEN (5 μM) in standard medium. MTT assays were performed at the time-points indicated as described in Materials and methods. Bars represent standard deviations of triplicate measurements.

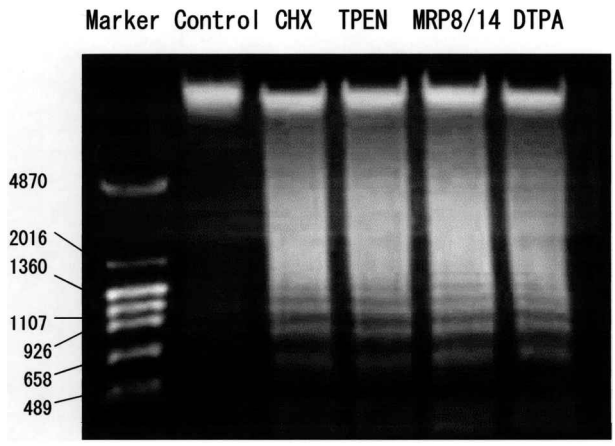


FIG. 5. Agarose gel electrophoresis of DNA extracted from EL-4 cells. Cells were cultured in standard medium without or with rhMRP8/rhMRP14 complex (5 μM) on DTPA (20 μM) for 24 h, or with TPEN (10 μM) or cycloheximide (CHX) (200 μg/ml) for 6 h. DNA was extracted and electrophoresed as described in Materials and methods.

reduction in EL-4 cells at a concentration of 2.5 μM in normal medium and at 0.63 μM in divalent-cation-depleted medium. Like calprotectin, the cytotoxic activity of DTPA was more potent in divalent-cation-depleted medium than in normal medium. The membrane-permeable chelator, TPEN, which was earlier reported to have apoptosis-inducing activity,[27–30] also completely suppressed MTT reduction at 2.5 μM in normal medium and at 1.25 μM in divalent-cation-depleted medium (Fig. 3b). Calprotectin and DTPA also induced a decrease in MTT-reducing activity of human cell lines, namely MOLT-4 leukemia cells and MCF-7 mammary carcinoma cells, in quite similar concentration ranges as the case of EL-4 cells, respectively (data not shown).

Figure 4 shows a kinetic study of changes in MTT reduction of EL-4 cells cultured with calprotectin or zinc chelators. Calprotectin induced a decrease of about half in the MTT reduction after 27 h and a complete loss of activity at 48 h of incubation. A membrane-impermeable DTPA suppressed MTT reduction with similar kinetics as calprotectin, while membrane-permeable TPEN caused the suppression with more rapid kinetics.

To ascertain whether DTPA induces apoptosis of EL-4 cells, we analyzed the DNA profile of EL-4 cells cultured with DTPA since DNA fragmentation is a hallmark of apoptosis. As shown in Figure 5, in addition to TPEN and cycloheximide used as positive controls, recombinant human calprotectin and DTPA both induced ladder-type DNA fragmentation, and consequently apoptosis.

Evidence of whether zinc chelation is essential in the cytotoxic mechanism of calprotectin and DTPA was sought by examining the effect of the addition of zinc sulfate on the cytotoxic activities. As shown in Figure 6, the cytotoxicity of DTPA and calprotectin as well as TPEN was completely abrogated by the

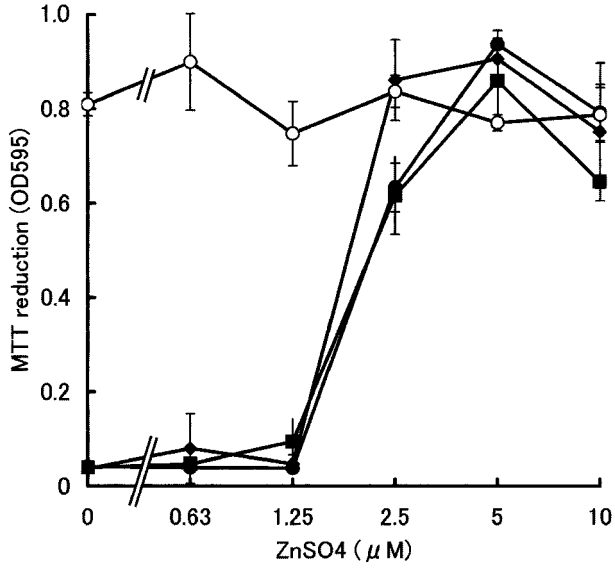


FIG. 6. Inhibitory effect of $ZnSO_4$ on apoptosis-inducing activity of rhMRP8/rhMRP14 complex, DTPA, or TPEN. EL-4 cells were cultured in divalent-cation-depleted medium (○) without, or (●) with rhMRP8/rhMRP14 complex (2.5 μM), with (■) DTPA (2.5 μM) or (♦) with TPEN (2.5 μM) for 48 h in the absence or presence of the indicated concentrations of $ZnSO_4$.

presence of zinc sulfate, suggesting that zinc chelation is truly implicated in apoptosis induction by DTPA and calprotectin.

Discussion

In the present report we have examined the apoptosis-inducing activities of the calprotectin heterodimer and its constituent subunits using recombinant human proteins. An equimolar mixture of rhMRP8 and rhMRP14 was found to have higher specific activity than either subunit alone. The effective concentrations of rhMRP8/rhMRP14 activities are similar to those previously reported for rat calprotectin.[5] Therefore, it is concluded that apoptosis-inducing activity is not unique to rat calprotectin.

There are several observations supporting a specific mechanism for the induction of apoptosis by calprotectin that involves the exclusion of extracellular zinc. First, the cell-death-inducing activity of rhMRP8/rhMRP14 (Fig. 6), as well as rat calprotectin,[4–6] was completely inhibited by the co-presence of zinc. Second, corresponding dose–response curves were shifted to lower concentration ranges in divalent-cation-depleted medium (Fig. 1).[6] Third, direct physical contact between calprotectin and tumor cells was not required to exert cytotoxicity. Finally, the membrane-impermeable zinc chelator DTPA decreased the MTT-reducing activity of EL-4 cells in a time-course similar to that of calprotectin, whereas membrane-permeable zinc chelator TPEN exerted cytotoxicity more rapidly. It was previously reported that rhMRP8 and rhMRP14 form a non-covalently associated heterodimeric complex with high preference over homodimers.[24,31] The mixture of MRP8 and MRP14 has higher specific activity than either protein individually, and these proteins appear to acquire higher zinc binding activity in the heterodimeric state. This hypothesis is supported by the X-ray crystal structure of Zn^{2+}-bound S100A7[32] and by molecular modeling of the Zn^{2+}-binding sites in S100A6 and S100A2.[33,34]

The results presented herein suggest that direct interaction is not required for the cytotoxic activity exerted by calprotectin on target cells. However, human leukemia cell lines[22,35] and endothelial cells[23] reportedly have binding sites for MRP8 and MRP14, suggesting that some cell types have receptor(s) for calprotectin. It is conceivable that membrane-bound calprotectin is more effective in inducing apoptosis than the freely soluble protein. One plausible explanation for this would be the effective localization of calprotectin to cells requiring its Zn-chelating activity.

Antifungal activity was reported to be an extracellular function of calprotectin.[18–21] Sohnle and coworkers reported that calprotectin exerted a candidastatic effect by eliminating zinc in a medium that is essential for *Candida* growth.[26] Recent studies of the antimicrobial activities of calprotectin using rhMRP8 and rhMRP14 examined Cys→Ser mutations, which avoid unwanted formation of disulfides.[21] These workers found that neither MRP8 nor MRP14 showed antimicrobial activity by itself, whereas equimolar concentrations of the two proteins potently inhibited growth. Our studies of the apoptosis-inducing activities of recombinant wild-type proteins revealed that MRP14 and MRP8 alone were able to cause target cell death, although their specific activities were lower than that of the mixture. This discrepancy prompted us to examine the suppression of the growth of *Candida albicans* by the wild-type proteins. Interestingly, we found that the isolated rhMRP14 and rhMRP8 (as well as the mixture) exhibited dose–response curves quite similar to those of their cytotoxicity against EL-4 cells (unpublished observation), including the enhancement of activity in divalent-cation-depleted media. The apparent difference between wild-type and mutated proteins is surprising. The most probable explanation is that the single cysteine residues in MRP14 and MRP8 play key roles in the binding of Zn^{2+} ions. Substitution of these residues by serine is anticipated to significantly reduce Zn^{2+} affinity, although a corresponding effect on the MRP14/MRP8 heterodimer would be anticipated. Clearly, further studies are required to establish the cause of these apparent discrepancies.

Recently, evidence has been accumulating that implicates zinc deficiency in induction of apoptosis. Zinc ions are observed to inhibit apoptotic nucleases[36] and caspase activation,[37] and chelation of intracellular zinc from cells induces apoptosis.[27–30] Martin *et al.* reported that human cell lines, Raji and HL-60, undergo apoptosis in zinc-deficient media.[38] However, the effect of extracellular zinc exclusion on induction of apoptosis has not been fully elucidated. To our knowledge, calprotectin is the first example that an endogenous factor present in body fluids induces apoptosis by zinc exclusion from target cells. Future studies will examine how extracellular zinc chelation by calprotectin or DTPA affects intracellular zinc concentration.

An intense increase in calprotectin concentration has been observed in body fluids at local sites of inflammation,[17] with an extremely high amount of calprotectin (1–20 mg/ml, i.e. 45–900 μM as a heterodimeric complex) in abscess fluids.[16] On the contrary, zinc concentrations in serum of healthy human subjects is about 15 μM on average,[39] and more than one-half of serum zinc binds with albumin or amino acids and is thought to be exchangeable with other ligands.[40] In areas where blood flow is limited in the inflammatory sites, zinc deficiency may be induced locally by calprotectin and may lead to apoptosis. This high abundance of calprotectin might be injurious to the cells in inflamed tissues if there is an insufficient

amount of zinc present. Calprotectin concentrations in local inflammatory sites such as abscesses, in which neutrophils extensively accumulate, may surpass the inhibitory effect of zinc or other inhibitory metals.[6] This is supported by the observation reported by Sohnle *et al.* that the supernatant fluids of abscess showed antifungal activity and that the activity was completely reversed by an addition of zinc.[41] The findings in the present paper may help to make a remedy for inflammation. For instance, the possibility can be raised that zinc supplement may suppress tissue destruction in local inflammatory sites, although *in vivo* activity of calprotectin awaits further research. Also, the mechanism underlying apoptosis induction by calprotectin may be related to the pathology of zinc deficiency.

The apoptosis-inducing activity of calprotectin by zinc chelation might also function in intracellular environments. Calprotectin is expressed abundantly in the cytosol of neutrophils on differentiation of the cell lineage, and the concentration of cytosolic calprotectin in neutrophils has been estimated at 3 mM.[12] Future research must examine whether calprotectin is an endogenous factor of neutrophils that contributes to, or perhaps even determines, the brief life span of this cell type.

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