Review

Calprotectin (S100A8/S100A9), an Inflammatory Protein Complex from Neutrophils with a Broad Apoptosis-Inducing Activity

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Calprotectin, a complex of two calcium-binding proteins that belong to the S100 protein family, is abundant in the cytosolic fraction of neutrophils. A high level of calprotectin reportedly exists in extracellular fluid during various inflammatory conditions, such as rheumatoid arthritis, cystic fibrosis and abscesses. However, the exact biological role(s) of the factor is now under investigation. We recently observed that neutrophils contain a factor that shows growth-inhibitory and apoptosis-inducing activities against various cell types including tumor cells and normal fibroblasts, and we identified that factor as calprotectin. The findings suggest that calprotectin exerts a regulatory activity in inflammatory processes through its effect on the survival or growth states of cells participating in the inflammatory reaction. It is also possible that calprotectin, at a high concentration, might have a deleterious effect on fibroblasts and influence the recovery of inflammatory tissue. Therefore, the protein factor may be a new drug target to control inflammatory reactions. We found that a few of the Amaryllidaceae alkaloids effectively inhibited the growth-inhibitory and apoptosis-inducing activities of calprotectin. In this article, we focus on the biological functions of calprotectin in extracellular fluids, focusing on its apoptosis-inducing activity.

Key words calprotectin; apoptosis; inflammation; zinc; S100A8/S100A9

1. INTRODUCTION

Neutrophils are known to be inflammatory cells which immigrate and accumulate in the early phase of an inflamed site. Since they secrete many protein factors which include enzymes, antibacterial proteins, and cytokines, it is the recent consensus that they play a role in controlling inflammation, in addition to killing microorganisms.[1,2] Examining the regulatory activity within neutrophils, we found that they contain a factor which has a novel cytostatic and cell death-inducing activity against various tumor cells and normal cell types, including macrophages and mitogen-activated lymphocytes.[3] We identified that factor as an abundant protein in the neutrophil cytosol, calprotectin,[4] and found that calprotectin exerts a cytotoxic effect on various tumor cell lines or normal fibroblasts by inducing apoptosis of these cells.[5,6]

Calprotectin is a calcium- and zinc-binding protein complex composed of 8 and 14 kD subunits. These 8 and 14 kD peptides belong to the S100 protein family which is a subfamily of proteins with Ca^{++}-binding motif, EF-hand in each molecule.[7] The subunits have also been termed L1 light and heavy chains,[8] p8 and p14,[9] migration inhibitory factor-related protein (MRP)8 and MRP14,[10] and calgranulin A and B.[11] The gene cluster of the S100 proteins is located on human chromosome 1q21, and the nomenclature of proteins was established according to the organization of these S-100 genes.[7] Therefore, the terms S100A8 and S100A9 are now common,[12] although the names MRP8 and 14, or calgranulin A and B, are still often used.

Neutrophils are the main producers of calprotectin. Therefore, it has been reported that the concentration of calprotectin increases in extracellular fluid in various inflammatory conditions (see below), suggesting that the factor has important functions influencing the inflammatory processes. Evidence that unravels the novel functions of calprotectin is now accumulating: its extracellular functions, including antimicrobial activity,[13,14] and its regulatory activities towards cells which participate in inflammation or immunological reactions,[15—19] were suggested [also see reviews 20—22]. The exact physiological role(s) of this protein complex, however, awaits further investigation.

Our findings concerning the growth-inhibiting and cell death-inducing activity of calprotectin suggest that the factor plays a regulatory role in inflammatory processes through its effect on the survival and/or growth state of fibroblasts and other cells involved in inflammation. It is also suggested that if a high concentration of calprotectin is present in the body fluid at local inflammatory sites, this might cause a delay in tissue repair and a deleterious effect on the inflamed tissue. Therefore, control of the apoptosis-inducing activity of calprotectin might be a new drug target.

As the findings regarding the biological functions of calprotectin have recently accumulated, it is natural to consider that in addition to cytokines and enzymes, calprotectin is a candidate for an novel inflammatory mediator released by neutrophils. In this article, we surveyed the functional aspects of calprotectin, focusing on our findings concerning the apoptosis-inducing activity. We then described the implication of the zinc-binding capacity of calprotectin in the apoptosis-inducing mechanism.

2. CALPROTECTIN-PRODUCING CELLS

Calprotectin is primarily expressed in neutrophils and macrophages, while it is not usually present in lymphocytes. It has been estimated to account for more than 40% and 5% of cytosolic and total proteins of neutrophils, respectively.[20—22] Calprotectin is also expressed in the cytosol of the cells of macrophage lineage, although the amount is less than in neutrophils. Blood monocytes and tissue macrophages in acute inflammation are positive in calprotectin expression, whereas resident macrophages and macrophages present in chronic inflammation are negative.[23—25] The expression of this protein complex may thus be related to the activity of

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macrophages in inflammation, although the exact relationship between calprotectin expression and cell activity remains unclear until the intracellular role of the factor is elucidated. The intracellular distribution of calprotectin, on the other hand, varies with the activation state of macrophages: normal macrophages contain the protein complex in the cytosolic fraction, but, once stimulated, it translocates to the cell membrane and localizes with proteins of the cytoskeleton,[26] which implies that calprotectin may be related to cell movement, phagocytosis or signal transduction.

Although calprotectin has been used as a specific marker for neutrophils and macrophages, it has been detected in some cell types in addition to the cells in myeloid cell lineages. As an example, it was detected in keratinocytes in inflammatory dermatoses,[27] a subset of epithelial cells, and squamous cell carcinoma.[11] Also, the 14 kD subunit of calprotectin is expressed in a subset of microglia in brain tissue with Alzheimer's disease.[28] Since the expression of calprotectin in these cell types seemed to be up-regulated by the inflamed state of the tissue, the functional relevance of the factor to each inflammatory process was suggested.[11,27,28]

3. PATHOLOGICAL STATES IN WHICH CALPROTECTIN IS UPREGULATED

The fact that calprotectin is abundant in inflammatory phagocytes makes it natural that the protein complex has often been found in many inflammatory sites, not only in intracellular compartments, but also in extracellular fluid.[11,29—41] Examples of the inflammatory states in which extracellular calprotectin reportedly increases are depicted in Table 1. The calprotectin-upregulated pathological states were also listed in more detail in a review by Johne *et al.*[21]

Berntzen *et al.* reported that in healthy human serum/plasma, the concentration of calprotectin was less than 1 μg/ml, while the plasma concentration of patients with rheumatoid arthritis (RA) was reportedly increased to approximately 10-fold higher concentrations as mean values.[30] In contrast, no substantial increase was observed in osteoarthritis.[30] An increase in blood calprotectin concentration was also reported in patients with Crohn's disease,[34] colorectal carcinoma,[35] cystic fibrosis,[36,37] multiple sclerosis,[38] human immunodeficiency virus (HIV) infection,[39] and patients who had undergone major surgery.[42]

A more intense increase in calprotectin concentration has been observed in body fluid from local inflammatory sites than in blood. For instance, the concentration in the synovial fluid of RA patients was reported to often increase by more than 100 μg/ml[30]; the cerebrospinal fluid with an opportunistic infection in HIV-infected patients reportedly contained 30—350 μg/ml of calprotectin.[44] The concentration of the factor thus often increases by more than 100-fold above the normal state. However, it is easy to imagine that there may be a higher concentration of calprotectin in the interstitial fluid of a limited inflammatory area in which neutrophils have extensively accumulated. This might be supported by the findings that $>$1 mg/ml of calprotectin is present in the abscess fluids of patients,[41] and which are possibly composed of very high amounts of fresh or effete neutrophils. In any event, calprotectin has been viewed as a good clinical marker for diagnosis, since there is a tendency for an increase in extracellular calprotectin correlate with the inflammatory state of a disease.[44—46]

Table 1. Examples of Pathological States in which Calprotectin is Extracellularly Upregulated

Pathological state	References
Rheumatoid arthritis	29—32
Sjögrens syndrome	33
Crohn's disease	34
Ulcerative colitis	34
Colorectal cancer	35
Cystic fibrosis	36, 37
Multiple sclerosis	38
HIV-infection	39
Gingivitis	40
Abscess	41

The next important problem is what type of cells contribute to extracellular calprotectin as the source of the factor. To answer this, we studied the course of intracellular and extracellular calprotectin expression of inflammatory cells using a rat peritonitis model. After an i.p. injection of heat-killed bacteria, *Enterococcus faecalis*, into Wistar rats, neutrophils immigrated into the peritoneal cavities after 6 h, and their number peaked around day 2. Thereafter, macrophage accumulation was observed (Fig. 1A). Although calprotectin was reportedly expressed in both types of exudate cells,[20] the course of change in the amount of the factor in the ascitic fluid was not linked with the course of the appearance of macrophages, but was closely linked with that of neutrophils (Fig. 1B, C). Neutrophils, rather than macrophages, were the most important source of extracellular calprotectin in this experimental model. It is possible that the factor is released from necrotic neutrophils, although an alternative possibility was that an active secretory mechanism may have been involved in the excretion.[48,49] The fact that calprotectin increases in many inflammatory conditions may signify its regulatory role in inflammation.

4. EXTRACELLULAR FUNCTION OF CALPROTECTIN

Reports dealing with the biological functions of extracellular calprotectin have recently become more numerous. The antifungal action of this protein complex was described earlier[13,14]: calprotectin suppresses the growth of yeast and fungi. The minimum effective concentration against the growth of *Candida albicans* is between 10 and 20 μg/ml. It was reported that the factor is also effective against bacteria, but the antibacterial action required a higher concentration than against fungal strains.[13]

The antifungal activity of calprotectin is inhibited by the presence of zinc ion and the factor has a zinc-binding property[50,51]; in fact, there are zinc-binding motifs in the subunit peptides.[52] This suggests that the factor withholds zinc from the growth media of fungi, because fungi strongly require zinc for their growth.[41] Several pieces of evidence now point to the deprivation of nutrient zinc as the mechanism of the antifungal action, and it seems that the direct contact of calprotectin with fungal cells is not required for the inhibition of growth.[41,53—55]

Calprotectin may have been a main antifungal factor in an

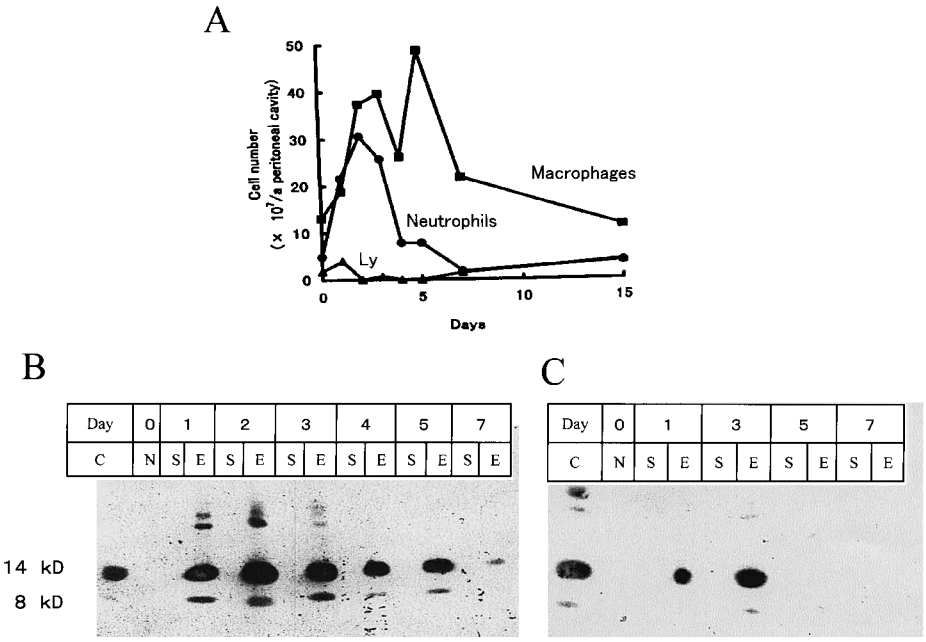

Fig. 1. Kinetic Analysis of Calprotectin Expression in Inflammatory Cells and Ascitic Fluids in Mouse Peritonitis Model

A. Changes in the population of peritoneal exudate cells (PECs) induced by heat-killed *Enterococcus faecalis*. Heat-killed *E. faecalis* (20 mg) was injected into the peritoneal cavity of Wistar rats and cell numbers were counted. (●; neutrophils, ■; macrophages, ▲; lymphocytes). B. Western blotting analysis of calprotectin in *E. faecalis*-induced peritoneal exudate cells. The peritoneal cells collected on the indicated days were lysed, and the lysates were separated in SDS-PAGE (4×10^4 cells/lane) and blotted on PVDF membranes. The protein signals were visualized by rabbit anti-calprotectin antiserum and horse raddish peroxidase-conjugated secondary antibody. Western blotting analysis of calprotectin in peritoneal fluid. Peritoneal fluid was collected after the injection of 1.5 ml saline into the peritoneal cavity. The supernatants of the obtained ascites were collected by centrifugation and 10 μl of each was analyzed as described above. B and C. C, standard rat calprotectin, N, peritoneal cells from untreated rats, S, peritoneal cells from saline-injected rats, E, peritoneal cells from *E. faecalis*-injected rats. The bands of the 8 kD subunit were very faint, possibly due to a low reactivity of the antiserum to the 8 kD subunit.

abscess *in vivo*, because the supernatant of the abscess fluid showed the antifungal activity and was also inhibited by the addition of zinc.[54] We also showed that calprotectin cooperates with neutrophils and/or their product, lactoferrin, to inhibit the growth of *C. albicans*.[56] Thus it can be concluded that calprotectin, in concert with other factors, contributes to the host defense mechanism against fungal infection.

Many biological activities of calprotectin, other than its toxic activities against fungi and mammalian cells, have recently been reported (Table 2). Chemotaxis of inflammatory cells is an important element in the pathology of inflammation. Geczy and her colleagues reported that murine MRP-8 (a light subunit of calprotectin), which they termed CP-10, had potent chemotactic activity for neutrophils and macrophages.[16,57] They also revealed that CP-10 enhances scavenger receptor and Mac-1 expression on murine macrophages.[58] It was also reported that the inoculation of agarose beads containing the 8 kD subunit, the 14 kD subunit, or their complex, into skin caused infiltration of neutrophils and macrophages *in vivo*, respectively. In this experimental system, the activity of the complex form was much stronger than that of the individual component.[17]

Calprotectin reportedly affects the function of inflammatory cells, besides those described above. The 14 kD subunit decreased the phorbol 12-myristate 13-acetate (PMA)-triggered H_2O_2 production of BCG-stimulated macrophages.[18] It was also reported that the 14 kD subunit enhanced the integrin-mediated adhesion of neutrophils without the activation of these cells, while the 8 kD subunit is known to inhibit the enhancement.[19] The 14 kD subunit was reportedly suggested to have an antinociceptic effect on inflammatory pain.[59] Calprotectin has an inhibitory activity against the immunoglobulin synthesis of lymphocytes,[15] suggesting that it might affect not only inflammatory processes, but immunological reaction. In any event, evidence has recently accumulated, that in addition to its cytotoxic activities, calprotectin has cytokine-like activity. It is, therefore, possible to suppose that the protein complex or the individual subunits are not only a useful marker of inflammatory states, but also an important mediator with multiple regulatory functions in inflammatory reactions.

Table 2. Proposed Activity of Calprotectin Regulating Inflammatory Cells

Activity	Active subunit	References
Chemotactic activity		
Chemotactic for macrophages and neutrophils	8 kD (CP-10)	16, 57
Cellular accumulation of macrophages and neutrophils	8/14 kD complex	17
Activity towards macrophages		
Macrophage deactivation	14 kD	18
Enhancement of scavenger receptor and Mac-1 expression	8 kD (CP-10)	58
Activity towards neutrophils		
Activation of neutrophil integrin	14 kD	19
Antinociceptive effect: control of inflammatory pain (possible contribution of neutrophils)	14 kD	59
Activity towards lymphocytes		
Inhibition of immunoglobulin synthesis	8/14 kD complex	15

5. GROWTH-INHIBITORY AND APOPTOSIS-INDUCING ACTIVITY OF CALPROTECTIN AGAINST MAMMALIAN CELLS

During the course of our search for a regulatory factor in neutrophils, we found that mouse or rat inflammatory neutrophils included an inhibitory activity of mitogen-induced thymocyte proliferation. The lysates of the neutrophils also induced lymphocyte death.[3] To elucidate the molecular nature of this factor, we performed purification studies using rat neutrophil-rich peritoneal cells. The results indicated that cytotoxic factor is a heterodimeric protein complex, of which subunits were assigned as 8 kD and 14 kD peptides of calprotectin, respectively.[4] The minimum effective concentration was about 50 μg/ml. Calprotectin also inhibits the growth factor-induced proliferation of macrophages and bone marrow cells. Interestingly, calprotectin by itself significantly enhanced macrophage DNA synthesis in its lower concentration range.[4]

Calprotectin also inhibited the growth of many tumor cells with broad specificity (Fig. 2): they included MM46 mouse mammary carcinoma, MH-134 mouse hepatoma, EL-4 mouse thymoma, L-929 mouse fibrosarcoma, B16 mouse melanoma, J774.1 mouse macrophage-like cells, Ros17/2.8, rat osteosarcoma, MCF-7 human mammary adenocarcinoma, and MOLT-4 human leukemia cells [ref. 4 and unpublished observation]. We have not yet encountered any cell line that is insensitive to calprotectin. It was again noticed that the marginal concentrations of calprotectin preparation enhanced ^{3}H-TdR incorporation of some tumor cell lines for unknown reason. In addition to the cytostatic effect, calprotectin induced the cell death of many of the tumor cells listed above.[4] The factor, therefore, seems to be one of the effector molecules of neutrophils against tumors. The growth-inhibitory or cytotoxic activities of calprotectin towards microorganisms and mammalian cells is depicted in Table 3.

To determine which subunit of calprotectin is responsible for the cytotoxic activity, we compared the activity of human recombinant 8 kD and 14 kD subunits of calprotectin and a complex of the two against EL-4 cells.[60] As a result, the cytotoxicity of the 14 kD subunit was detected from 10 μM, whereas that of the 8 kD subunit was marginal at the same concentration. On the other hand, the mixture of both induced almost complete cell death at 5 μM: calprotectin has higher specific activity than either subunit individually.

Apoptosis is a physiologically programmed cell death which is essential for the maintenance of normal tissue as well as pathological processes including cancer or inflammation.[61,62] As the cytocidal reaction of calprotectin requires at least a 20 h incubation, the cell death was thought to be caused by a programmed mechanism. Using the tumor cell lines EL-4, MOLT-4, and MM46, we found that calprotectin induced apoptosis.[5,63] As described earlier, the calprotectin concentration in body fluid increases in many inflammatory conditions. To learn the role of the factor in inflammation, we examined its growth-inhibitory and apoptosis-inducing activities against normal fibroblasts, because fibroblasts are a cell type constituting inflammatory tissue. They regulate the repair of a wound site through cell growth and the production of the materials covering the intercellular matrix. We found that calprotectin exerted an inhibitory effect on the growth of

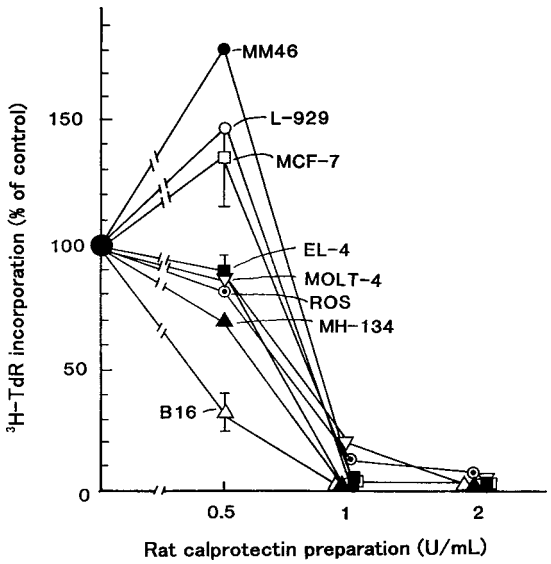

Fig. 2. Effect of Calprotectin on the Growth of Various Tumor Cells

Each cell line was cultured with rat partially purified calprotectin for 18 h, except the MCF-7 cells, which were cultured for 45 h. DNA synthesis of each cell line was measured by pulsing ^{3}H-TdR for an additional 4 h. To signify the concentration in the partially purified samples, it was expressed as U/ml (U/ml initially defined as number of minimum dilution folds having a complete growth inhibition for the MM46 cells under a serious two-fold dilution. One U/ml contained *ca.* 50 μg/ml calprotectin).

Table 3. Growth-Inhibitory and Cytotoxic Functions of Calprotectin

Function	References
Antimicrobial activity	
Antibacterial activity	13
Antifungal activity	13, 14
Growth-inhibitory activity against mammalian cells	
Macrophages (growth factor-stimulated)	4
Bone marrow cells (M-CSF-stimulated)	4
Lymphocytes (mitogen-stimulated)	3
Fibroblasts	6
Tumor cell lines	3—5
Cytocidal or apoptosis-inducing activity	
Lymphocytes (mitogen-stimulated)	3
Tumor cell lines	4,5
Fibroblasts	6

mouse embryonic fibroblasts and human dermal fibroblasts.[6] Moreover, the factor induced the apoptosis of both cell types, although the reaction was much slower and to a lesser extent than that of the EL-4 and MOLT-4 tumor cells. Incubation of mouse embryonic and human dermal fibroblasts with calprotectin for 3 d caused only 23 and 13% of apoptotic cells, respectively, whereas a great majority of the above tumor cells underwent apoptosis under similar conditions. Figure 3 shows the morphological appearance of the embryonic fibroblasts cultured with calprotectin for 3 d, in which the typical apoptotic features, namely nuclear condensation and segregation, were observed.

6. INHIBITION OF CALPROTECTIN CYTOTOXICITY BY ZINC

Like its antifungal action, the cell death (apoptosis)-inducing activity of calprotectin is inhibited by the presence of

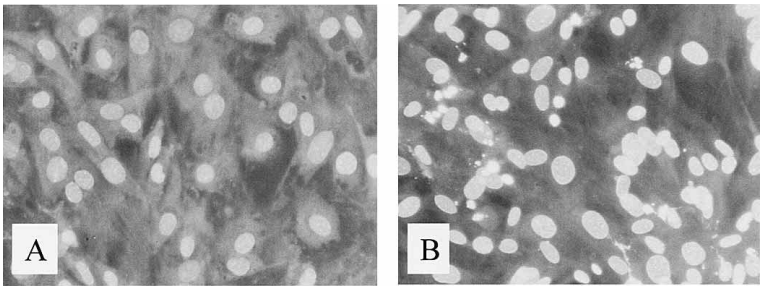

Fig. 3. Morphological Appearance of Mouse Embryonic Fibroblasts Treated with Calprotectin

Mouse embryonic fibroblasts were cultured without (A) or with (B) 2 U/ml of calprotectin for 3 d. Chromatin were visualized with 4,6-diamidino-2-phenylindol, and their morphology was observed by phase contrast microscopy. Original magnification: ×200. About half of the cells had the apoptotic morphology which is characterized by chromatin condensation. Chromatin of some cells were segregated.

zinc: the activity of 50—100 μg/ml of calprotectin was completely inhibited by 10 μM of zinc sulfate.[5,6] In addition to zinc ion (Zn^{2+}), Cu^{2+} also effectively inhibited the apoptosis, whereas Mn^{2+} and Fe^{2+} showed only moderate inhibition. In contrast, Ca^{2+}, Mg^{2+}, Fe^{3+}, and Al^{3+}, showed no inhibition at all.[6] It was reported that zinc inhibits apoptosis which is induced by various signals.[64,65] To analyze whether the zinc action was due to a direct interaction between zinc and the calprotectin molecule, we compared the dose-response relationships of its apoptosis induction in standard medium and that in the divalent metal ion (other than Ca^{2+}, Mg^{2+})-depleted medium. The dose-response curves of both cell growth-inhibitory and apoptosis-inducing activities of calprotectin were found to be shifted to about a 10-fold lower concentration range than that in standard medium[6] (Fig. 4). These results strongly suggest that zinc (or other divalent metal ions) may bind to the calprotectin molecules to attenuate the activity of the molecule. In other words, the point of action of zinc may not be located in the intracellular cascade reactions to accomplish apoptotic cell death.

We observed that not only the viability but also the growth state of EL-4, MM46 cells, and fibroblasts, did not change in the zinc-depleted medium for long culture periods, while the growth of *C. albicans* was markedly attenuated in such a medium [unpublished observation]. However, as in the case of the antifungal action of calprotectin, exclusion of zinc from the target cells might be one mechanism, since it was reported that apoptosis was induced by the depletion of intracellular zinc with the cell membrane-permeable zinc cheletor, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN).[66—68] To examine this possibility, the cytotoxic activity of calprotectin against EL-4 cells was tested under conditions where physical contact between the factor and the cells was precluded by a dialysis membrane.[60] As the result, the factor induced cell death even when contact was hindered. The membrane-impermeable zinc cheletor, Diethylenetriaminepentaacetic acid (DTPA), also induced cell death in a similar way to calprotectin. Taken together, the cell death-inducing mechanism was the exclusion of zinc from EL-4 cells.

However, we recently observed that several cell lines, including MM46 cells, were insensitive to the action of DTPA, but were sensitive to calprotectin, in spite of the fact that the calprotectin effect on MM46 cells was also inhibited by zinc. The activity of the factor was severely attenuated by inhibiting the contact between the protein complex and the MM46

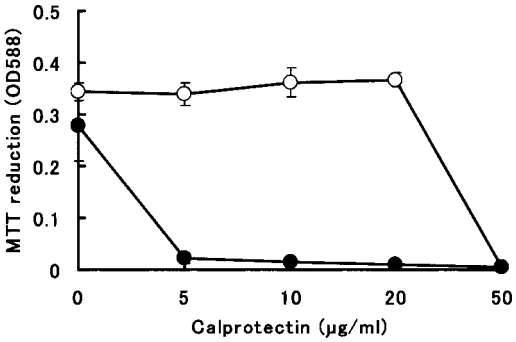

Fig. 4. Dose–Response Relationship of the Cytotoxic Effect of Calprotectin

EL-4 cells were cultured with calprotectin in standard RPMI 1640 medium (○) or divalent metal ion (other than Ca^{++} and Mg^{++})-depleted medium (●) for 40 h. After the culture, percentage of dead cells was estimated by a trypan blue dye exclusion test.

cells by a dialysis membrane, suggesting that the factor induced cell death by binding to the MM46 cells [Y. Nakatani, M.Yamazaki, S. Yui, manuscript in preparation]. These observations imply that calprotectin induced apoptosis through a dual mechanism: one was zinc exclusion from the target cells, and the other was an undefined mechanism originating from the binding of the factor to the cell surface of the target cells, possibly in a ligand-receptor fashion.

We observed that a substantial amount of calprotectin binds not only to MM46 cells but also to EL-4 cells. Several authors also reported that calprotectin binds to mammalian cells.[19,69] It was reported that a receptor for advanced glycation end products (RAGE) on macrophages or other cells is also the receptor for the polypeptides of the S100 family.[70] It was reported that calprotectin interacts with carboxylated glycan[71] or sulfated glycosamininoglycans[72] on endothelial cells. It was also proposed that α_2-macroglobulin is a ligand on endothelial cells for calprotectin.[73] Determination of the mode of interaction between calprotectin and cell surface receptor for tumor cells is an important hurdle in solving the apoptosis-inducing mechanism of the factor, in addition to identifying the mechanism of zinc inhibition.

The intracellular event of apoptotic cell death in the execution step is a cascade reaction which involves caspase activation, transition of mitochondrial permeability, production of reactive oxygen species, and activation of specific endonuclease.[74,75] This execution step to accomplish apoptotic cell death does not require protein synthesis in the target cells; the induction of apoptosis by signaling through the receptor

of tumor necrosis factor or Fas does not require protein synthesis.[74] On the other hand, several apoptosis-inducing reactions, such as glucocorticoid, calcium ionophore, and γ-irradiation-induced lymphocyte apoptosis were reported to be inhibited by the inhibitors of RNA or protein synthesis.[76—78] These stimuli may induce newly synthesized protein factors to introduce apoptotic signals into the second execution step. We found that an apoptosis inducing reaction against MM46 cells by calprotectin was inhibited by an inhibitor of protein synthesis, cycloheximide. We also observed that the addition of cycloheximide in a later phase of the reaction no longer inhibited the accomplishment of apoptosis, suggesting that the target protein synthesis in the early phase of the reaction is important.[63] To analyze the event of the later phase, we examined the effect of antioxidative reagents and found that *N*-acetyl-L-cysteine or propyl gallate, which were added at an even later period, significantly impaired the emergence of cell death.[63] We therefore concluded that protein synthesis and the generation of a reactive oxygen species may be essential elements in the early or later phases of the cell death-inducing reaction of calprotectin, respectively.

The above results also imply that the effective concentration of calprotectin to induce apoptosis *in vivo* is determined on a balance with the zinc concentration. The zinc concentration in the serum of healthy human subjects is about 15 μM on average, and more than half of the serum zinc binds with albumin or amino acids and is thought to be exchangeable with other ligands.[79] Since 10 μM of zinc is capable of neutralizing 50—100 μg/ml calprotectin, the systemic blood flow is inhibitory for the factor to exert apoptosis-inducing activity. Zinc may be a negative regulator restricting the systemic effect of the toxic protein. However, it is probable that in a local inflammatory site where a great number of neutrophils have accumulated, the calprotectin concentration is high enough to exert these effects. In this respect, it is to be noted that abscess sites of contain 1—20 mg/ml of calprotectin,[41] which may inhibit growth and induce the apoptosis of fibroblasts or other cells surrounding the abscess *in vivo*.

Calprotectin seems to cause tissue destruction when it is present in an excess amount in local inflammatory tissue for a long period. The protein complex is an injurious factor of neutrophils. If so, is there any beneficial aspect of calprotectin activity, or is it merely a toxic substance that nature capriciously endowed to the body? At least in an abscess condition, it seems probable that growth inhibition or apoptosis of the surrounding fibroblasts by calprotectin has a positive effect in curing of the abscess, because destruction of fibroblasts constituting connective tissue may hasten the excretion of the abscess contents which include dead and viable inflammatory cells (the source of calprotectin) and microorganisms. After these harmful materials are cleared out (which decreases the local calprotectin concentration), the tissue repair can start by the cooperation of fibroblasts and other cells. However, if the process of inflammation is prolonged, calprotectin might have a the deleterious effect on the body. Other than in an abscess, calprotectin may regulate the inflammatory reaction through its growth-inhibitory and apoptosis-inducing activity against a broad type of cells.

7. PHARMACOLOGICAL MANIPULATION OF CALPROTECTIN-INDUCING APOPTOSIS

As the novel functions of this inflammation factor are elucidated, the pharmacological manipulation of the factor may open the way to new means of regulating inflammation. Since calprotectin might cause tissue destruction through its growth inhibitory- or cytotoxic effects under severe inflammatory conditions, we searched for drugs to suppress the cytotoxic effect of the factor. We screened plant products that have been used in Chinese medicines for antiinflammatory activity. Using a tumor cell line as a target, we found that the extract of *Crinum asiaticum* had a strong inhibitory activity against calprotectin induced cell death among hot water extracts of 59 plant species.[80] Through purification studies, the molecule responsible for the inhibition was identified as the Amaryllidaceae alkaloid, lycorine (Fig. 5). Lycorine inhibited not only apoptosis induction by calprotectin, but also its growth-inhibitory effects at a half effective concentration of 0.1—0.5 μg/ml. Although lycorine has inhibitory activity against protein synthesis on ribosomal translation,[81—83] the inhibition occurs at a more than 5-fold higher concentration than that required by the inhibition of calprotectin activity. Since lycorine seemed to act in the early induction phase of the apoptosis reaction of calprotectin, it might be a good tool

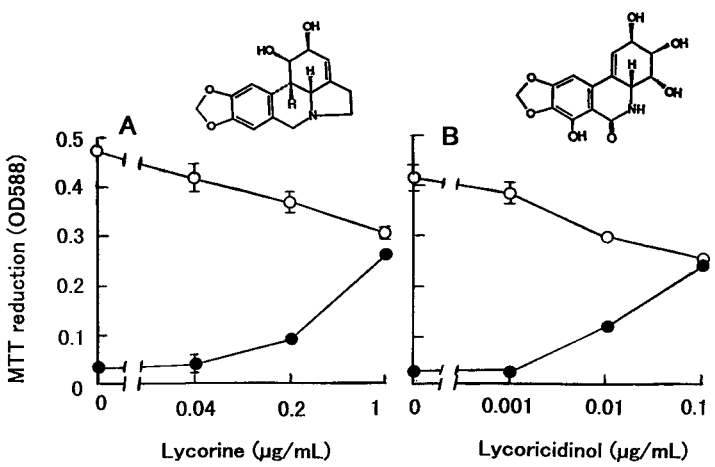

Fig. 5. Inhibitory Activity of Lycorine and Lycoricidinol on Calprotectin-Induced Cell Death

MM46 cells were cultured with or without calprotectin (2 U/ml) in the presence of lycorine (A) or lycoricidinol (B) for 16 h, and MTT-reducing activity of the cells was assessed. Structures of Amaryllidaceae alkaloids, lycorine, and lycoricidinol, are shown at the top of the figure.

for the analysis of a common pathway in the signal transduction of cell death.

The group of Amaryllidaceae alkaloids contains many biologically active compounds, which include cytotoxic and antiviral substances.[84] Among these lycoricidinol (narciclasine) has a very potent inhibitory activity for the protein synthesis of mammalian cells.[81] To identify the compounds that are more effective than lycorine in inhibiting the cytotoxic activity of calprotectin, we studied the inhibitory activity of lycoricidinol. We found that lycoricidinol inhibited calprotectin-induced cytotoxicity at a more than 10-fold lower concentration than lycorine: its concentration causing 50% inhibition was 1—10 ng/ml (Fig. 5).[85]

In the next step, we examined the prophylactic effect of lycoricidinol on the rat adjuvant arthritis model, since calprotectin is reported to increase in the local inflammatory sites of rheumatoid arthritis patients.[30—32] We found that lycoricidinol had a significant prophylactic effect on the arthritis model.[85] These results suggested that lycoricidinol might be a candidate as a drug with a suppressive activity for inflammation which might be influenced by calprotectin. However, it is, of course, necessary to examine whether calprotectin inhibition is the true mechanism of the prophylactic effect.

8. CONCLUSION

As the knowledge regarding calprotectin accumulates, the protein complex is strongly expected to gain a position as a mediator in inflammation. To find the drugs that modify the functions of calprotectin might be an important goal in regulating inflammatory reactions.

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