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A Presumed Human Nuclear Autoantigen That Translocates to Plasma Membrane Blebs during Apoptosis

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The structure and subcellular localization of a number of molecules change during apoptosis. These molecules are recognized by the immune system, leading to the development of autoimmunity when apoptotic cells fail to be effectively cleared by phagocytosis. We searched for such molecules by analyzing sera from 12 individuals who suffered from autoimmune diseases and from 3 patients with amyotrophic lateral sclerosis. One serum sample, designated 681, detected an antigen that fulfilled the above criteria. In Western blotting of lysates of human Jurkat T cells, the 681 antigen appeared as a distinct signal with a molecular mass of 60 kDa in normal cells, and 2 additional signals with faster mobilities were detected in apoptotic cells. The results of subcellular fractionation and immunofluorescence experiments revealed this antigen to be strictly localized in the nucleus of normal cells, but to be translocated to a region near the plasma membrane, to membrane blebs in particular, after the induction of apoptosis. Under conditions in which membrane blebbing was inhibited in apoptotic cells, the antigen still moved away from the nucleus, but its accumulation at the periplasmic region was completely abolished. The apparent partial cleavage and intracellular redistribution of the 681 antigen in apoptotic cells mimics changes previously reported for the nuclear autoantigen La, but the 681 antigen was clearly distinct from La. These results suggest that cleavage-dependent exit from the nucleus during apoptosis is a phenomenon common to nuclear autoantigens.

Key words: apoptosis, autoantigen, autoimmune disease, membrane bleb.

Phagocytic clearance of cells undergoing apoptosis plays roles not only in preventing organisms from being exposed to the noxious contents of dying cells but also in more actively maintaining tissue homeostasis by directly eliminating microbe-infected cells, producing anti-inflammatory substances, and presenting antigens to T lymphocytes (Refs. 1–3 for reviews). Dysregulation of the clearance of apoptotic cells could result in the development of serious diseases such as acquired immunodeficiency syndrome, neurodegenerative diseases, and autoimmune diseases.

Autoimmune diseases are characterized by the production of autoantibodies that induce inflammatory responses in organisms. Most antigens recognized by autoantibodies are molecules that exist within cells under normal conditions. However, once cells are broken, they are recognized by immune surveillance and evoke autoimmunity. Many types of aged, spent, or non-self cells routinely die in great numbers via apoptosis. These cells are rapidly and selectively eliminated through apoptosis-dependent phagocytosis by both circulating and tissue-restricted phagocytes. Apoptotic cells exhibit necrotic characteristics sometimes called secondary necrosis when they are left unremoved. Necrosis is a mode of cell death in which the cellular contents leak out through broken plasma membranes. It is therefore reasonably anticipated that a failure of apoptotic cells to undergo phagocytosis could lead to autoimmune diseases (Ref. 4 for a review). In fact, a deficiency of phagocytosis of apoptotic cells mediated by complement C1q (5–7) or the tyrosine kinase c-mer (8, 9) results in the development of autoimmunity. In addition, the injection of apoptotic cells induces the production of autoantibodies in mice (10, 11).

Both the structure and composition of the cell surface change during apoptosis. The most obvious change is the occurrence of membrane blebbing, in which parts of the plasma membrane protrude, and cells eventually come to look like raspberries (Ref. 12 for a review). As a less dynamic change, the structures of surface sugar chains are altered by the action of glycosidases that cleave off terminal sugars during apoptosis (Ref. 13 for a review). Another type of fine tuning observed in many types of apoptotic cells is the redistribution of membrane phospholipids; phospholipids confined to the inner leaflet of the membrane bilayer in normal cells, such as phosphatidylycerine and phosphatidylethanolamine, translocate to the outer leaflet and are exposed on the cell surface (Ref. 14 for a review). These changes make apoptotic cells distinguishable from normal cells. Selective recognition and
engulfment of apoptotic cells by phagocytes is defined by the specific binding of phagocytosis markers, which exist at the surface of apoptotic cells, to their receptors present on phagocytes. Until now, various molecules have been proposed as such phagocytosis markers and their receptors (Refs. 2, 14, and 15 for reviews). In most cases, pre-existing molecules acquire the function of phagocytosis markers after cellular molecules are externalized or surface molecules are structurally modified upon the induction of apoptosis. Those phagocytosis markers may at the same time be recognized by the immune system as non-self antigens. If the phagocytic clearance of apoptotic cells is inhibited or delayed, phagocytosis markers present at the surface of apoptotic cells might evoke autoimmunity (16). In fact, some autoantigens move to a region near the cell periphery during apoptosis (Ref. 4 for a review): the nuclear autoantigen La translocates from the nucleus to the cytoplasm or plasma membrane upon infection with herpes simplex virus type 1 (17) or adenovirus 2 (18); fragmented endoplasmic reticulum, ribosomes, the ribonucleoprotein Ro, nucleosomal DNA, La, and small nuclear ribonucleoproteins are relocalized to plasma membrane blebs in UV-irradiated cells (19); Ro and La become detectable at the surface of staurosporine-treated cells (20, 21); La is cleaved and relocalized to the cytoplasm in UV-irradiated cells (22); ribosomal proteins S15, P0, L5, L6, L36a, and L41 are externalized at the surface of cellular antigens to the plasma membrane plays a role in autoimmunity.

In the present study, we analyzed sera of autoimmune disease patients searching for molecules whose subcellular localizations change during apoptosis.

MATERIALS AND METHODS

Cell Culture and Apoptosis Analysis—Jurkat cells, a human leukemia T-cell line, were grown in RPMI 1640 containing 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2 in air. The cells were treated with doxorubicin (Sigma, St. Louis, MO, USA) (0.3 μg/ml) or CH11, an agonistic anti-human Fas antibody (0.1 μg/ml) (MBL, Nagoya), for the induction of apoptosis as described previously (24, 25). The occurrence of plasma membrane blebbing was morphologically determined by phase contrast microscopy. For inhibition of membrane blebbing, cells were pre-treated with the ROCK-I inhibitor Y-27632 (0.1 mM) (Mitsubishi Welpharma, Tokyo) 1 h before the induction of apoptosis (26). To examine the occurrence of chromatin condensation, cells were stained with the DNA-binding fluorochrome Hoechst 33342 and analyzed by fluorescence-phase contrast microscopy.

Human Sera—Serum samples were obtained with informed consent from 17 donors: 12 patients suffering from autoimmune diseases including myasthenia gravis (6 patients), multiple sclerosis (1 patient), systemic lupus erythematosus (1 patient), chronic inflammatory demyelinating polyradiculoneuropathy (2 patients), acute inflammatory demyelinating polyradiculoneuropathy (1 patient), and Sjögren’s syndrome (1 patient). In addition, serum samples from 3 patients with amyotrophic lateral sclerosis and from 2 healthy individuals were obtained. Either unfractionated serum or the IgG fraction prepared by protein A-Sepharose chromatography was used in the study. Another serum sample from a Sjögren’s syndrome patient, which was positive for the Ro antigen and negative for the La antigen, was used to detect Ro in Western blotting.

Preparation of Cell Lysates—To obtain whole-cell lysates, Jurkat cells were lysed in buffer containing 62.5 mM Tris-HCl (pH 6.8), 2.5% (w/v) SDS, and 2.5% (v/v) 2-mercaptoethanol, incubated on ice for 30 min, and centrifuged at 18,000 × g for 15 min at 4°C, and the supernatants were collected. For subcellular fractionation, Jurkat cells were disrupted using a glass-Teflon homogenizer in buffer consisting of 0.13 M KCl and 25 mM Tris-HCl (pH 7.5), centrifuged at 900 × g for 5 min at 4°C, and the supernatants and pellets (nuclear fraction) were collected. The supernatants were further centrifuged at 100,000 × g for 60 min at 4°C, and the supernatants (cytosol fraction) and pellets (membrane fraction) were collected. The membrane fraction was solubilized as for the preparation of whole-cell lysates and analyzed by Western blotting.

Western Blotting—Protein samples were separated by 12% (w/v) SDS–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millipore, Bedford, MA, USA). The membrane was blocked with 5% (w/v) dry skim milk, reacted first with primary IgG antibodies, and then with horse-radish peroxidase–conjugated anti-human IgG (Immunotech, Marseilles, France) or alkaline phosphatase-conjugated anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA, USA), and subjected to a chemiluminescence reaction with Renaissance substrates (NEN Life Science Products, Boston, MA, USA) or Immun-Star substrates (Bio-Rad Laboratories). The primary antibodies used in this study were IgG preparations purified from human serum samples and mouse anti-La monoclonal IgG (22).

Immunofluorescence and Flow Cytometry—For immunofluorescence analysis, cells were smeared on 3-amino-propyltriethoxysilane–coated glass slides and incubated with IgG from human serum samples or anti-La monoclonal antibody. Cells incubated with human IgG were treated with fluorescein isothiocyanate–labeled anti-human IgG (Immunotech), and those incubated with the anti-La antibody were successively treated with biotinylated anti-mouse IgG antibody (Zymed, San Francisco, CA, USA) and Alexa 488–labeled streptavidin (Molecular Probe, Eugene, OR, USA). The samples were then examined under a fluorescence-phase contrast microscope or a confocal laser-scanning microscope (LSM510; Carl Zeiss, Jena, Germany). To locate autoantigens and DNA simultaneously, cells subjected to immunofluorescence were further stained with Hoechst 33342, and examined by fluorescence-phase contrast microscope. For flow cytometry, cells were first incubated with IgG from human serum or anti-La antibody, successively treated with fluorescein isothiocyanate–labeled secondary antibodies, and analyzed with a flow cytometer (Epics-XL; Coulter, Hialeah, FL, USA).
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RESULTS

Detection of Candidate Autoantigens—We first examined sera collected from 11 individuals suffering from autoimmune diseases (8 patients) or amyotrophic lateral sclerosis (3 patients) (see “MATERIALS AND METHODS”) for the presence of antibodies that detected antigens localized in the membrane fractions of apoptotic cells. For this purpose, membrane-rich lysates prepared from either normal or doxorubicin-treated Jurkat cells were subjected to Western blotting with IgG fractions prepared from sera of 11 patients (shown as arbitrary numbers at the top of each panel). The positions of molecular mass markers are shown on the left. Signals that were detectable only with lysates of apoptotic cells are indicated by arrowheads. A: Whole-cell lysates (50 μg of protein) and membrane fractions (50 μg of protein) were analyzed as above with 3 antibodies (681, 111, and S-250). The positions and calculated molecular masses of the 3 signals detected in lysates of doxorubicin-treated cells with the 681 antibody are indicated.

Fig. 1. Detection of putative antigens corresponding to serum antibodies of autoimmune disease patients. A: Membrane fractions (50 μg of protein) of Jurkat cells that were treated (+) or not treated (−) with doxorubicin for 24 h were analyzed by Western blotting with IgG fractions prepared from sera of 11 patients (shown as arbitrary numbers at the top of each panel). The positions of molecular mass markers are shown on the left. Signals that were detectable only with lysates of apoptotic cells are indicated by arrowheads. B: Whole-cell lysates (50 μg of protein) and membrane fractions (50 μg of protein) were analyzed as above with 3 antibodies (681, 111, and S-250). The positions and calculated molecular masses of the 3 signals detected in lysates of doxorubicin-treated cells with the 681 antibody are indicated.

Fig. 2. Time course of changes of the 681 antigen. Whole-cell lysates (50 μg of protein) of Jurkat cells that had been treated (+) or not treated (−) with CH11 for the indicated lengths of time were analyzed by Western blotting with the 681 antibody. The positions of molecular mass markers are shown on the left, and the positions and molecular masses of the 3 signals detected in lysates of CH11-treated cells are indicated on the right.
were similarly observed when whole-cell lysates and membrane fractions were compared (Fig. 1B). These results indicate that these apoptosis-specific proteins are associated with membranes. We decided to perform more extensive analyses of the signals detected by the 681 antibody.

Apoptosis-Dependent Change of the 681 Antigen—We next examined whether or not the above-described phenomenon was restricted to a particular type of apoptosis inducer. To test this, Jurkat cells were incubated with CH11, an agonistic anti-Fas antibody, and whole-cell lysates of these cells were analyzed by Western blotting using the 681 antibody. The results clearly showed that both 43- and 28-kDa signals became detectable in the lysates of Jurkat cells upon treatment with CH11 (data not shown, see Fig. 2). We next determined the time course of the change in the 681 antigen during apoptosis. Whole-cell lysates of Jurkat cells that had been treated with CH11 for various lengths of time were analyzed by Western blotting (Fig. 2). An increase in the intensity of the 43- and 28-kDa signals became apparent as early as 2 h after the addition of the apoptosis inducer, at which time Jurkat cells were positive for phosphatidylserine externalization, chromatin condensation, and plasma membrane blebbing (data not shown), and the signal intensities continued to increase thereafter. The intensity of the 60-kDa signal, in contrast, decreased as the culture period was extended. These results indicate that the change in the structure of the 681 antigen does not depend on the apoptosis inducer and occurs at an early stage of the apoptosis pathway. In addition, the 43- and 28-kDa proteins may be degradation products of the 60-kDa protein.

Since 681 serum was derived from a myasthenia gravis patient, we asked whether the 681 antigen is a common antigen for this particular type of autoimmune disease. To do so, whole-cell lysates of normal and apoptotic Jurkat cells were analyzed by Western blotting with sera of 4 more patients with the same disease. All the tested sera gave the 60-kDa signal with normal cell lysates, although its intensity was weak relative to that obtained with 681 serum and many other signals were also detectable (data not shown). The presence of various signals made the appearance of the apoptosis-specific 43- and 28-kDa signals in apoptotic Jurkat cells ambiguous (data not shown). These results suggest that the 60-kDa protein is a common antigen in myasthenia gravis, but no conclusion was made as to the generality of its cleavage during apoptosis.

Relocalization of the 681 Antigen during Apoptosis—We next examined whether the subcellular localization of the 681 antigen changes during apoptosis. Normal Jur-
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kat cells that had been fixed and membrane permeabilized were subjected to immunofluorescence analysis with the 681 antibody and examined by fluorescence microscopy (Fig. 3A) or confocal laser scanning microscopy (Fig. 3B). The signal derived from the 681 antigen was localized within the nucleus, which was identified by staining cells with Hoechst 33342, a DNA-binding fluorescent dye (top panels in Fig. 3A). Treatment with CH11 caused a relocalization of the 681 antigen: signals were no longer detectable in the cell nucleus, and instead cell bodies, in particular the region near plasma membrane blebs, became positive in apoptotic Jurkat cells (bottom panels but one in Fig. 3A and middle panels in Fig. 3B). Not all membrane blebs in CH11-treated cells appeared to contain the 681 antigen, and this could reflect a differential distribution of the antigen in small and large membrane blebs as reported for other nuclear autoantigens (4, 19), although the physiological consequences of this phenomenon remain to be determined. Similar results were obtained with Jurkat cells undergoing doxorubicin-induced apoptosis (data not shown). To examine the role of membrane blebs in the redistribution of the 681 antigen upon the induction of apoptosis, Jurkat cells were induced to undergo apoptosis in the presence of the ROCK-I inhibitor Y-27632 (27), which inhibits plasma membrane blebbing. The presence of this drug completely abrogated membrane blebbing although the cells underwent apoptosis, as indicated by nuclear fragmentation (bottom panels in Fig. 3, A and B), as previously observed (26). In those cells, the 681 antigen did not move to a position near the plasma membrane, but did move away from the cell nucleus. Treatment with Y-27632 did not affect the localization of the 681 antigen in normal cells (second panels from the top in Fig. 3A). These results indicate that the 681 antigen is a nuclear protein whose localization moves to a place near plasma membrane blebs upon the induction of apoptosis. The accumulation of the antigen in the periplasmic region, but not its exit from the cell nucleus, seems to be dependent on the occurrence of membrane blebbing.

A similar change in subcellular localization during apoptosis has been reported for another nuclear autoantigen, La (17–22). To confirm this, we examined the localization of La in normal and apoptotic Jurkat cells using a monoclonal antibody specific for La (Fig. 3, C and D). The results were similar to those obtained with the 681 antibody: signals were restricted to the cell nucleus in normal cells, moved to a place near plasma membrane blebs upon the induction of apoptosis, and stayed in the middle of apoptotic cells in the presence of Y-27632. The 60-kDa 681 antigen is clearly distinct from known nuclear autoantigens La and Ro as assessed by Western blotting analyses using anti-La monoclonal antibody and human serum positive for Ro (Fig. 4). This indicates that two distinct nuclear proteins, the 681 antigen and La, change their intracellular distributions during apoptosis in a similar manner. To correlate signals detected by immunofluorescence with those detected by Western blotting, Jurkat cells either treated or not treated with CH11 were subjected to subcellular fractionation and analyzed by
Western blotting with the 681 antibody (Fig. 5). In line with the results of the immunofluorescence analyses, the 60-kDa protein was predominantly recovered in the nuclear fraction prepared from normal Jurkat cells. Significant portions of the 43- and 28-kDa proteins, which were specifically detected in lysates of CH11-treated cells, were present in the membrane fraction but not in the cytosol fraction. In contrast, the 60-kDa signal was restricted, at a reduced level, to the nuclear fraction prepared from apoptotic cells. These results indicate that signals detected by immunofluorescence outside the nuclei in apoptotic Jurkat cells reflect the 43- and 28-kDa proteins associated with membranes, and suggest that the apoptosis-dependent exit of the 681 antigen from the nucleus requires proteolytic cleavage.

Association of the 681 Autoantigen with Membrane Blebs on the Cytoplasmic Side—We next used flow cytometry to examine whether the 681 antigen is exposed on the cell surface during apoptosis. The relevancy of the flow cytometric detection of the 681 antigen was shown...
by the fact that normal Jurkat cells were positively stained only when the plasma membranes were permeabilized (Fig. 6A). Jurkat cells treated or not treated with CH11 for varying lengths of time were incubated with the 681 antibody and analyzed by flow cytometry. The antibody did not bind to apoptotic Jurkat cells with non-permeabilized plasma membranes while it efficiently bound to cells positive for staining with propidium iodide (Fig. 6B). Furthermore, the externalization of La during apoptosis was not obviously detected under the conditions adopted in this study (Fig. 6C). These results indicate that the 681 antigen changes its structure and subcellular localization during apoptosis, but is not effectively externalized and exposed on the cell surface. Results of subcellular fractionation experiments showed that the 43- and 28-kDa proteins were mostly associated with membranes in apoptotic cells (see Fig. 5). It is thus likely that the 681 antigen undergoes proteolytic cleavage, exits the nucleus, and becomes associated with membrane blebs on the cytoplasmic side after the induction of apoptosis.

**DISCUSSION**

We searched for molecules whose structure and subcellular localization change during apoptosis by analyzing sera obtained from 15 individuals including 12 patients with autoimmune diseases. One serum sample, obtained from a patient (anonymously designated 681) suffering from myasthenia gravis, bound to an interesting 60-kDa nuclear antigen that was distinct from the known nuclear autoantigens, La and Ro. Serum samples from 4 more patients with the same disease detected this antigen in Western blotting. An antibody recognizing the nuclear La antigen plays roles in the development and pathogenesis of systemic lupus erthematosis and Sjögren’s syndrome. It can thus be speculated that an antibody to the 60-kDa nuclear antigen is involved in the pathogenesis of myasthenia gravis, at least in some of its subtypes, in addition to acetylcholine receptor-specific autoantibodies (28).

Portions of the 60-kDa antigen appeared to be broken down into 43- and 28-kDa proteins during apoptosis, and, at the same time, the antigen translocated from the nucleus to the cytoplasm in apoptotic cells after the induction of apoptosis. It will be necessary to identify the 681 antigen and determine the molecular basis for its structural alteration during apoptosis in order to resolve the above issues.

Many autoantigens appear to accumulate in the vicinity of plasma membrane blebs (4, 16). We show in the present study that a putative nuclear autoantigen, designated 681, is localized near the plasma membrane in apoptotic cells and that membrane blebbing is required for this relocalization. The fact that membrane blebbing as well as the redistribution of the 681 antigen were inhibited by a ROCK-I inhibitor suggests that the redistribution of the 681 antigen during apoptosis is mediated by a signaling pathway involving this protein kinase. Cocca et al. recently suggested that plasma membrane blebbing causes autoantigens to become exposed on the surface of apoptotic cells (29). However, our results indicate that the 681 antigen remains on the cytoplasmic side of membrane blebs in apoptotic Jurkat cells. Regardless of cell surface exposure, it is important to determine the functional significance of the subcellular relocalization of nuclear autoantigens.

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