Observation of Neuronal Death *In Vitro* by SEM and Optical Microscopy*

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(Received 9 January 2014; Accepted 25 February 2014; Published 19 April 2014)

We observed neurons cultivated on an indium tin oxide (ITO) substrate using scanning electron microscopy (SEM) whose high spatial resolution allows us to observe neuronal morphological details. First, we optimized the fixation condition of cultivated neurons for SEM observation. The first fixation with paraformaldehyde and glutaraldehyde, and the second fixation with OsO₄ were both necessary to avoid cell removal during the preparation before SEM observation. After optimization, we examined the morphological changes of neurons under an apoptotic condition, induced by staurosporine, by using both SEM and an immunochemical technique. Interestingly, the addition of staurosporine induced both apoptosis and a necrotic phenomenon. Immunostaining analysis revealed late-stage apoptosis after early-stage apoptosis, which was observed induced in cortical neurons by staurosporine for the first time. We confirmed that the SEM imaging of neurons is very useful as regards observing the apoptotic process. It is also a promising tool for understanding such neuronal activities as synaptic formation and axonal growth. Further examination of the interaction between neurons and substrates will contribute to the implementation of the artificial neurological devices. [DOI: 10.1380/ejssnt.2014.179]

Keywords: Scanning electron microscopy (SEM); Bioimaging and engineering; Cortical neurons; Apoptosis

I. INTRODUCTION

The last century saw a huge number of studies related to the patterning of neurons *in vitro* [1] and in recent decades the focus has been on controlling neuronal axonal growth, mostly for fabricating artificial neuronal circuits with a view to understanding the signaling mechanisms of neurons, neuronal circuits and the brain [2, 3]. A more recent target has been the examination of synaptic formation in *in vivo* neurons [4] and in neuronal circuits *in vitro* [5]. To control synaptic formation, it is essential to grow neurons on a substrate in a controlled manner and thus form synapses at a certain position in an artificial device when neurons are still in an active and healthy condition.

Scanning electron microscopy (SEM) provides very high spatial resolution, and so would be an effective tool with which to observe the morphology of the neurons [6], whose cell bodies are about 10 μm in size. SEM is also useful for evaluating neuronal growth on a substrate by observing the interface between the neurons and the substrate [2, 7]. Synapse scale, namely tens of nanometer scale, observation can also be developed to assist the realization of an artificial synapse. Many SEM observation studies have incorporated the freeze-fracture method [8] or the embedding of samples into resins and sections [9]. Both methods allow us to observe functioning synapses in detail, especially the internal structure of the synapses with a spatial resolution as high as a nanometer. Compared with these techniques, conventional SEM can obtain surface morphological images that are closer to the actual image (*in situ*) because it needs relatively less sample treatment, however the spatial resolution is lower.

In this study, as a first examination, we used SEM to observe the morphology of neurons grown on indium tin oxide (ITO) coated glass. ITO is a transparent and highly conductive material, which makes it suitable for both optical examination using an inverted microscope and the SEM observation of the interface between neurons and a substrate for further examination of the neuronal growth. We examined the optimized the sample preparation conditions for SEM imaging. Then, as a model of the cell condition, we induced apoptosis (one type of cell death) in the cells by employing staurosporine, a commonly used PKC inhibitor [10–12], and observed the neuronal morphological changes including blebbing, cell shrinkage, and nuclear fragmentation using the optimum cell fixation condition.

This morphological observation will help us to establish technology for examining the morphology of neurons grown *in vitro* using SEM to understand neuronal growth and synapse formation.

II. EXPERIMENTAL

A. Neuron culture

Cells were prepared from Wister rat cortex (embryonic 18 days), and cultivated on ITO glass (ITO thickness: 10 nm) coated with 20 $\mu\text{g}/\text{ml}$ laminin and 100 $\mu\text{g}/\text{ml}$ poly-D-lysine for 7–14 days in a neurobasal medium with L-glutamine, glutamate, gentamycin, and 2% B27 supplement, at 37°C in 5% CO₂ with saturated humidity.

B. Sample preparation and SEM observation

After cultivation, the cells were fixed in two steps; the first was fixation with paraformaldehyde (PFA, Sigma Aldrich) and glutaraldehyde (GA, Sigma Aldrich), and the second fixation was with OsO₄ (Sigma Aldrich), all prepared with HEPES buffer (pH 7.4). PFA and GA form crosslinks mainly with the lysine residue in proteins in cytoplasm, and OsO₄ forms crosslinks mainly with lipids

*This paper was presented at the 12th International Conference on Atomically Controlled Surfaces, Interfaces and Nanostructures (ACSIN-12) in conjunction with the 21st International Colloquium on Scanning Probe Microscopy (ICSPM21), Tsukuba International Congress Center, Tsukuba, Japan, November 4–8, 2013.

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FIG. 1: Identification of cultivated cells by immunostaining. Cells were cultivated on a glass-bottom dish. The image was a merged image with a phase contrast image and an immunostained image of neurons stained with pan-neuronal marker (green).

by reacting with unsaturated carbon-carbon bonds via strong oxidation.

Next the cells were dehydrated using ethanol (Kanto Chemical Co., Inc.) with a gradient of 50% to 99.5% and then dried in a freeze dryer (Tokyo Rikakikai Co. Ltd.) in *t*-butanol (Sigma Aldrich) at -50°C for two hours. The samples were coated with a 5 nm thick layer of Au. SEM (AURIGA60, Carl Zeiss or S-4300SE, Hitachi High-Technologies Corporation) was used for morphological observation. The samples were observed at angles of either 90° (top view) or 54° . The selected acceleration voltages were either 5 or 10 kV to avoid charging effects.

C. Immunostaining

The cells in the samples were identified using immunostaining. The samples were fixed with 4% PFA in PBS for 30 min, and blocked with a mixture of 5% NBA and 1% BSA in PBS at 4°C . The neurons were stained with Pan Neuronal Marker (Millipore).

D. Apoptosis

Apoptosis was induced by staurosporine (Sigma Aldrich), which is a nonspecific apoptosis inducer. Annexin V-FITC (Beckman Coulter) or anti-caspase-3 (Casp) antibody (Cell Signaling Technology) was adopted to detect apoptosis in cultivated cells. Cell death was detected by using propidium iodide (PI, Sigma Aldrich), which is a DNA-intercalating reagent. A confocal laser microscope (LSM 510, Carl Zeiss) was used to examine both immunostaining and apoptosis.

FIG. 2: Determination of fixation condition of cultivated cells. All the samples were freeze-dried in *t*-butanol, and coated with Au before the SEM observation. (A) SEM images of cells fixed only in GA without a secondary fixation with OsO₄. Under this condition, the number of neurons was very small and only small spheres were observed. (B) SEM image of cells fixed in GA and OsO₄. A few neurons and neurites were observed. (C) SEM image of cells fixed in GA, PFA and OsO₄. Neurons and neurites were successfully observed. (D) Relative neuron density observed with SEM under different fixation conditions. By increasing the OsO₄ concentration, the neuron density was increased (blue bar). By increasing PFA from 2 to 4%, the neuron density was also increased in the same OsO₄ concentration. Error bars show standard errors.

III. RESULTS

First, we undertook an investigation using immunostaining to determine the existence of neurons and their size in the cultivated samples. Figure 1 shows that neurons were observed, and the cell body size was about 10 μm .

Then we examined the optimum fixation condition as regards cell preparation for SEM observation. Figure 2(A) shows typical SEM images of cells cultivated for 10 days on ITO, fixed with primary fixation using GA (3%, 30 min) and without secondary fixation using OsO₄. Only small 2-5 μm spheres were seen and no neuronal feature was observed. Figure 2(B) shows the result of fixation with GA and OsO₄ (1%, 30 min). Some neurons (indicated with a white arrow) and neurites were observed with SEM, however, not as many as expected from Fig. 1. Figure 2(C) shows the result of fixation with GA, PFA (4%) and OsO₄. In this condition, the neurons (indicated with white arrows) with neurites grown on their surface were observed by being fixed when PFA, GA, and OsO₄ were all present. These results suggest that GA alone is insufficient to fix samples during the freeze-drying process, and better fixation results were obtained using both GA and OsO₄, which could crosslink and stabilize cells and organelle membrane lipids [13]. The best fixation was obtained when PFA, GA and OsO₄ were all used for fixation.

FIG. 3: Consecutive images to examine the apoptosis of neurons by staurosporine. Apoptotic cells were stained with PS by annexin V (green), and the DNA of dead cells or necrotic cells were stained with PI (red). Staurosporine was added at time zero (100 nM final). These images indicate that neuronal apoptosis mainly started at 0.5 or 1 hour (white arrows), and uneven cell surfaces were observed (yellow dotted circles). At 3 hours, blebs were observed by PS and a phase contrast image (yellow arrows). White bar: 50 μm .

FIG. 4: Morphology of cultivated cells obtained from rat brain observed using SEM. Apoptosis was induced by the addition of staurosporine (100 nM final) and the morphology changed with the time from the addition. (A) and (B) show two typical different types of change in the cell as large as 10 μm (white bar: 5 μm). Figure 4(C) shows closer images of (B) for cell surface details (dotted bar: 1 μm).

However, an increase in OsO_4 concentration can destroy the superstructure of proteins and the actin filament [14]. It can also damage the protein structures in antibody recognition sites, which can prevent further immunostaining for identifying the proteins in the cells [13]. Therefore, we examined the condition for achieving a higher cell density at a lower OsO_4 concentration. Figure 2(D) shows the relative neuronal density under various fixation conditions using PFA, GA and OsO_4 obtained from five SEM images for each condition. The cell densities were standardized with the density at fixation with GA 3%, PFA 4% and OsO_4 0.2%. As the OsO_4 concentration increased, the relative cell density increased steadily. There was also a significant increase in the density when the PFA concentration was doubled. This suggests that both PFA and OsO_4 were necessary to avoid cell removal during the freeze-drying process.

After finalizing the optimized parameter for SEM imag-

ing, we investigated the neuronal surface morphology when apoptosis was induced. Staurosporine was added to the cultivation medium of the neuron after the 12 day *in vitro* (DIV) with a final concentration of 100 nM, and incubation was continued at 37°C for 0.5, 1 and 6 hours. Then samples were rinsed with HEPES buffer and subjected to a first fixation with GA 2.5% and PFA 4%, and a second fixation with OsO_4 0.3%.

Figure 3 shows fluorescent images of an early-stage apoptosis marker, phosphatidyl serine (PS) with annexin V-FITC (green), and a cell death marker, PI (red), that we used to examine the details of cell death. PS exists in a plasma membrane as one of phospholipids mainly on the intracellular side, however, apoptosis induces the translocation of PS from the inner to the outer leaflet of the plasma membrane, where annexin V binds to PS. PI binds to DNA where the permeability of the cell membrane is increased by swelling or other factors. In the con-

FIG. 5: Change in the density of Casp (+) PI (-) cells and Casp (+) PI (+) cells induced by staurosporine addition. Immunostaining images are also shown both for 3 and 6 hours stimulation. The numbers of cells or cell-like-particles were all divided by the number of neurons observed from phase contrast images for standardization. As the staurosporine exposure time increased, the density of the Casp (+) PI (-) cells increased at 0.5 and 1 hour, and subsequently returned closer to the zero level (green bars). The density of the Casp (+) PI (+) cells also increased at 0.5 and 1 hour, and further increased through 6 hours (red bars). The green bar indicates the increase in apoptotic cells induced by staurosporine. The red bar increased after 3 hours suggesting late-stage apoptosis. A further increase in the cell number from 3 to 6 hours suggests nuclear fragmentation caused by late-stage apoptosis, which could be confirmed in the immunostaining images. Error bars show standard errors, but only either their positive or negative sides to make the difference clearer. White bar: 50 μm .

trol, which contained no staurosporine, the neurons did not show any annexin V signals indicating the absence of apoptosis. However, the small particles were stained with both annexin V and PI. At 0.5 and 1 hour, the fluorescent signal of annexin V was observed in the neuronal surfaces as shown by the white arrows. Also, an uneven cell surface, probably caused by small blebs, began to be observed in the regions marked with yellow dotted circles. After 3 hours, the formation of blebs was observed with both fluorescent signals from annexin V and the phase contrast image shown by the yellow arrows. The time-lapsed images also revealed that shortly after the blebbing, newly formed blebs did not show PI signals.

Figure 4 shows typical SEM images of the morphological changes in the neurons, 12 DIV, induced by staurosporine addition. The solid white bars indicate 5 μm , and the dotted white bars indicate 1 μm . Before the staurosporine stimulation, the control, neurons were observed with several neurites including axons and dendrites, and many fine filaments on the substrate surface. Then after 0.5 hour or more, the neurons exhibited two different types of morphological changes. The first change, type (A) in Fig. 4, was induced by changing the cytoplasmic membrane, where small spheres appeared to be removed from the cell body (indicated by red triangles). After 6 hours, the neurites degenerated and the cell body became spherical. Several small spheres were also observed suggesting the possibility of removal from the cell body.

The second changes, type (B) in Fig. 4, involved swelling and an increase in small concavities or a roughening of the surface. Soma swelling was observed after 6 hours although there was no clear increase in the cell volume at 0.5 and 1 hour. The magnified cell surfaces shown in Fig. 4(C) indicate that small concavities began to be observed on the cellular surfaces at 0.5 or 1 hour (yellow triangles) through 6 hours.

We then examined the cell density quantitatively at different times after the staurosporine stimulation. Figure 5 shows the change in the ratio of cells or cell-like particles to the number of neurons with the time after staurosporine addition. Data were obtained from the immunostaining images using anti-caspase-3 antibody (Casp) and PI at each incubation time with staurosporine. The numbers of cells or cell-like particles larger than 2 μm in diameter were counted to quantify those that were Casp (+) and PI (-) (green line) and those that were Casp (+) and PI (+) (red line). All the numbers were divided by the numbers of neurons observed in the phase contrast images for standardization. At time zero, namely before staurosporine addition, the cells smaller than 2 μm were all PI (+) cells and all Casp (-), hence, they were not counted to exclude the dead cells before the stimulation. From the result, the number of Casp (+) PI (-) cells increased at 0.5 hour, and 1 hour, and approached the zero level after 3 hours. The number of Casp (+) PI (+) cells increased to a level almost the same as that of the Casp (+) PI (-) cells at 0.5 and 1 hour. Then, their numbers increased steadily from 3 through 6 hours. Figure 5 also shows the immunostaining results for caspase-3 (green) and PI (red) after 3 and 6 hours stimulation. Compared with the image obtained at 3 hours, the PI (+) cells were smaller and their numbers were larger in the image obtained at 6 hours.

IV. DISCUSSION

A. Apoptosis and necrosis

In the above results we observed both apoptosis and necrosis, namely programmed and unprogrammed cell death, respectively. Apoptosis involves blebbing, chromosomal DNA fragmentation, nuclear fragmentation and soma shrinkage [15], and can generally be observed during an organism's life cycle as, for example, differentiation in the developmental stage. After apoptosis has occurred, phagocytic cells can remove them quickly before the contents of the cell are able to spill out onto surrounding cells and cause damage. Staurosporine was also observed to induce apoptosis including blebbing, size reduction, and a rounded shape in cardiocytes [11].

Necrosis has many possible causes including injury, infection, cancer, infarction, toxins and inflammation and involves the swelling of an entire cell and its constituent organelles. In contrast to apoptosis, the cleanup of cell debris by phagocytes in the immune system is generally more difficult. The cell swelling causes permeabilization of the cell membrane (lysis), therefore, necrosis is easily detected *in vitro* by exposure to a DNA binding dye such as PI. Viable cells and cells in the early and middle stages of apoptosis exclude the DNA binding dye, suggesting

that PI can detect necrotic cells or late-stage apoptotic cells.

The observation of PS translocation by annexin V assay in Fig. 3 reveals early-stage apoptosis, and PI staining shows late-stage apoptosis or necrosis. Small particles stained with both annexin V and PI that were observed even before staurosporine addition probably indicate the presence of apoptosis before the stimulation that occurs during the cultivation process. The progression of apoptosis is evident after 0.5 hour, because the blebbing morphology can be observed in both phase contrast images and immunostaining images. However, at this stage, these blebs did not show PI signals, suggesting a lack of DNA molecules, or they are in the early stages of apoptosis. The blebbing-like morphology was also observed in SEM images obtained 0.5 hour after the staurosporine stimulation (data not shown).

B. Apoptotic body

The small spherical particles attached to the cell surface or removed from the cell bodies observed in Fig. 4(A) were probably apoptotic bodies. Apoptotic bodies are sealed membrane vesicles that are produced by cells undergoing cell death by apoptosis. They also send signals to immune cells allowing them to detect the apoptotic phenomenon. The formation of apoptotic bodies is a mechanism that prevents both the leakage of the potentially toxic cellular contents of dying cells and inflammation reactions as well as tissue destruction. These SEM images suggest that apoptotic bodies might be removed from apoptosis-induced neurons. Figure 3 might include apoptotic bodies as well as blebs.

C. Late-stage apoptosis

The increase in the Casp (+) PI (−) cells in Fig. 5 at 0.5 and 1 hour suggests that the apoptotic phenomenon has occurred in the staurosporine stimulated neurons. In contrast, the increase in the Casp (+) PI (+) cells after 3 hours suggests the induction of apoptotic bodies removed from the neurons or the existence of late-stage apoptosis. Late-stage apoptosis is a necrotic process after the apoptotic stage that has many of the morphological features of necrosis [16] as stated previously, but it also involves nuclear fragmentation and chromatin condensation. In many early-stage apoptotic cells, PS exposure to the cell surface can be a signal for cell removal by phagocytic cells. The cells that fail to be removed by phagocytosis move into a late-stage apoptosis state. The same phenomena were observed in both myoblast [12] and cortical neurons [17]. These morphological changes, namely

blebbing, varicosity formation, and neurite swelling, are similar to those observed as a result of glutamate stimulation in stem cell derived neurons [18]. Furthermore, the increase in the number of Casp (+) PI (+) cells from 3 to 6 hours suggests that the fragmentation of nuclei containing DNA molecules. This is not an indication of necrosis but an indication of late-stage apoptosis.

V. CONCLUSIONS

In this study, neurons and other cells from rat cortex cultivated on ITO substrates were successfully observed using SEM by optimizing the fixation condition for SEM observation. We examined the change in the surface morphology of neurons with and without staurosporine, which stimulates neurons to induce apoptosis. Blebbing-like morphological changes, the removal of apoptotic bodies and cytoplasmic swelling were observed. Furthermore, an immunostaining examination suggested the possibility of late-stage apoptosis induced by staurosporine, which was first observation in cortical neurons.

There are several issues regarding the SEM observation of biological samples because observation in a vacuum can alter the surface morphology of samples. (1) Observation has to be in a vacuum, which can modify the morphology, and (2) fixation precludes us from the simultaneous observation of the same neurons at different stages of apoptosis, which provides us with limited information on the precise progression of cell death. However, both of the above issues can be alleviated by employing specific approaches; (a) examination of sample treatment, (b) statistical analysis using data from a relatively large number of cells and (c) combination with other methods for single-live cell observation. Thus, this study demonstrated that the above three approaches could obtain morphological details of neurons, which is a merit of SEM observation.

As a next step we must obtain morphological details by examining the interface between neurons and substrates using SEM combining with the focused-ion beam (FIB) milling technology. In this way we will be able to obtain more detailed information about neuronal growth and axon guiding. We will also be able to observe the morphology of synapse formation using SEM. These techniques will be important for realizing biological devices with living neurons.

Acknowledgments

We thank Dr. Yoshiaki Kashimura for his assistance with the SEM observations. We also thank Ms. Yuriko Furukawa for undertaking the immunostaining and Ms. Aki Hirao for preparing the samples.

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