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Autoimmune diseases caused by defects in clearing dead cells and nuclei expelled from erythroid precursors

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Summary: Apoptotic cells are recognized and subsequently engulfed by macrophages and immature dendritic cells. The engulfed dead cells are transported to the lysosomes of macrophages, and their components are degraded into amino acids and nucleotides for reuse. In mammals, macrophages also engulf nuclei expelled from erythroid precursors in the final stage of definitive erythropoiesis. Failure to swiftly engulf dead cells at the germinal centers of lymphoid organs causes systemic lupus erythematosus-type autoimmune diseases. In contrast, failure to efficiently degrade the DNA of dead cells or erythroid cell nuclei activates innate immunity, causing lethal anemia in the fetus and chronic arthritis in adults.

Keywords: *apoptosis, definitive erythropoiesis, DNA degradation, phosphatidylserine, macrophages, MFG-E8*

Introduction

Every day, a large number of cells (10^8–10^9 cells) in the human body undergoes apoptosis (1, 2). In the last stage of this process, apoptotic bodies, which are fragmented cell bodies carrying cellular components, present 'eat-me' signals and are engulfed by phagocytes such as macrophages, immature dendritic cells, and microglia (3–5). Endosomes containing the engulfed dead cells fuse with lysosomes, where the dead cell components are degraded into amino acids and nucleotides. In adult humans, about 10^{11} red blood cells are produced per day. Red blood cells in mammals do not carry nuclei, and they are produced in a process called definitive erythropoiesis (6). Definitive erythropoiesis takes place in anatomical units called 'erythroblastic islands' in the fetal liver or bone marrow, where erythroblasts proliferate and differentiate in contact with the macrophages, that reside in the center of each island (7, 8). In the last stage of this process, nuclei are expelled from the erythroid precursors and are engulfed by the macrophages.

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Unlike most cellular components that are degraded during apoptosis, the degradation of chromosomal DNA occurs in two steps. First, the chromosomal DNA of apoptotic cells is cell-autonomously cleaved into nucleosomal units (9) by caspase-activated DNase (CAD) (10, 11). Following engulfment by macrophages, the DNA of the dead cells is further degraded into nucleotides by DNase II, an acid DNase located in the lysosomes of macrophages (12). DNase II is also responsible for degrading nuclear DNA from erythroid precursor cells in the macrophages of bone marrow and fetal liver (13). In DNase II-deficient mice, macrophages in various tissues, particularly fetal liver and bone marrow, accumulate undigested DNA in the lysosomes (13, 14), which activate them to produce cytokines such as interferon β (IFNβ) and tumor necrosis factor α (TNFα) (12, 15). IFNβ thus produced from the macrophages in erythroblastic islands kills erythroblasts directly or indirectly, leading to lethal anemia in embryos (15). However, TNFα goes to the joints, activates synovial cells, and triggers chronic polyarthritis in adults (16).

The engulfment of apoptotic cells has been thought to prevent the release of noxious materials from dying cells, which otherwise might cause inflammation (17). However, there has been no clear evidence for this notion. We recently identified a molecule that works as a bridge between apoptotic cells and phagocytes (18). A deficiency in this molecule causes inefficient engulfment of apoptotic cells in the germinal centers of the spleen and induces systemic lupus erythematosus (SLE)-type autoimmune diseases (19, 20).

The identification of molecules involved in the engulfment and degradation of dead cells suggests that failure of this process leads to autoimmune diseases such as rheumatoid arthritis and SLE. In this review, I discuss the final stage of programmed cell death and erythropoiesis (engulfment and degradation of dead cell components as well as the erythroid nuclei) and the diseases caused by its failure.

Apoptosis

During animal development, many useless and toxic cells are generated and removed (2, 21). Cells also die in adults: that is, every cell has its own life-span, and senescent cells die and are replaced by newly generated ones. When cells are infected by bacteria or viruses, they are swiftly removed to prevent the pathogen from spreading. These physiological cell deaths, also known as programmed cell death, consist of two steps: cell death and the clearing of the corpse.

There are at least two death processes, necrosis and apoptosis, and they can be distinguished morphologically (22). In necrosis, the nuclei and mitochondria swell, the cell membranes rupture, and all the cellular contents are released. During the apoptotic process, the cell membranes convolute, and the nuclei condense and become fragmented. In the final stage of this process, the cells themselves become fragmented, and the fragments retain the cellular contents. These condensed and fragmented cells (called apoptotic bodies) are engulfed by phagocytes, and therefore the contents of the dying cells are not released. This clean apoptotic death process, regulated by gene products, is likely to account for most of the cell death that occurs during development (23). In contrast, interdigital cells, chondrocytes in bone, and intestinal epithelial cells may be removed by necrosis during development (24–27), but the molecular mechanism of necrosis is not well understood.

Most animal cells carry the machinery for apoptosis (1, 2, 28–30). During animal development, some factor(s) that triggers the death machinery is transcriptionally upregulated in certain cells and then activated at the appropriate developmental point (intrinsic cell death) (1). Cytotoxic T lymphocytes (CTLs), anti-cancer drugs, γ-irradiation, and factor deprivation also turn on the death machinery. Among these death triggers, CTLs and natural killer cells express Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL), which are type II-membrane proteins of the TNF family, and trigger non-cell-autonomous apoptosis (extrinsic cell death) (31–33). Whatever the trigger, apoptotic cell death is mediated in most cases by caspases (cysteine proteases). Caspases comprise a family of 12 members in human, and they take part in an apoptotic cascade when activated. Caspases 3 and 7 are the downstream effectors of the cascade, and they specifically cleave a set of cellular enzymes and proteins (more than 300 substrates), thereby inactivating or activating them (34). The cleavage of these enzymes and proteins is responsible for the morphological and biochemical changes that occur during apoptosis and for killing the cells. There are many excellent reviews on apoptosis (1, 28, 29, 31, 32, 35–38) that describe the details of the apoptotic pathways.

Apoptotic DNA degradation

In addition to the morphological changes in the cells, apoptosis is accompanied by DNA fragmentation (9, 39, 40). At the early stage of apoptosis, chromosomal DNA is degraded into multimers of 180 bp, a nucleosomal unit. This cleavage is accomplished by CAD, also called DFF-40 (DNA fragmentation factor 40) (10, 41–43). In proliferating cells, CAD is complexed with its inhibitor, ICAD (inhibitor of CAD)

or DFF-45 (10, 11, 43). ICAD also works as a chaperone for CAD during its synthesis (44, 45), and functional CAD cannot be synthesized without ICAD (41, 46). DNA can bind to the CAD–ICAD complex, but it cannot be cleaved because the active site of CAD that is necessary for its enzymatic activity is masked by ICAD (47). Caspases 3 and 7 cleave ICAD at two positions, inactivating ICAD's ability to inhibit CAD's DNase (48). Similar to DNase I and DNase II, CAD is an endonuclease containing histidine residues in its active site (49, 50), and it cleaves double-stranded DNA with some preference for A–T-rich regions (51). CAD carries a nuclear localization signal and cleaves chromosomal DNA when the nucleosomal structure is intact. It exists as a homodimer with a scissors-like structure, and the histidine-containing active site is located in the deep cleft between the 'blades' (52). This structure prevents CAD from accessing the DNA on the nucleosomes but allows CAD to access DNA at the spacer regions between the nucleosomes. This explains why chromosomal DNA is degraded into nucleosomal units during apoptotic cell death.

There are reports claiming that residual DNA degradation (high molecular weights or nucleosomal units) takes place in ICAD-deficient cells (53, 54). However, in our hands, cells expressing caspase-resistant ICAD or cells lacking ICAD or CAD show no DNA degradation in response to any apoptotic stimuli we have tested (12, 41, 55), indicating that CAD is solely responsible for the cell-autonomous apoptotic DNA degradation. Other DNases, such as endonuclease G, DNase I, and DNase γ, have been proposed to play a role in this process (53, 56–58). However, the absence of DNA degradation in CAD-null cells and the lack of effect on apoptotic DNA degradation when the genes for *endonuclease G* or *DNase I* are deleted (59–61) indicate that these nucleases are not involved in apoptotic DNA degradation or that their role in this process is limited (62, 63).

In contrast to the lack of apoptotic DNA fragmentation observed *in vitro* in *CAD*-null cells, apoptotic DNA degradation, assessed by staining with TdT-mediated dUTP-nick end labeling (TUNEL), occurs normally *in vivo* in *CAD*-deficient mice (64). Further *in vitro* studies indicated that the DNA of apoptotic cells is degraded by DNase II, a lysosomal enzyme, after the dying cells are engulfed by macrophages (12, 65). Thus, the chromosomal DNA of apoptotic cells is degraded in two steps (12, 64, 66) (Fig. 1). First, the DNA is cleaved by CAD in the dying cells, leaving the nucleosomes intact. Next, the apoptotic cells are engulfed by macrophages and transferred into their lysosomes. Lysosomes carry not only DNase (DNase II) but also proteases, lipases, and glycosidases, and the cellular components of apoptotic cells are degraded by these enzymes into amino acids, nucleosides, fatty acids, and glycosides.

Degradation of nuclear DNA during erythropoiesis

Early in normal mouse embryogenesis, the red blood cells are produced in the yolk sac, and these cells are nucleated (67). This process is called primitive erythropoiesis. From embryonic day 12.5 (E12.5) onward, erythropoiesis takes place in the fetal liver instead of in the yolk sac. Like the erythroid cells produced in the bone marrow of the adult, those produced in the fetal liver are enucleated; this process is known as 'definitive erythropoiesis' (68). Definitive erythropoiesis takes place in anatomical units called 'erythroblastic islands' in the bone marrow and fetal liver. Each island contains a macrophage at the center, surrounded by erythroid precursor cells at different developmental stages (erythroblast, reticulocytes, and mature enucleated erythrocytes). It seems that the macrophage in the blood island supports the proliferation and differentiation of erythroid precursor cells (69, 70). Erythroid cells cell-autonomously undergo enucleation, and the expelled nuclei are engulfed by the central macrophage (Fig. 1). Like the DNA of engulfed apoptotic cells, the engulfed erythocytic nuclei are degraded in the lysosomes of the macrophages by DNase II (13).

Lethal anemia in *DNase II*-null embryos

DNase II is an endonuclease that works efficiently under acidic conditions (71). There are two subtypes of DNase II, DNase IIα (DNase II) and DNase IIβ [DNase II-like acid DNase (DLAD)] (72, 73). The expression of DLAD is limited to the lens-fiber cells of the eye (74), in particular, the fiber cells close to the organelle-free zone of the lens (75). DLAD is responsible for cell-autonomously degrading nuclear DNA during the differentiation of the fiber cells (Fig. 1). Thus, $DLAD^{-/-}$ mice accumulate undigested DNA in the cytoplasm of lens-fiber cells and suffer from cataract (Fig. 2).

DNase II or DNase IIα is ubiquitously expressed in various tissues, particularly in macrophages. Cell lysates prepared from the fetal livers of $DNase\ II^{-/-}$ mice do not show DNase activity under acidic conditions, indicating that lysosomes carry a single DNase, DNase IIα. The null mutation of the *DNase II* gene is embryonic lethal; $DNase\ II^{-/-}$ mice die late in embryogenesis from severe anemia (13). In these embryos, the number of primitive erythrocytes at E12.5 is normal, but the number of definitive erythrocytes at E17.5 is severely reduced (< 10% of the number in wildtype mice). This finding suggests that definitive erythropoiesis is specifically

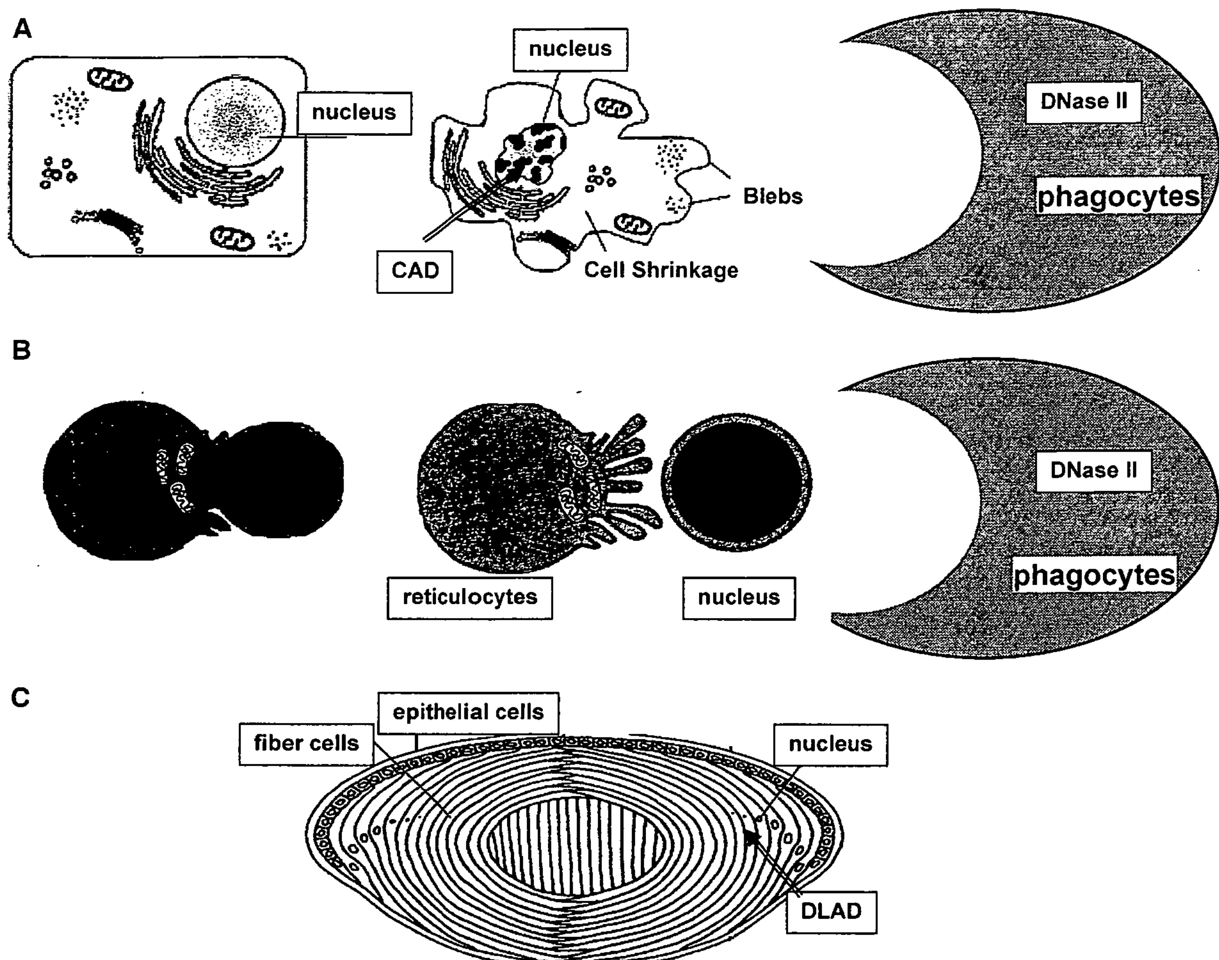

Fig. 1. DNA degradation during three developmental stages. (A) Two-step DNA degradation in programmed cell death. Cells die through apoptosis, in which the cells and nucleus are fragmented. In this process, chromosomal DNA of apoptotic cells is cleaved first by caspase-activated DNase (CAD) into nucleosomal units. Dead cells are then engulfed by phagocytes, such as macrophages and immature dendritic cells, and DNase II in their lysosomes digest DNA of the dead cells into nucleotides. (B) DNA degradation in definitive erythropoiesis. Red blood cells lack a nucleus and are produced in a process called definitive erythropoiesis, which occurs in erythroblastic islands that have a single, central macrophage. In the last stage of erythropoiesis, nuclei are extruded by the erythrocytes and engulfed by the macrophage. DNase II in the lysosomes of the macrophage digests DNA. (C) DNA degradation in lens-cell differentiation. The eye lens is composed of fiber cells that are derived from epithelial cells. At the equator, epithelial cells move toward the lens nucleus and differentiate into fiber cells. During this differentiation process, all the cell organelles, including the nucleus, mitochondria, and endoplasmic reticulum, are degraded. DNase II-like acid DNase (DLAD) in the lysosomes of fiber cells digests chromosomal DNA.

affected in the *DNase II*-null mutant, which leads to fatal anemia. When the fetal liver cells of *DNase II*$^{-/-}$ mice are transferred into an irradiated host, the *DNase II*-null precursor cells differentiate into mature enucleated erythrocytes. This finding indicates that DNase II is not necessary in the erythroid cells but in the cells that support erythropoiesis.

A large number of macrophages carrying undigested DNA are present throughout the *DNase II*$^{-/-}$ embryos (13, 14) (Fig. 2). These macrophages are found not only in tissues where many cells undergo programmed cell death, such as the thymus, brain, and kidney, but also in the fetal liver, where definitive erythropoiesis takes place, confirming that DNase II is responsible for degrading the DNA of apoptotic cells as well as the nuclear DNA from erythroid precursor cells. The gene expression profile, determined by microarray analysis, indicated that the expression levels of genes that support erythropoiesis, such as erythropoietin, stem cell factor, erythropoietin receptor, and GATA-1, are similar between the wild-type and *DNase II*$^{-/-}$ fetal livers. In contrast, a set of IFN-inducible genes are strongly activated in the fetal liver and thymus of *DNase II*$^{-/-}$-embryos (12, 15). In fact, IFNβ and IFNγ mRNAs were found in the fetal liver of *DNase II*$^{-/-}$ but not in that of wild-type mice. *In situ* hybridization showed a high level of IFNβ mRNA in macrophages carrying undigested DNA, while IFNγ mRNA was found not only in macrophages but also in other cells, such as erythroid cells, indicating that IFNβ is primarily produced by the macrophages carrying undigested DNA (15, 76).

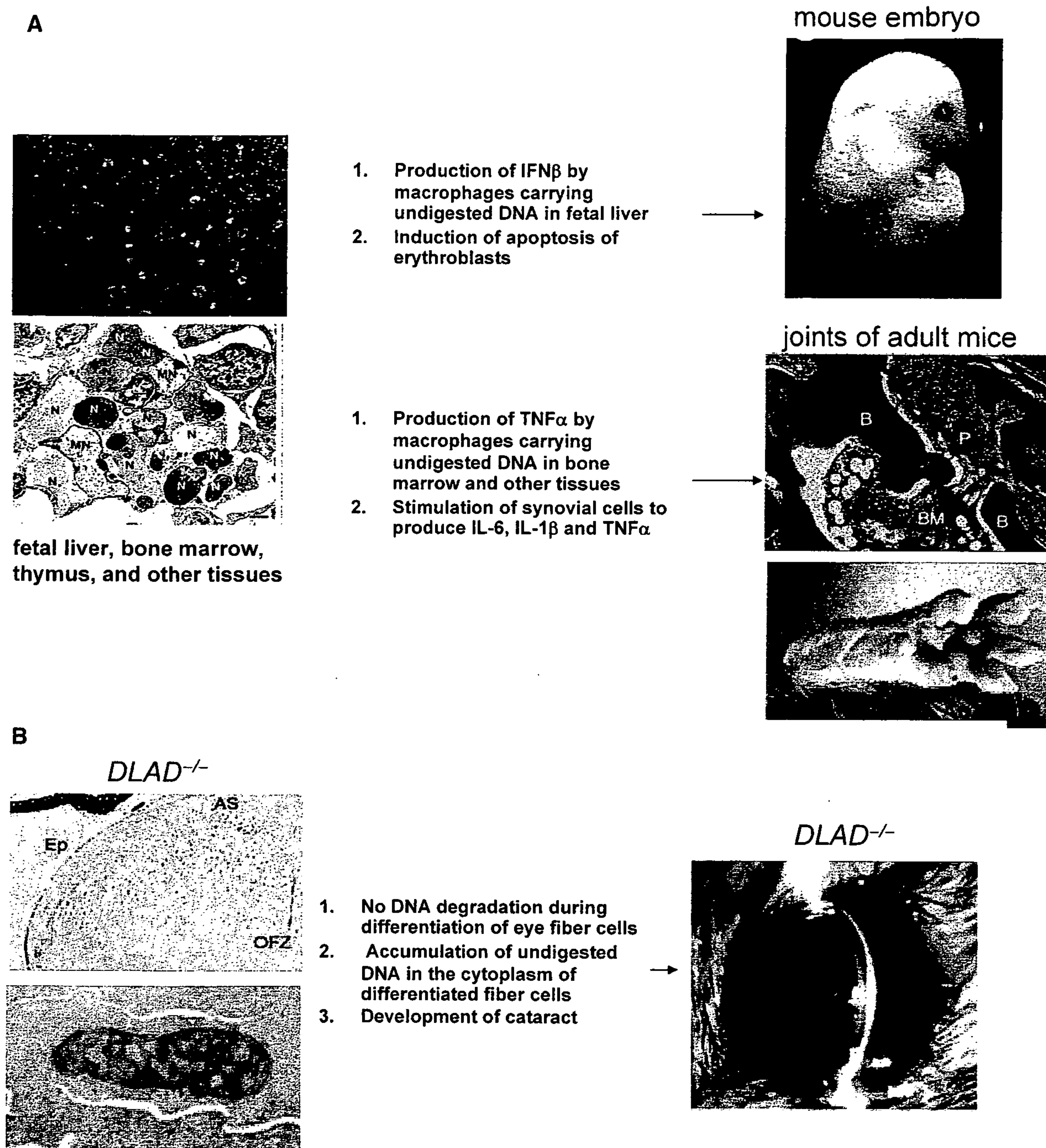

Fig. 2. Diseases caused by inefficient DNA degradation. (A) Anemia and chronic polyarthritis caused by DNase II deficiency. In *DNase II*$^{-/-}$ embryos, the macrophages in various tissues, particularly in the fetal liver, accumulate a large amount of undigested DNA in their lysosomes. These macrophages produce inflammatory cytokines such as interferon β (IFNβ) and tumor necrosis factor α (TNFα). IFNβ induces apoptosis of erythroblasts, leading to severe anemia. When the DNase II gene is deleted after birth, TNFα produced by the macrophages seems to be responsible for the activation of synovial cells in the joints, leading to chronic polyarthritis. (B) Cataract caused by the lack of DNase II-like acid DNase (DLAD). In *DLAD*$^{-/-}$ mice, chromosomal DNA is undigested during the differentiation of fiber cells in the eye lens. Thus, DNA is left in the cytoplasm of the differentiated fiber cells, where it disturbs the light path and leads to cataract formation.

Involvement of IFNβ in the lethality of *DNase II*$^{-/-}$ embryos

IFNβ is a cytokine belonging to the type I IFN family (77, 78). It protects cells from virus, but it also has strong cytotoxic effects in newborn mice (79, 80). There are two types of IFN receptors, type I (IFN-IR) and type II (IFN-IIR). The IFN-IR is responsible for mediating the signals of IFNα and IFNβ, while the IFN-IIR mediates the IFNγ signal. Mice that are doubly deficient in the genes for *DNase II* and *IFN-IR* (15), but not for *DNase II* and *IFN-IIR* (K. Kawane and S. Negata, unpublished

results), are apparently normal at birth, confirming that the IFNβ produced in the *DNase II*$^{-/-}$ mice is responsible for the lethality. Many erythroblasts in the fetal liver of *DNase II*$^{-/-}$ embryos are positive for TUNEL staining, indicating that erythroblasts undergo apoptosis in these mice (15). Because IFN-induced apoptosis depends on protein or RNA synthesis, it is likely that one or more factors induced by the elevated IFNβ indirectly activate the apoptosis program in the erythroblasts.

IFNβ is normally produced by cells infected by a virus or bacteria and is regarded as a marker gene for innate immunity (81). Toll-like receptors (TLRs) and their adapters [myeloid differentiation factor 88 (MyD88) and TIR-domain-containing adapter-inducing IFNβ (TRIF)] are involved in the activation of the IFNβ gene in the innate immune reaction to bacterial or viral infection (82–84). In *DNase II*$^{-/-}$ mice, the macrophages accumulate DNA in their lysosomes and express IFNβ (15). When *DNase II*$^{-/-}$ macrophages engulf apoptotic cells *in vitro*, they also accumulate undigested DNA in their lysosomes and produce IFNβ (76), indicating that mammalian DNA that escapes degradation in lysosomes activates the innate immune system. However, a null mutation of *TLR9* or *TLR3* or of their adapters (*MyD88* and *TRIF*) had no effect on the lethality of the *DNase II*$^{-/-}$ genotype (76), suggesting that mammalian DNA activates the innate immune system in a TLR-independent manner. For bacterial components to activate the innate immune system, IRF3 (IFN-regulatory factor 3) and IRF7 are activated downstream of MyD88 or TRIF and bind to the IFN-regulatory element in the promoter region of the *IFNβ* gene (85, 86). The deletion of the genes for both *IRF3* and *IRF7* abolished the expression of IFNβ gene in the fetal liver of *DNase II*$^{-/-}$ embryos and rescued the lethality (Y. Okabe and S. Negata, unpublished results), indicating that an unidentified sensor for mammalian DNA is present upstream of IRF3/IRF7.

Chronic polyarthritis in *DNase II*-null mice

DNase II$^{-/-}$-*IFN-RI*$^{-/-}$ mice are born normal, but they develop polyarthritis as they age (16). When the *DNase II* gene alone is deleted after birth in an inducible conditional knockout mouse, the mice do not die but develop polyarthritis in a time-dependent manner (Fig. 2). Two to 3 months after the deletion of the *DNase II* gene, the forelimbs and hindlimbs start to swell. Similar to the development of rheumatoid arthritis in human patients, the joints affected first are at the periphery (digit), and those affected subsequently are increasingly central (foot and wrist). Eventually, all joints are affected within 8 months. The swollen joints show severe synovitis with aggressive pannus formation. Tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts are at the leading edge of the pannus, which fills the joint cavity, erodes cartilage, and often destroys the bones. Genes for inflammatory cytokines, such as interleukin-1β (IL-1β), IL-6, and TNFα, are strongly activated in the affected joints. An anti-TNFα antibody and a soluble form of the TNF receptor that antagonize TNFα action have been successfully used to treat human patients with rheumatoid arthritis (87, 88). Similarly, the administration of an anti-TNFα antibody significantly improved the clinical score for the polyarthritis developed by the *DNase* II-null mice (16).

Inflammatory cytokines (IL-1β, TNFα, and IL-6) stimulate the proliferation of synovial cells in the joints, and these abnormally proliferated synovial cells form pannus and destroy the joints (89). In human rheumatoid arthritis, not only TNF antagonists but also molecules that neutralize IL-6 or IL-1β have clinically beneficial effects (90–92). Patients who respond to the TNF antagonist often respond to the IL-6 or IL-1β antagonists as well. Because the inflammatory cytokines TNFα, IL-1β, and IL-6 stimulate each other's genes (93, 94), it is likely that blocking one cytokine's action will cause the shutdown of gene expression for all the inflammatory cytokines. In this regard, it will be interesting to study whether molecules that neutralize IL-6 or IL-1β block the development of arthritis in *DNase II*$^{-/-}$ mice.

Although inflammatory cytokines are known to play an essential role in the maintenance of rheumatoid arthritis in human (95), what triggers the disease is still unknown, and the anti-TNF therapy does not cure it. Previously, Keffer *et al.* (96) showed that mice carrying human TNFα gene as a transgene develop polyarthritis. In the *DNase* II-null mice, the macrophages carrying undigested DNA in their lysosomes are activated as part of the innate immune reaction, and the IFNβ and TNFα genes are activated as well. In fact, *in situ* hybridization indicates that bone marrow macrophages carrying undigested DNA in *DNase II*$^{-/-}$ mice express TNFα mRNA (16). A low but significant level of TNFα is found in sera of *DNase II*$^{-/-}$ mice, at the stage before joints show any abnormality. Neutralization of TNFα by injecting anti-TNFα antibodies into young *DNase II*$^{-/-}$*IRF-IR*$^{-/-}$ mice prevented the development of polyarthritis.

Programmed cell death occurs extensively during development and then continues constitutively at a steady rate. Erythropoiesis is also strongest during development (97). It is likely that macrophages loaded with DNA from dead cells or erythroid precursors constitutively produce TNFα in *DNase* II-null mice, which triggers the development of arthritis (Fig. 2). Some patients with rheumatoid arthritis can be cured by bone marrow transplantation (98), suggesting that these patients

have a defect(s) in bone marrow-derived cells. It will be useful to learn whether these patients have defects in lysosomal DNA digestion by macrophages.

DNase II is a lysosomal enzyme, and the diseases caused by its deficiency are essentially lysosomal storage diseases, which result from defects in lysosomal function (99). Lysosomal storage diseases include more than 40 disorders with inactive or malfunctioning lysosomal enzymes, including proteases, glycosidases, and lipases. Innate immunity is activated by bacterial or viral proteins, polysaccharides, DNA, and RNA (100). As described above, mammalian DNA activates the innate immunity via a TLR-independent system. Other cellular components that escape degradation in lysosomes may also activate *TNFα* and *IFN* genes, leading to the symptoms of storage diseases. The finding that cytokines are constitutively secreted by fibroblasts derived from patients with Niemann–Pick disease type C (101), an inherited lipid storage disorder, may support this idea.

Engulfment of apoptotic cells

Although a large number of cells undergo apoptosis during mammalian development, it is difficult to detect them *in situ*, because they are quickly engulfed by professional phagocytes such as macrophages and immature dendritic cells (4, 5, 17). In animals such as *Caenorhabditis elegans*, which does not have professional phagocytes, non-professional fibroblasts or endothelial cells in the neighborhood of the apoptotic cells engulf the dead cells instead, although their efficiency of engulfment may be low (102, 103).

Macrophages engulf apoptotic cells but not healthy living cells. It is therefore thought that apoptotic cells present an eat-me signal(s) on their surface, and phagocytes recognize it. Many molecules have been proposed as the eat-me signal and its receptor. These include phosphatidylserine, intercellular adhesion molecule-3 (ICAM-3), Annexin I, and cardiolipin as the eat-me signal (17, 104, 105) and CD44, CD14, CD36, CD68, scavenger receptor, and phosphatidylserine receptor (PSR) as the receptor. However, understanding how these molecules are exposed on the cell surface of apoptotic cells and how they are involved in the cell's engulfment has been very elusive and rather confusing.

As soon as apoptotic dead cells are engulfed by phagocytes, the dead cell's components are degraded in the phagocyte's lysosomes. It is not easy, therefore, to assay the engulfment of apoptotic cells or to distinguish cells engulfed by macrophages from those that are simply attached to them. Using our knowledge that *CAD*-deficient cells undergo apoptosis without DNA fragmentation and are efficiently engulfed by macrophages (12), which efficiently cleave their DNA and produce TUNEL-positive cells (64), we established a reliable assay system for the engulfment of apoptotic cells (18). A hamster hybridoma library of monoclonal antibodies against mouse macrophages composed of more than 3000 clones was screened with this assay, and one monoclonal antibody was found to affect engulfment. Purification of the antigen recognized by the monoclonal antibody led to its identification as 'milk fat globule EGF factor VIII' (MFG-E8) (18). Mouse MFG-E8 is a secreted protein that has a signal sequence, two EGF domains, and two factor VIII-homologous domains (106, 107). MFG-E8 was first identified as a protein expressed in mammary glands and is particularly abundant when the mammary glands undergo involution (108, 109). It is also secreted from thioglycollate-elicited peritoneal macrophages but not resident peritoneal macrophages (18).

Among the many molecules proposed as eat-me signals, phosphatidylserine, a glycerophospholipid, is located inside healthy cells but exposed to the outer leaflet of the plasma membrane when the cells undergo apoptosis (110). When erythrocyte ghosts (erythrocytes whose contents have been removed) are loaded with phosphatidylserine, they are phagocytosed by macrophages (110, 111). Thus, phosphatidylserine has been a strong candidate for the eat-me signal (*Fig.* 3). MFG-E8 binds phosphatidylserine via its factor VIII-homologous domain and binds the $\alpha_v\beta_3$- or $\alpha_v\beta_5$-type integrin through an RGD motif in the EGF domain (18, 112–114). Because macrophages and immature dendritic cells express the $\alpha_v\beta_3$- or $\alpha_v\beta_5$ integrin, it seems likely that MFG-E8 works as a bridge between apoptotic cells and phagocytes. In fact, NIH3T3 fibroblasts, which normally do not engulf apoptotic cells, efficiently engulf apoptotic cells in the presence of MFG-E8 when they are transformed with $\alpha_v\beta_3$ integrin.

Autoimmunity caused by a defect of MFG-E8

In addition to mammary epithelial cells, MFG-E8 is expressed in thioglycollate-elicited peritoneal macrophages and granulocyte macrophage-colony-stimulating factor (GM-CSF)-induced bone marrow-derived immature dendritic cells (115). These macrophages and immature dendritic cells from *MFG-E8*-deficient mice have severe defects in engulfing apoptotic cells, indicating that these cells mainly use MFG-E8 for engulfment (19). Langerhans cells, immature dendritic cells of the skin, also express abundant MFG-E8, although its physiological role in the Langerhans cells is not clear. The secondary lymphoid

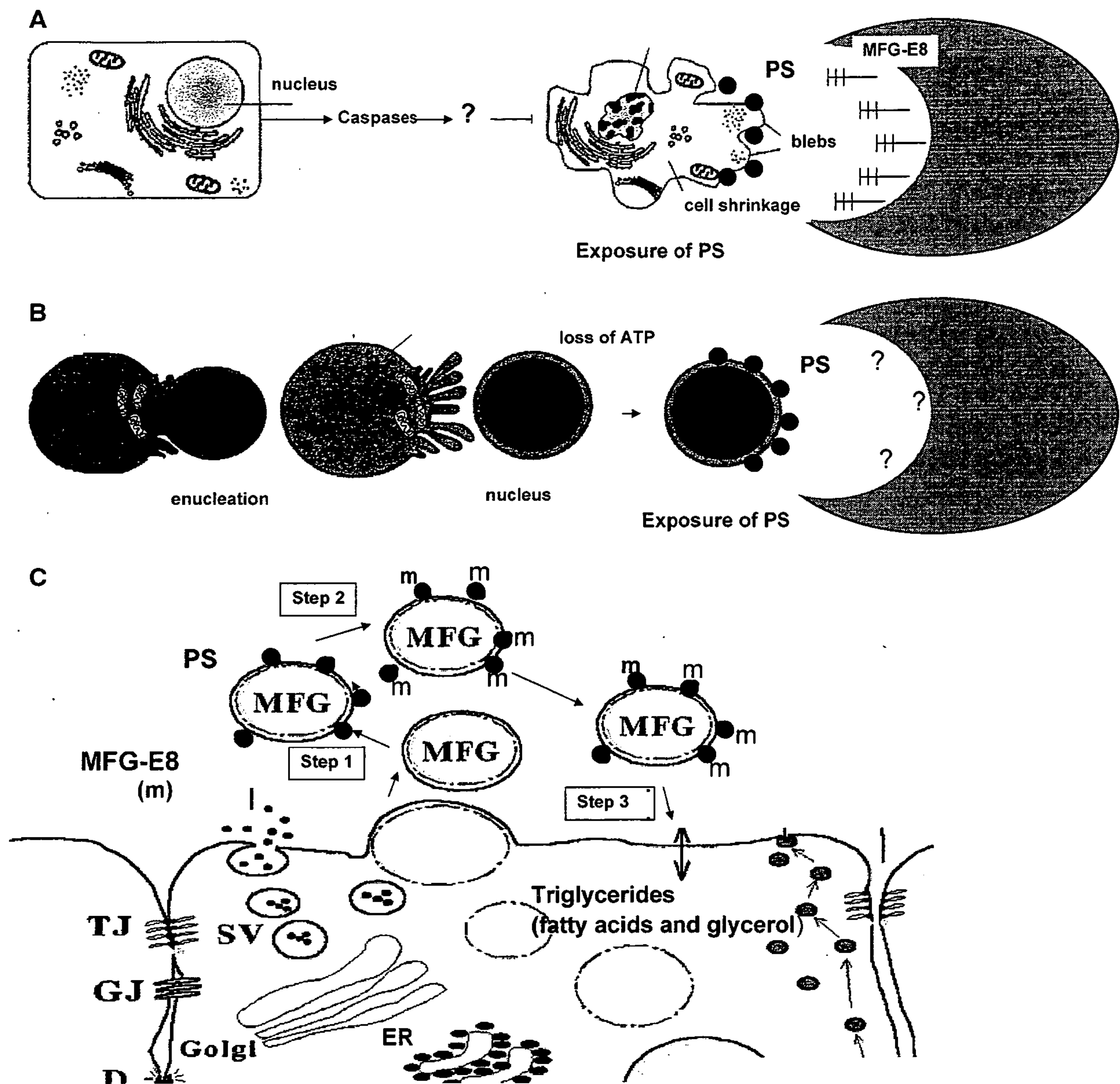

Fig. 3. Phosphatidylserine-dependent engulfment of apoptotic cells, erythroid cell nuclei, and milk fat globules (MFGs). (A) Phosphatidylserine-dependent engulfment of apoptotic cells. When cells undergo apoptosis, they expose phosphatidylserine on their surface. This process is caspase-dependent, but the detailed molecular mechanism is unknown. Macrophages use various molecules to recognize phosphatidylserine as an eat-me signal. The molecule used by tingible-body macrophage in the spleen is MFG-E8, which binds to phosphatidylserine on apoptotic cells and $\alpha_v\beta_3$ integrins on phagocytes, thus acting as a bridge between them. (B) Phosphatidylserine-dependent engulfment of nuclei expelled from erythroid precursors. When nuclei are expelled from erythroid precursor cells, they quickly lose adenosine triphosphate (ATP), because they do not have the machinery to make it. This loss of ATP inactivates the ATP-dependent translocase and activates the Ca-dependent scramblase, thus leading to the exposure of phosphatidylserine on the plasma membrane covering the nucleus. An unidentified molecule(s) on macrophages in the bone marrow or fetal liver recognizes nuclei for engulfment. (C) MFG-E8-dependent recycling of MFG. MFGs that are released from mammary epithelial cells are a kind of exosome that contains triglycerides. Because MFGs do not have an ATP-generating system, phosphatidylserine is exposed on the surface as soon as they are released from cells. MFG-E8 is also secreted by mammary epithelial cells and binds to phosphatidylserine on MFGs. When mammary glands undergo involution, MFGs left in mammary ducts are apparently re-absorbed by epithelial cells in an MFG-E8-dependent manner.

organs, the spleen and lymph nodes, contain diverse subsets of macrophages, with regard to the expression of CD68, F4/80, and MOMA-1. MFG-E8 is expressed in the tingible-body macrophages, which engulf apoptotic lymphocytes and reside at the germinal centers in the spleen and lymph nodes (19). In the germinal centers, many B lymphocytes are activated, undergo apoptosis (116), and are then engulfed. In MFG-E8-deficient mice, many TUNEL-positive apoptotic cells were

found associated with tingible-body macrophages in the germinal centers, particularly when the mice were challenged with keyhole limpet hemocyanin (KLH) to activate the B lymphocytes. These findings indicate that MFG-E8 plays an essential role in the engulfment of apoptotic cells at least in the tingible-body macrophages. However, the fact that apoptotic cells were associated with *MFG-E8*$^{-/-}$ macrophages indicates that one or more molecules other than MFG-E8 must be involved in the binding of apoptotic cells to macrophages.

MFG-E8-null mice are normal at birth. However, as they grow older, they develop splenomegaly with an enlarged white pulp and numerous germinal centers (19, 20). Many immunoglobulin G (IgG)-producing plasma cells are found in the germinal centers. Accordingly, *MFG-E8*-deficient mice develop SLE-type autoimmune diseases. At the age of 40 weeks, *MFG-E8*-deficient mice produce a large quantity of anti-double-stranded DNA and anti-nuclear antibodies (ANAs), develop glomerulonephritis from a massive deposition of Igs in the glomeruli, and suffer from proteinuria. As found in human SLE patients, the female mice exhibit more severe phenotypes than the male mice (19). When the mice were immunized with KLH, *MFG-E8*-null female mice, even young ones, quickly generated ANAs. These results confirm that if apoptotic cells, in this case B lymphocytes activated in the germinal centers, are not efficiently engulfed by macrophages, the animal will develop autoimmune disease. Recent reports on the involvement of MFG-E8 in atherosclerosis and Alzheimer disease (117, 118) also point out pathological roles of unengulfed dead cells in these diseases.

Defect in involution of mammary glands in MFG-E8-deficeint mice

Mammary epithelial cells express a high level of MFG-E8 during involution of the mammary glands (108, 109). The MFGs produced by the epithelial cells are plasma membranes covering triglycerolipids (119), and they associate with MFG-E8, which gave this molecule its name. MFGs expose phosphatidylserine because they lack an adenosine triphosphate (ATP)-generating system, and MFG-E8 binds to the MFGs through the phosphatidylserine on the plasma membrane (109) (*Fig.* 3). During the involution of mammary glands, epithelial cells undergo extensive apoptosis, and macrophages enter the mammary glands to engulf them (120). In addition, many of the MFGs that remain in the mammary glands are reabsorbed by epithelial cells in an MFG-E8-dependent manner. Thus, in *MFG-E8*$^{-/-}$ mice, a large number of MFGs remains in the mammary glands after the pups are weaned. This condition prevents the re-development of mammary glands for the next generation of pups, and the pups therefore starve.

Engulfment of nuclei expelled from erythroid precursors

During the differentiation of erythroid cells, nuclei are expelled from the erythroid precursors, leaving reticulocytes that further mature into red blood cells. Nuclei that are expelled from the erythroid precursor cells are engulfed by macrophages, whereas reticulocytes are not, indicating that nuclei present an 'eat nucleus' signal on their surface. As described above, apoptotic cells often use phosphatidylserine as an eat-me signal. To clarify the eat nucleus signal, a system for enucleating erythroid precursor cells was developed using erythroblasts from the spleen of phlebotomized mice (121). Nuclei expelled from erythroblasts are surrounded by a plasma membrane and are efficiently engulfed *in vitro* by macrophages from the fetal liver. This engulfment is blocked when phosphatidylserine is masked, indicating that as for apoptotic cells, phosphatidylserine is an eat-me signal for erythroid nuclei. In fact, immediately after the nuclei are disconnected from the reticulocytes, the surrounding plasma membrane exposes phosphatidylserine.

The asymmetrical distribution of phosphatidylserine on plasma membranes is thought to be maintained by an as-yet unidentified ATP-dependent aminophospholipid translocase that catalyzes the inward movement of aminophospholipids (122, 123). Another enzyme thought to contribute to the exposure of phosphatidylserine is a Ca^{2+}-dependent, ATP-independent scramblase that catalyzes both the inward and outward movements of lipids. Nuclei extruded from reticulocytes quickly lose their ATP, because they do not contain mitochondria and the glycolysis system that generates it. This inactivates the ATP-dependent aminophospholipid translocase and ATP-dependent Ca pump, which transports Ca^{2+} out of the cells against its large concentration gradient. Therefore, the Ca^{2+} concentration increases in the nuclei, and the Ca^{2+}-dependent scramblase is activated, leading to the exposure of phosphatidylserine on the nuclear plasma membrane surface (*Fig.* 3). Although candidate molecules for scamblases and translocases have been identified (124, 125), whether these enzymes are in fact involved in the exposure of phosphatidylserine on the plasma membranes is still controversial (126). In addition, the mechanism for exposing phosphatidylserine during apoptotic cell death remains a mystery.

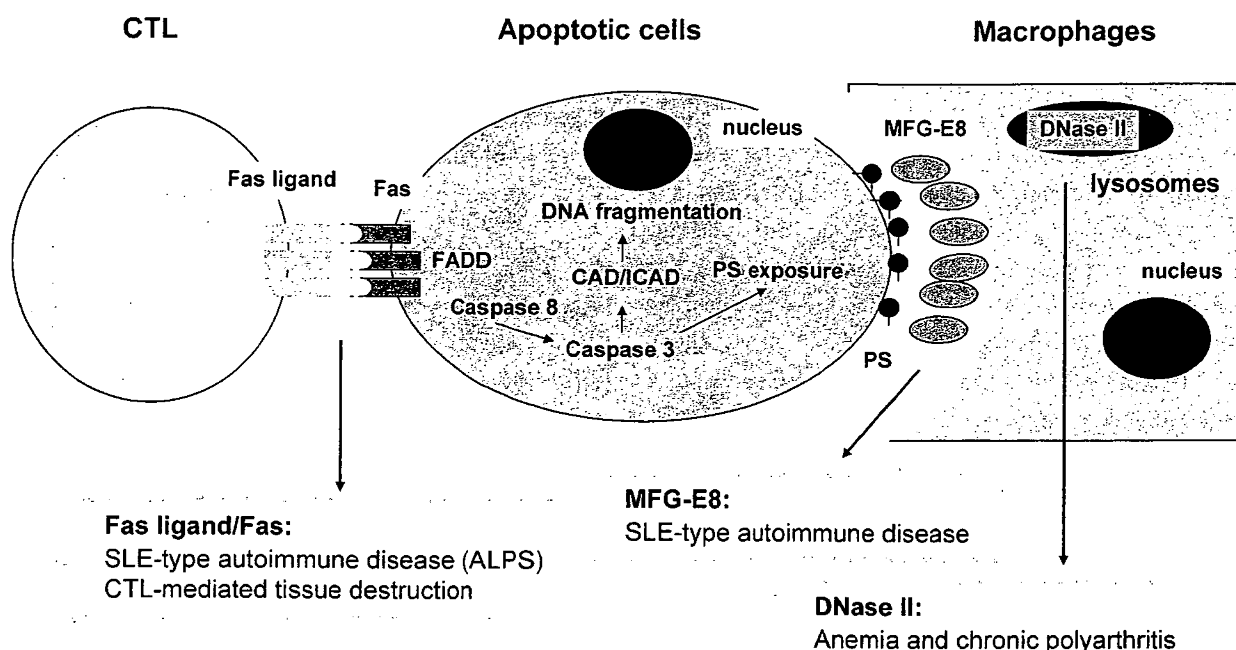

Fig. 4. Fas-mediated cell death, followed by clearance of dead cells. Fas ligand (FasL) is a type II-membrane protein expressed on cytotoxic T lymphocytes (CTLs). Binding of FasL to its receptor, Fas, activates the apoptosis program in the target cells. This program includes caspase-activated DNase (CAD)-dependent DNA fragmentation and the exposure of phosphatidylserine on the membrane surface. Macrophages engulf the apoptotic cells by recognizing phosphatidylserine and degrade all the cellular components. One molecule that recognizes phosphatidylserine is MFG-E8, which is expressed by tingible-body macrophages. DNase II in the lysosomes of macrophages degrades the DNA of apoptotic cells. Loss-of-function mutations in Fas or FasL cause an systemic lupus erythematosus (SLE)-type autoimmune disease, and exacerbation of FasL-induced apoptosis leads to tissue destruction. Inefficient engulfment of apoptotic cells because of the lack of MFG-E8 also leads to SLE-type autoimmune diseases. When DNA of apoptotic cells is left undigested in the lysosomes of macrophages because of deficiency of DNase II, the result is anemia in embryos and chronic polyarthritis in adults.

Exosomes

The engulfment of apoptotic cells, nuclei from erythroid precursor cells, and MFGs all follow the exposure of the same eat-me signal (phosphatidylserine) on the surface. Exosomes are small vesicles that are secreted by a variety of cell types (e.g. activated lymphocytes and cancer cells) (127, 128). They associate with MFG-E8 (129), are ingested by macrophages and immature dendritic cells, and seem to play a role in antigen presentation. The apoptotic bodies that are generated during apoptosis and are composed of membranous vesicles carrying cellular components are a kind of exosome.

Exosomes are implicated in activating the immune system, at least in the case of exosomes from transformed cells. The engulfment of apoptotic cells by immature dendritic cells also has an immunoregulatory role. If the dead cells are the organism's 'own healthy cells' that die during development or due to senescence, their engulfment will not activate the immune system. In contrast, if the cells undergoing apoptosis are virally infected or transformed, their engulfment may activate the immune system. The exact role played by exosomes in the immune system and whether similar mechanisms are used by different kinds of exosomes including apoptotic cells to affect immune responses remain an interesting subject for future studies.

Conclusion and perspective

All the cells in our body die sooner or later. The clearance of apoptotic cells is a normal process for recycling cellular components and maintaining homeostasis in mammals. Defects in apoptosis lead to cancer and autoimmune diseases (130–132). In particular, the autoimmune diseases caused by defects in the Fas and FasL system have been well characterized in human and mice (133–137) (Fig. 4). As described above, recent progress in understanding the clearance of apoptotic cells indicates that a defect in clearance or degradation of apoptotic cell components causes autoimmune disease, anemia, and chronic arthritis. The number of red blood cells produced per day in our bodies is about 10 times higher than the number of apoptotic cells, and an equal number of nuclei are expelled by erythroid precursor cells during red cell differentiation. A large number of exosomes seem to be produced in various processes in our bodies. Failure to engulf

and degrade these nuclei and exosomes are likely to cause problems similar to those observed in the inefficient engulfment of apoptotic cells.

MFG-E8 functions as a bridge between apoptotic cells and macrophages. However, it is expressed only in macrophages in just a few tissues. How macrophages in the thymus or microglia in the brain engulf apoptotic cells and how macrophages in the fetal liver or bone marrow engulf erythroid cell nuclei have not been elucidated. The development of specific diseases, anemia and polyarthritis, as a result of the inefficient digestion of DNA of apoptotic cells was a surprise to us. Elucidation of the detailed molecular mechanism for clearing apoptotic cells will provide more surprises and certainly contribute to the understanding of human disease.

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Degradation of nuclear DNA by DNase II-like acid DNase in cortical fiber cells of mouse eye lens

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The eye lens is composed of fiber cells that differentiate from epithelial cells on its anterior surface. In concert with this differentiation, a set of proteins essential for lens function is synthesized, and the cellular organelles are degraded. DNase II-like acid DNase, also called DNase IIβ, is specifically expressed in the lens, and degrades the DNA in the lens fiber cells. Here we report that DNase II-like acid DNase is synthesized as a precursor with a signal sequence, and is localized to lysosomes. DNase II-like acid DNase mRNA was found in cortical fiber cells but not epithelial cells, indicating that its expression is induced during the differentiation of epithelial cells into fiber cells. Immunohistochemical and immunocytochemical analyses indicated that DNase II-like acid DNase was colocalized with Lamp-1 in the lysosomes of fiber cells in a relatively narrow region bordering the organelle-free zone, and was often found in degenerating nuclei. A comparison by microarray analysis of the gene expression profiles between epithelial and cortical fiber cells of young mouse lens indicated that some genes for lysosomal enzymes (cathepsins and lipases) were strongly expressed in the fiber cells. These results suggest that the lysosomal system plays a role in the degradation of cellular organelles during lens cell differentiation.

The lens is an avascular tissue that focuses light onto the retina in the eye. It consists of closely packed fiber cells that are bounded at the anterior by a monolayer of epithelial cells. Fiber cells are produced continuously by the differentiation of the epithelial cells near the lens equator. New fiber cells are added steadily, and as there is no cell turnover, the lens grows throughout life [1,2]. The differentiating fiber cells are overlaid by new layers of younger cells, and when they have fully differentiated, the fiber cells are incorporated as part of the lens nucleus. As the epithelial cells differentiate into fiber cells, they change morphologically to become fiber-like, and they synthesize lens-specific proteins such as crystallins. One of the most notable changes is the elimination of all organelles, including the endoplasmic reticulum, mitochondria, Golgi apparatus, and nucleus, at the last stage of differentiation [3]. The adult lens thus contains two populations of fiber cells in the cortex: a cortical layer of differentiating fiber cells, which contain organelles, and the non-nucleated fiber cells, which do not contain organelles. Several degradation mechanisms have been proposed, most prominently apoptosis [4], cytosolic degradation [5], and autophagy [6,7]. However, the mechanism that operates during the differentiation of fiber cells has been very elusive.

In mammals, there are two acid DNases, DNase II and DNase II-like acid DNase (DLAD) or DNase IIβ [8–10]. DNase II is ubiquitously expressed in various tissues, particularly in macrophages [11,12]. The

Abbreviations
DLAD, DNase II-like acid DNase; rDLAD, recombinant DNase II-like acid DNase.

DNase II expressed in macrophages is responsible for degrading the DNA of apoptotic cells and the nuclei expelled from erythroid precursor cells [13,14]. In contrast, DLAD is uniquely expressed in the eye lens [15]. The targeted mutation of the *DLAD* gene in mice causes cataract of the nucleus lentis [15] from a large amount of naked DNA that accumulates in the cytoplasm of the differentiated fiber cells, indicating that DLAD is responsible for cell-autonomously digesting its own chromosomal DNA in lens fiber cells during lens cell differentiation.

In this study, we found that the expression of mouse DLAD is strongly induced during lens fiber cell differentiation. DLAD resides in the lysosomes of cortical fiber cells, close to the organelle-free zone. Small lysosomal vacuoles carrying DLAD were present surrounding nuclei, and degenerating nuclei were localized with DLAD. In addition to DLAD, a set of lysosomal enzymes was strongly upregulated in the cortical fiber cells, suggesting an involvement of lysosomes in degradation of organelles during lens cell differentiation.

Results

Localization of DLAD to lysosomes

DLAD was identified as a protein homologous to DNase II [9,10]. An alignment of the amino acid sequence of murine DLAD with that of murine DNase II (Fig. 1A) showed 37.9% identity at the amino acid sequence level. In particular, three histidine residues that are necessary for the DNase activity of porcine DNase II [16] and their flanking regions were well conserved. There is some controversy about the subunit structure of DNase II [17,18]: the DNase II from porcine spleen is reported to be composed of three subunits (α_1, α_2 and β) [18], whereas human DNase II is proposed to be a single polypeptide with a clipped signal peptide [17]. To determine the subunit structure of murine DLAD and DNase II, they were tagged with Flag at the C-terminus, synthesized in human 293T cells, and purified to homogeneity. Both the purified recombinant DLAD and DNase II were composed of a single polypeptide (Fig. 1B). An Edman degradation analysis for N-terminal amino acids indicated that mouse DLAD and DNase II start from Thr1 and Leu1, respectively, leaving signal sequences of 22 and 19 amino acids, respectively (Fig. 1A). These cleavage sites conform to von Heijne's criteria for signal sequences, and the overall structures of mouse DLAD and DNase II are consistent with that proposed for human DNase II [17]. As shown for human DLAD [10], mature murine DLAD is a basic protein with a pI of 9.56, which contrasts with murine DNase II's pI of 7.2. As reported for DNase II [19], mouse DLAD is a glycoprotein, because treatment of rDLAD with *N*-glycosidase reduced its molecular mass from 67 to 40 kDa (data not shown).

Proteins carrying a signal sequence can be secreted from cells or imported into lysosomes. To determine whether DLAD is secreted or kept inside the cells, the DLAD expression plasmid was introduced into 293T cells, and the DLAD protein levels in the culture supernatant and cells were analyzed by western blotting. As shown in Fig. 1C, compared with mouse DNase II, which could be efficiently secreted into the culture supernatant, mouse DLAD was mainly found in the cell lysates. HeLa cell transformants expressing mouse DLAD were then established, and stained with a mAb to mouse DLAD. As shown in Fig. 1D, very strong staining with granular signals was found adjacent to the nucleus. The parental HeLa cells were not stained with the mAb, confirming its specificity. Signals from an antibody to Lamp-1, a lysosomal membrane protein, colocalized with the anti-DLAD signals, confirming that DLAD could be present in lysosomes.

Expression of DLAD in lens cortical fiber cells

During the differentiation of lens epithelial cells into fiber cells, the gene expression profile greatly changes [20]. An RT-PCR analysis of RNA from epithelial and fiber cells indicated that E-cadherin, a marker protein for epithelial cells [21], was expressed in lens epithelial cells, but was almost completely downregulated in the fiber cells (Fig. 2A). On the other hand, gene expression for crystallin γD, which is needed to maintain the transparency of the lens, was strongly activated in the fiber cells. Filensin, an eye-specific intermediate filament, was also expressed only in fiber cells. DLAD is necessary to degrade nuclei in the lens fiber cells [15]. Accordingly, a western blotting analysis of the epithelial and cortical fiber cells indicated that the cortical fiber cells but not epithelial cells expressed a DLAD protein of about 50 kDa (Fig. 2B). This specific expression of DLAD in the cortical fiber cells could also be observed at the RNA level. That is, northern hybridization with DLAD cRNA showed a major band of 1.8 kb and a minor band of 4.0 kb in the RNA from cortical fiber cells but not from epithelial cells (Fig. 2B). The size of the minor band was consistent with it being a DLAD precursor RNA carrying introns. A real-time PCR analysis indicated that the DLAD mRNA level in the cortical fiber cells was 17 times higher than in epithelial cells (Fig. 2B).

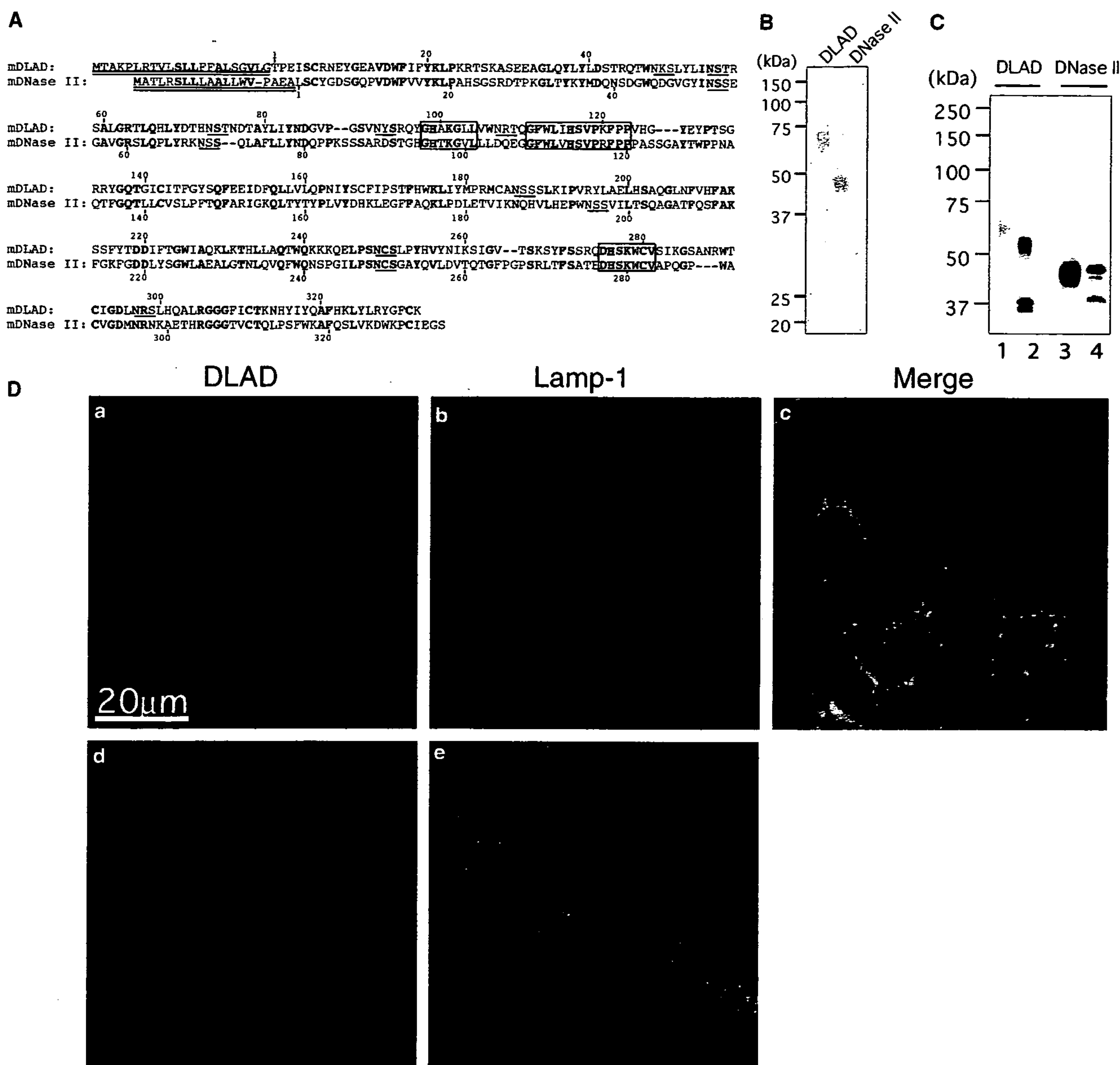

Fig. 1. Localization of murine DLAD to lysosomes. (A) Alignment of mouse DLAD and DNase II amino acid sequences. The amino acid sequences of mouse DLAD and DNase II were aligned to give maximum homology by introducing several gaps (–) using GENETYX-MAC genetic information-processing software (version 12) (Genetyx, Tokyo, Japan).The amino acid residues conserved between the two proteins are shown in bold. The amino acids are numbered from the N-terminus, which was determined by Edman degradation of mature rDLAD and rDNase II. The signal sequences are marked by double underlines. Putative active sites carrying the conserved histidine residues (red) are boxed. Potential N-glycosylation sites (Asn-X-Ser/Thr) are underlined. (B) Production of mouse rDLAD and rDNase II. Mouse rDLAD and rDNase II were produced in human 293T cells transfected with their respective expression plasmid. The DLAD and DNase II secreted into the culture medium were purified, and 0.5 µg of protein was subjected to SDS/PAGE followed by staining with Coomassie Brilliant Blue. The molecular masses of standard proteins are shown in kDa on the left. (C) Transient expression of rDLAD and rDNase II in 293T cells. Human 293T cells were transfected with the expression vector for Flag-tagged DLAD or DNase II, and cultured for 2 days. The culture supernatants were collected, and the cells were lysed. The Flag-tagged recombinant proteins in the culture supernatants were immunoprecipitated with anti-(Flag) protein A–sepharose. Aliquots of the immunoprecipitates (lanes 1 and 3) and cell lysates (lanes 2 and 4) corresponding to 5×10^4 cells were separated by SDS/PAGE (10%), followed by western blotting with the antibody to Flag. (D) Stable expression of DLAD in HeLa cells. Human HeLa cells were stably transformed with the mouse DLAD expression plasmid. The parental HeLa cells (d, e) or their transformants (a, b, c) were stained with hamster mAb to mouse DLAD (a, d; red) or mouse mAb to human Lamp-1 (b, e; green). In (c), the images obtained with anti-DLAD and anti-Lamp-1 are merged. Scale bar: 20 µm.

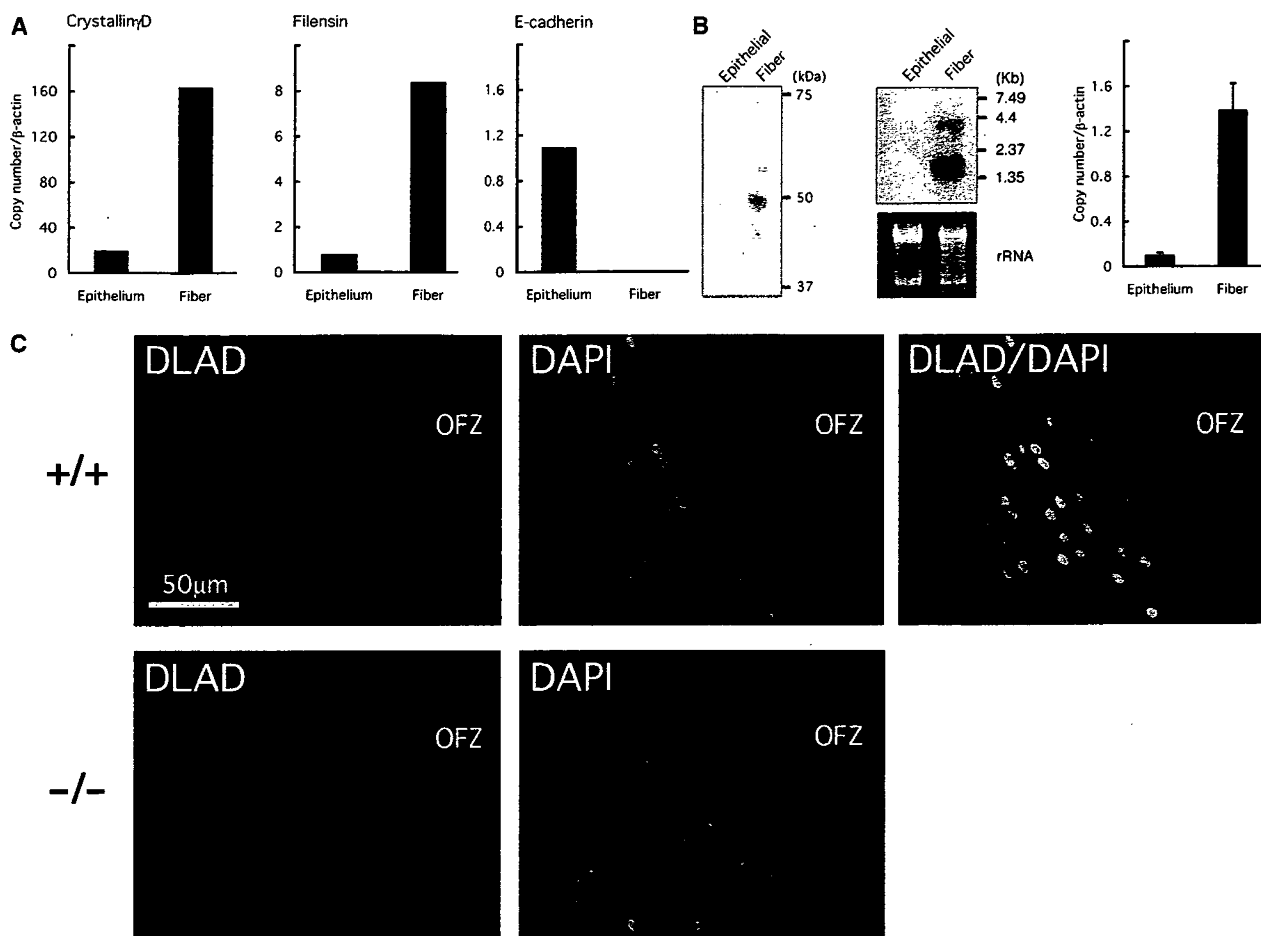

Fig. 2. Expression of DLAD in lens cortical fiber cells. (A) Different gene expression between epithelial cells and fiber cells in the lens. The mRNA levels for crystallin γD, filensin and E-cadherin in lens epithelial and fiber cells were determined by real-time PCR. As an internal control, the level for actin mRNA was determined, and the mRNA expression levels for crystallin γD, filensin and E-cadherin were expressed as ratio to actin mRNA. Experiments were performed in duplicate, and the average values are shown. (B) Specific expression of DLAD in lens fiber cells. Left panel: cell lysates (7.5 μg of protein) prepared from the epithelial (Epithelial) or cortical fiber (Fiber) cells of mouse eye lens were subjected to western blot analysis with the mAb to DLAD. The sizes of standard proteins are shown in kDa on the right. Middle panel: total RNA (1.0 μg) prepared from the lens epithelial or fiber cells was analyzed by northern hybridization using mouse DLAD cRNA as the probe. The sizes of the marker RNAs are shown in kb on the right. The lower panel shows a gel stained with SYBR Gold Nucleic Acid Gel Stain (Molecular Probe) for rRNA. Right panel: DLAD mRNA levels in epithelial and fiber cells were determined by real-time PCR, and the expression levels are shown as ratios to that of actin mRNA. The experiment was performed three times, and average values are plotted with SD (bars). (C) Localization of DLAD in mouse eye lens. Midsagittal lens slices from $DLAD^{+/+}$(+/+) or $DLAD^{-/-}$ (−/−) mice were stained with the mAb to DLAD (red), and this was followed by DAPI staining (blue). Scale bar: 50 μm. OFZ, organelle-free zone.

We then used immunohistochemistry to locate DLAD expression in the mouse lens. As shown in Fig. 2C, strong signals for DLAD were observed only in the layers of cortical fiber cells that surround the organelle-free zone. The signals were not detected in sections from $DLAD^{-/-}$ lens, verifying the authenticity of this result. These results indicated that the *DLAD* gene is transiently activated at the final stage of lens cell differentiation, and the expressed DLAD protein is degraded immediately after the organelles are destroyed. The lens sections were then costained for DLAD and Lamp-1, and analyzed by light and electron microscopy. As shown in Fig. 3A, Lamp-1 was ubiquitously expressed in the lens fiber cells, which contrasts with the limited expression of DLAD in the fiber cell layers adjacent to the organelle-free zone. The immunoelectron microscopy showed prominent signals for Lamp-1 in the lysosomes of fiber cell in the cortical zones, where the signals for DLAD were not frequent (Fig. 3Ba,b). On the other hand, the DLAD signals could be frequently detected in the fiber cell layers close to the organelle-free zone, and were colocalized to lysosomes

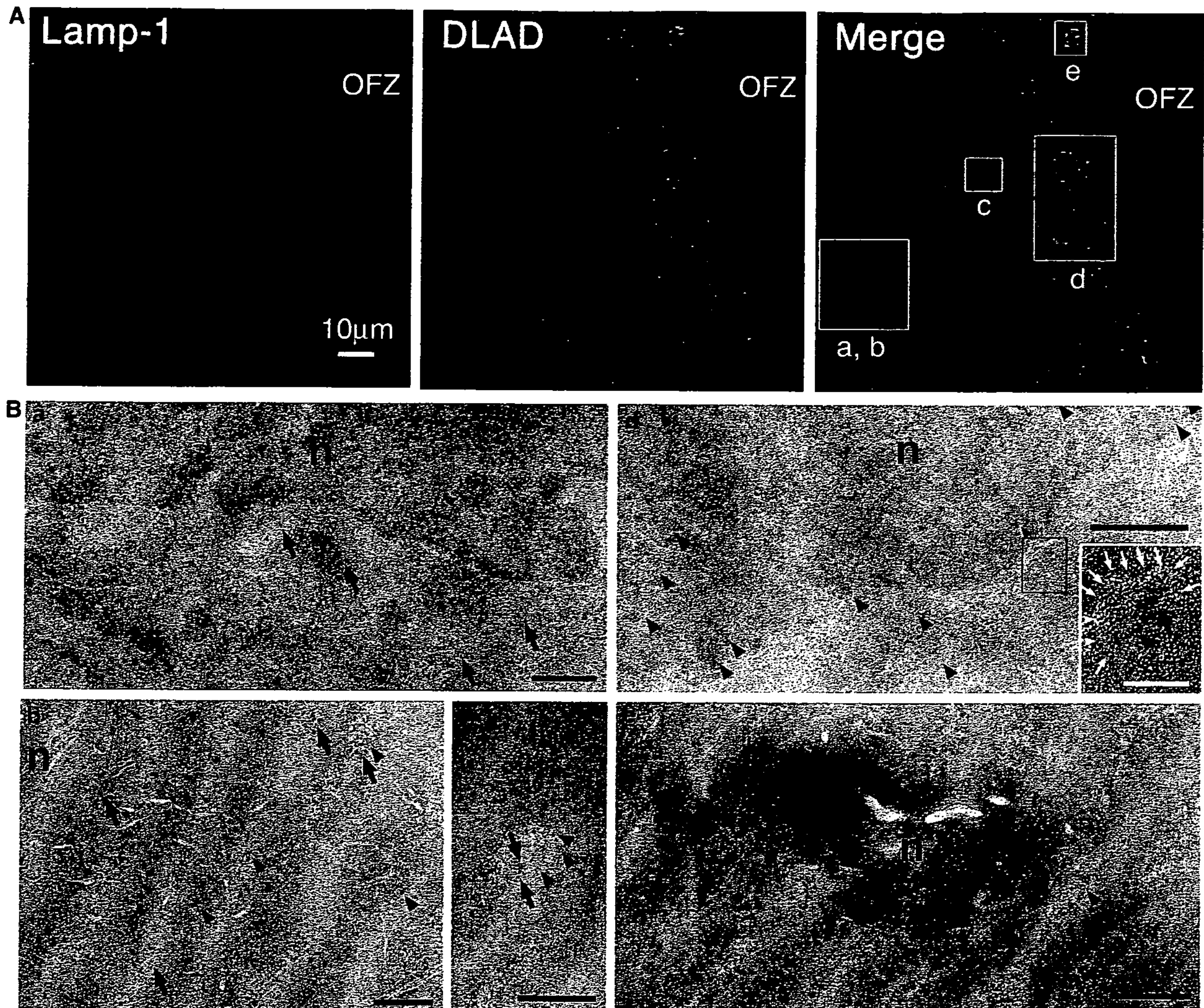

Fig. 3. Immunohistochemical and immunocytochemical staining for Lamp-1 and DLAD in mouse lens fiber cells. (A) Immunohistochemical staining. Lenses from 6-day-old mice were stained with Lamp-1 (red, left panel) and DLAD (green, middle panel). In the right panel, the staining profiles for Lamp-1, DLAD and DAPI are merged. OFZ, organelle-free zone. Scale bar: 10 μm. The fields analyzed by electron microscopy are indicated. (B) Immunocytochemical staining. The thin cryosections from the fields indicated in (A) were stained for Lamp-1 (10 nm gold particles, arrows) and DLAD (5 nm gold particles, arrowheads). n, nucleus. Scale bars: 0.25 μm. In (d), the boxed area is enlarged in the inset. White arrows indicate membranous structure. The scale bar in the inset is 0.1 μm.

together with Lamp-1 (Fig. 3Bc,d). Lysosomes carrying DLAD in this region were relatively small and associated with nuclei, and many DLAD-specific signals were found scattered in the nuclei (Fig. 3A,Bd). In particular, the degenerating nuclei carried DLAD (Fig. 3Be). These results suggested that DLAD is transferred from lysosomes to nuclei to degrade DNA.

Expression of other lysosomal enzymes in cortical fiber cells

To examine whether other enzymes and factors were induced during fiber cell differentiation, the gene expression profiles of the epithelial and fiber cells were compared by gene array analysis. The expression of about 500 genes was upregulated more than five-fold in the fiber cells, and a similar number of genes was downregulated at least five-fold. Major upregulated genes were the crystallins, ion transporters, and sugar transporters, as well as transcription factors that may regulate the differentiation of fiber cells (GEO GSE7533). In addition, several lysosomal hydrolases were strongly upregulated in the fiber cells (Table 1). That is, the mRNA levels for lysosomal proteases such as cathepsin G, cathepsin R and legumain were 5–25 times higher in fiber cells than in epithelial cells. A

Table 1. Hydrolases and their related genes upregulated in the lens fiber cells. Gene array analysis was performed using a Codelink Bioarray System. Signals were obtained by dividing the raw hybridization data by the median value. The median values were 25.0 and 7.7 for the epithelial and fiber cell RNAs, respectively. The increase in gene expression (Fold increase) was determined by dividing the signal obtained with fiber cell RNA by the signal obtained with epithelial cell RNA, and is shown as the fold increase.

Gene	Epithelial cell signal	Fiber cell signal	Fold increase
DNase II-like acid DNase (*dlad* or *dnase2b*)	3.3	28.4	8.6
Cathepsin G (*ctsg*)	9.3	50.6	5.4
Cathepsin R (*ctsr*)	0.6	16.2	24.7
Cathepsin J (*ctsJ*)	0.5	6.3	10.9
Legumain (*lgmn*)	3.9	27.7	7.1
Pancreatic lipase-related protein 2 (*pnliprp2*)	18.4	1400.7	76.3
Phospholipase a2, group vii (*pla2g7*)	1.0	79.7	78.4
Fatty acid-binding protein 5 (*fabp5*)	218.5	3014.9	13.8
Autophagy atg3p/aut1p-like (*atg3*)	15.2	112.7	7.4
Autophagin-1 (*atg4b*)	13.5	65.5	4.8
Caspase 7 (*casp7*)	5.7	58.8	10.9

specific lipase (pancreatic lipase-related protein 2) and a phospholipase (phospholipase a2, group vii) were also strongly upregulated in the lens fiber cells, suggesting that these enzymes are involved in degrading cellular organelles in the fiber cells. In this regard, it is noteworthy that the expression of some proteins involved in autophagy (atg3 and atg4b) was significantly (five- to eight-fold) upregulated in the fiber cells.

Discussion

The removal of cellular organelles during differentiation of fiber cells in the lens is essential to maintain the transparency of the eye lens, and a defect in this process can lead to cataract formation [15]. Among many models proposed for organelle degradation in the lens, three models (apoptosis, cytosolic degradation, and autophagy) predominate [4–7]. That is, when fiber cells differentiate, they undergo a series of morphologic and biochemical changes (condensation and fragmentation of chromatin, and generation of TdT-mediated dUTP-biotin nick end labeling (TUNEL)-positive DNA fragments) that are similar to those found in apoptotic cells [22]. Findings including the temporal activation of caspase-like activities [23], the degradation of some canonical caspase substrates [24] and the downregulation of antiapoptotic Bcl-2 members [25] in differentiating fiber cells also supported the idea of apoptosis being involved in lens cell differentiation. On the other hand, caspase-activated DNase, which is responsible for the apoptotic DNA fragmentation [26], is not expressed in lens fiber cells, and there is no abnormality in the lens of mice deficient in caspase-activated DNase [15]. Moreover, Zandy *et al.* [27] recently reported that the organelle-free zone is correctly formed in the eye lens of mice lacking caspase 3, caspase 6, or both, indicating that the elimination of fiber cell organelles does not require apoptosis or a caspase-dependent process. However, the expression of caspase 7 specifically increases in the fiber cells (Table 1), and some caspase 3-deficient mice transiently develop cataracts [27]. Therefore, it is possible that some caspases may regulate the development of the eye lens.

In the model in which organelles undergo cytosolic degradation, 15-lipoxygenase integrates into organelle membranes, permeabilizing the organelles to create access for cytosolic proteolytic machinery, such as the ubiquitin–proteasome system [28]. As 15-lipoxygenase was found in differentiating fiber cells, van Leyen *et al.* [5] proposed the involvement of lipoxygenase-mediated cytosolic degradation in programmed organelle degradation in the eye lens. However, the expression levels of 15-lipoxygenase and 12-lipoxygenase determined by our gene array analysis were comparable between epithelial and fiber cells. Furthermore, expression of proteases in the ubiquitin-dependent degradation system was not activated during differentiation into fiber cells, suggesting that the cytosolic degradation system may not be involved in the removal of organelles during lens cell differentiation. No lens abnormality was reported in mice deficient in 15-lipoxygenase or 12-lipoxygenase [29,30], supporting this idea.

We previously reported that DLAD is specifically expressed in the eye lens, and the null mutation of *DLAD* causes cataract due to the accumulation of undigested DNA in the cytoplasm of fiber cells [15]. The nuclei of the fiber cells are degraded in the fiber cell layers near the organelle-free zone. Consistent with this finding, DLAD mRNA was more abundant in