Multinucleated Giant Cells Undergoing Apoptosis in Experimental Autoimmune Myocarditis*

Keisuke SUZUKI1,2, Tohru IZUMI1, Toshihiko IWANAGA2, Tsuneo FUJITA2 and Akira SHIBATA1

First Department of Internal Medicine1 and Department of Anatomy2, Niigata University School of Medicine, Niigata, Japan

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Summary. This study used an experimentally induced myocarditis model in rats to demonstrate the formation and fate of multinucleated giant cells which are known to occur in different kinds of inflammatory lesions. Multinucleated giant cells were frequently recognized in the inflammatory foci, being intermingled with numerous ED1-positive inflammatory macrophages rich in phagosomes. The giant cells reacted with the ED1 antibody but not with ED2, and ultrastructurally resembled the inflammatory macrophages. Multinucleated giant cells possessing less than 5 nuclei in an ultrathin section were rich in phagosomes, whereas those with more nuclei contained numerous lipid droplets and only few phagosomes in their cytoplasm.

Light microscopic observation of hematoxylin-eosin stained sections revealed that some multinucleated giant cells displayed variously sized dark bodies which likely corresponded to condensed and fragmented nuclear chromatin. Such multinucleated giant cells were positively stained with the TUNEL method. Under the electron microscope, all nuclei in one multinucleated giant cell showed an eccentric mass of homogeneously condensed chromatin.

These observations suggest that multinucleated giant cells are formed by aggregation and also likely by fusion of inflammatory macrophages; gradually loosing the phagocytic activity characteristic of the latter cells, they then die by apoptosis.

It is well known that multinucleated giant cells occur in several inflammatory diseases (CHAMBERS, 1978). They appear in the heart in a certain type of myocarditis, namely giant cell myocarditis, which typically is a rapidly progressing fatal disease whose etiology is unknown (WILSON et al., 1984; WYNNE and BRAUNWALD, 1992). KODAMA et al. (1990) established an experimental model of autoimmune myocarditis by immunizing Lewis rats with human cardiac myosin. As in the case of human giant cell myocarditis, this model was characterized by the occurrence of multinucleated giant cells at the center of inflammatory foci, where extensive myocardial necrosis with marked cellular infiltration was recognized. According to KODAMA et al. (1991), the multinucleated giant cells were observed from Day 16 to Day 35 after immunization. On Day 42, when cell infiltration had almost disappeared, no giant cells were located. Although there has been general agreement in that multinucleated giant cells might be formed by the fusion of monocytes/macrophages (CHAMBERS, 1978; THEAKER et al., 1985; MCINNES and RENNICK, 1988), the precise mechanism of their formation has been unclear. Furthermore, no morphological reports are available dealing with the process of their degeneration and disappearance.

Recently, it has been reported that some infiltrating cells in inflammatory lesions die by programmed cell death or apoptosis (PENDER et al., 1992; MANGAN et al., 1993; SCHMIED et al., 1993). In contrast to necrosis, a pathological fate of cells, apoptosis indicates a physiological process of cell death which has been shown to be associated with DNA fragmentation (WYLLIE et al., 1980; DUKE et al., 1983). It is morphologically characterized by the condensation and fragmentation of nuclear chromatin and a positive reaction in the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method invented by GAVRIELI et al. (1992). This report therefore aims to investigate the life, from birth to death, of multinucleated giant cells, especially as to whether their death is apoptotic or

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necrotic in nature in the rat autoimmune myocarditis model.

MATERIALS AND METHODS

Animals

Male Lewis rats, weighing 190–200 g, were purchased from Charles River Japan Inc. (Atsugi, Japan).

Immunization and pathological examination

Myosin fraction was isolated from pig hearts according to the method by Murakami et al. (1976). The cardiac myosin fraction was injected into 7-week-old rats at a dosage of 5 mg/kg in an equal volume of complete Freund's adjuvant. The immunized rats were sacrificed under ether anesthesia on Days 17, 21, 28 and 35. The hearts were removed and several transverse sections were obtained; these were immersed in 10% formalin for 12 h and embedded in paraffin according to conventional procedures. Paraffin sections were cut at 5 µm in thickness and stained with hematoxylin-eosin.

Immunohistochemistry

The hearts were removed immediately after sacrifice, and were horizontally cut at the mid-ventricular level. Samples were embedded in OCT compound (Miles, Inc., Elkhart, USA) and rapidly frozen in liquid nitrogen. Cryostat sections were sequentially cut at 10 µm in thickness using a Coldtome CM-41 (Sakura, Tokyo, Japan). After fixation in acetone for 10 min, the sections were incubated with mouse monoclonal antibodies against rat-derived antigens ED1 (Serotec, UK) and ED2 (Serotec, UK) for the detection of inflammatory and resident macrophages, respectively, to then be processed according to the indirect enzyme-immunohistochemical method. The antigen-antibody reactions were visualized with dianaminobenzidine.

DNA nick end labeling of tissue sections (TUNEL)

Paraffin sections, 5 µm in thickness, were deparaffinized by a xylene-ethanol series, and washed in PBS. The tissue sections were stripped from proteins by incubation with 1 µg/ml proteinase K (Sigma, USA) for 10 min at room temperature, and the slides were washed in PBS. The sections were incubated at room temperature overnight with terminal deoxynucleotidyl transferase (Takara, Japan) 50 u/50 µl and biotin-16-2'-dUTP (Boehringer Mannheim GmbH, Germany) 5 nmol/50 µl in 0.5 ml TdT buffer (30 mM Trizm base, 140 mM sodium cacodylate, 1 mM cobalt chloride, 0.5 g bovine albumin/1000 ml distilled water). After washing with PBS, the sections were covered with streptavidine-peroxidase for 30 min, and the reactions were visualized with dianaminobenzidine. The method was according to Gavriel et al. (1992).

Electron microscopy

The immunized rats were perfused with a Locke solution via the cardiac apex, followed by the administration of 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The hearts were removed, chopped into 1 mm cubes, and immersed into the same fixative for 3 h. After washing in the buffer, the tissues were post-fixed in 1% OsO4 for 2 h. After dehydration through the ethanol-propylene oxide series, they were embedded in Araldite resin. Ultrathin sections were double-stained with uranyl acetate and lead citrate, to be examined in a Hitachi H-7000 electron microscope.

RESULTS

Pathological and immunohistological study

Microscopic observations showed multinucleated giant cells in the inflammatory nest at all stages from Days 17 to 35 after immunization. Multinucleated giant cells were frequently located at the peripheral area in the inflammatory foci (Fig. 1a). They were usually oval in shape, with lengths ranging from 30 to 40 µm. They possessed many nuclei, counting from 5 to 20 in a section, which were arranged in a rosette or horseshoe configuration. At the center of the inflammatory area was extensive myocardial necrosis with numerous infiltrating cells. Although these were composed of several types of inflammatory cells, the central region of every necrotic nest was occupied by ED1-positive cells (Fig. 1b). On the other hand, ED2-positive cells, which were numerous in normal rat hearts, were scarcely detected at the center of the necrosis (Fig. 1c). Immunohistochemical studies showed that the giant cells were immunostained with ED1, but were negative for ED2 antibody (Fig. 1b, c).

Cells containing 10–20 nuclei of concentrated chromatin occasionally appeared in the inflammatory foci (Fig. 2a). All the nuclei were affected, leaving no intact ones in the cytoplasm. Their cytoplasm was roughly oval in shape and smaller than the multinucleated giant cells which possessed morphologically normal nuclei, because of an apparent condensation of cytoplasm. They were usually located near the multinucleated giant cells which apparently were lively (Fig. 2b). Some mononuclear cells also revealed a mass of condensed chromatin, being intermingled with infiltrating cells in the inflammatory area.
The TUNEL method demonstrated positively stained cells scattered in the inflammatory lesions. Most of these were mononuclear inflammatory cells, but some of the positive cells possessed 5 to 15 nuclei (Fig. 3a). The positively stained multinucleated giant cells in the TUNEL method varied in size, with one of them showing a similar cell size as the live-looking multinucleated giant cells. Although all the nuclei in the cell were positively stained, some of them were faintly positive in the TUNEL method. The horseshoe-shaped arrangement of nuclei, which was usually characterized by live multinucleated giant cells, disappeared (Fig. 3a). On the other hand, those cells possessing strongly positive-stained nuclei showed their cytoplasm to be condensed and appear smaller than that of the former cell (Fig. 3a, b). There were a number of macrophages around them, but no image could be obtained indicating that multinucleated giant cells were phagocytosed by those macrophages. The altered cells were frequently located near live multinucleated giant cells (Fig. 3b). The cells positive in the TUNEL reaction precisely corresponded in shape and distribution to the cells which contained homogeneously condensed nuclei in hematoxylin-eosin staining. Cardiocytes showed neither a positive reaction in the TUNEL method, nor condensed chromatin when...
Fig. 2  a. Day 21, hematoxylin-eosin staining. Six multinucleated giant cells are undergoing apoptosis, showing chromatin-concentrated nuclei (arrowheads). Note that their cytoplasm is also condensed. b. Day 35. An apoptotic multinucleated giant cell (arrowhead) located near a live-looking multinucleated giant cell (G). a, b: \times 1,000
Fig. 3. Day 35. a. Positively stained cells in the TUNEL method are scattered in an inflammatory area. Some are mononuclear cells (arrows), while others are multinucleated giant cells (asterisks). One of the latter cells possessing a large-sized cytoplasm (arrowheads) is weakly positive, and another cell with a small cytoplasm shows strongly-stained nuclei in the TUNEL method. b. A TUNEL-positive multinucleated cell (asterisk) is seen near a live multinucleated giant cell (G). All the nuclei contained in a shrunken cytoplasm are affected. a, b: ×1,000
Electron microscopy

Multinucleated giant cells were present at the inflammatory foci, surrounded by macrophages which possessed many phago-lysosomes (Fig. 4). The ultrastructural features of the former cells such as rich phagosomes, short processes on the cell surface, and irregular shaped nuclei closely resembled those of the latter. The giant cells contained, as counted in a thin section, 3 to 15 nuclei. Multinucleated giant cells with smaller numbers of nuclei, i.e. no more than 5, possessed large-sized phagosomes with a few lipid droplets (Fig. 5). On the other hand, cells with more numerous, i.e., over 5 or 6 nuclei, contained many lipid droplets, well-developed Golgi apparatus and only few phagosomes (Fig. 6a). Their chromatin masses were arranged along the nuclear membrane, and some of their nuclei possessed a nucleolus at the center. Their nuclei were roughly oval in shape, whereas inflammatory macrophages and the multinucleated giant cells with smaller numbers of nuclei tended to possess irregular-shaped nuclei. The cytoplasm of the multinucleated giant cells was rounded, and there were many short processes like microvilli on the cell surface (Fig. 6a). Although the present report failed to find an image suggesting the cell fusion of inflammatory macrophages, it was often demonstrated on Day 17 that inflammatory macrophages were in close contact with a multinucleated giant cell, intertwining the processes of both cells (Fig. 6b).

Cells possessing over 10 nuclei in the cytoplasm occasionally exhibited all their nuclei as round in shape, with their chromatin condensed into a crescent, uniformly dense mass on one side of the nuclear membrane (Fig. 7). In their cytoplasm, cytoplasmic organelles were rather scanty but were morphologically intact, except for a slight expansion of rough
surfaced endoplasmic reticulum. Their cytoplasm was condensed, and the short processes on the cell surface had disappeared. These findings, especially the changes in the nuclear chromatin, were characteristic of apoptotic figures, while necrotic features of multinucleated giant cells could not be observed.

**DISCUSSION**

In the present autoimmune myocarditis, multinucleated giant cells frequently appeared in the clusters of infiltrating cells, which were mainly composed of ED1-positive inflammatory macrophages. On the other hand, ED2-positive cells, corresponding to resident macrophages, were only rarely seen at the center of the inflammatory regions. Multinucleated giant cells in autoimmune myocarditis have been established as immunoreactive with OX42 and ED1 antibodies, but not with anti-desmin antibody (KODAMA et al., 1991). On the basis of their ultrastructure and the positive activity of acid phosphatase, SAEKI et al. (1994) suggested that they might be derived from macrophages by cell fusion. The present study showed that the multinucleated giant cells were stained with ED1, but not with ED2 antibodies. They resembled the inflammatory macrophages in their ultrastructural features such as numerous lipid droplets, lysosomes and short processes on cell surface. Moreover, the latter cells were closely located around them. Although the present study failed to discover actual instances displaying fusion between both cells, intermediate forms between them could occasionally be found. These immunohistological and ultrastructural findings strongly suggest that the multinucleated giant cells are formed by the fusion of inflammatory macrophages and not of resident macrophages. Several studies support this view by showing that certain cytokines such as IL-4, IFN-γ induce cultured monocytes/macrophages to...
Fig. 6. Day 17. a. A multinucleated giant cell (G + asterisk) and a macrophage (M) closely associated at an inflammatory focus. Few phagosomes are seen in the former cell. They possess short processes like microvilli on the cell surface (arrows). Another multinucleated giant cell (G) is also present. Lp lipid droplets. $\times 2,000$. b. Closer view of a part of Fig. 6 a. The processes of the giant cells (G) and macrophage (M) are closely interwined. $\times 8,000$
form multinucleated giant cells in vitro (McInnes and Rennick, 1988; Takashima et al., 1993). Since various types of cytokines are released by infiltrating cells in viral myocarditis (Shioi et al., 1994), it is possible that the formation of multinucleated giant cells might have been influenced by certain cytokines in autoimmune myocarditis.

The roles of multinucleated giant cells in the inflammatory process remain unknown. The present study showed that the giant cells with less than 5 or 6 nuclei possessed large-sized phagosomes, whereas the cells possessing more nuclei tended to contain only few phagosomes in their cytoplasm. Thus, phagocytic activity seems to be lost during the maturation process of multinucleated giant cells, in disagreement with Schlesinger et al. (1984), who reported that the multinucleated giant cells were able to kill and ingest Candida albicans as efficiently as macrophages.

The present study demonstrated that the formation of the multinucleated giant cells was followed by their apoptotic cell death. Therefore, fusion of the inflammatory macrophages might represent the first step of their disappearance from the inflammatory lesion. However, it is possible that the giant cells play other unknown roles because of their possessing well-developed Golgi apparatuses as compared with the macrophages. The physiological significance of the giant cell formation remains to be elucidated.

Morphological studies, especially electron microscopic observations, have shown that apoptosis is characterized by the condensation and margination

Fig. 7. Day 17. An electron micrograph showing typical apoptotic changes in a multinucleated giant cell (G), which possesses at least 12 nuclei (asterisks). The chromatin of all nuclei in its cytoplasm has aggregated and forms semilunar, uniformly dense masses on one side of the nuclear membrane. The surface of the cell has lost the short processes (arrowheads), and the cytoplasm is shrunken. Cytoplasmic organelles are scanty, but remain well-preserved except for a slight expansion of rough-surfaced endoplasmic reticulum (arrow). ×2,000
of chromatin to form homogeneously dense masses that abut on the nuclear membrane (Wyllie et al., 1980; Kerr et al., 1987; Schmied et al., 1993). The TUNEL method, which is based on the direct and specific labeling of DNA breaks in nuclei in situ (Gavrieli et al., 1992), has been useful to detect apoptotically altered cell nuclei. The present study demonstrated that the multinucleated giant cells in the model of autoimmune myocarditis undergo apoptosis based on both the electron microscopic criteria and the TUNEL reaction. As far as we know, this report offers the first evidence by light and electron microscopy that the cells in question die by apoptosis after maturation.

Interestingly, all the nuclei contained in one multinucleated giant cell showed apoptotic features at the same time. This in situ finding supports the view that the signals which trigger the onset of apoptosis of multinucleated giant cells might exist extranuclearly and not intranuclearly. Apoptosis is associated with endogenous endonuclease activity (Wyllie, 1980), and it may possibly be induced by a variety of different signals which may be intrinsic as well as extrinsic to the cells (Kerr et al., 1987). Although the exact nature of the signal transmission causing apoptosis from the cell surface to the nucleus remains unclear, several reports in vitro are available which suggest that apoptosis is profoundly influenced by the extracellular microenvironment such as the occurrence of glucocorticoid, cytokines, NO synthase, and silica (Wyllie, 1980; Mangan et al., 1992; Sarith et al., 1993 a, b).

As has been demonstrated to occur in certain apoptotic cells in inflammatory regions (Savill et al., 1989), it seems reasonable to propose that they might be removed by phagocytic macrophages. The present study, however, could not obtain information concerning the process of disappearance of the multinucleated giant cells after apoptosis, leaving this point for future investigations.

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REFERENCES


Dr. Keisuke SUZUKI
First Department of Internal Medicine
Niigata University School of Medicine
Asahimachi-dori 1, Niigata
951 Japan

鈴木 昭介
951 新潟市旭町通1
新潟大学医学部
第一内科学教室