Lecture 9

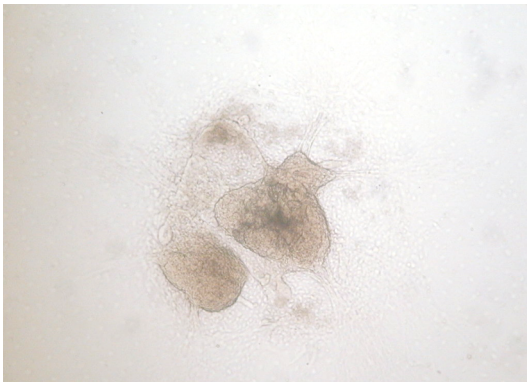
Application of Synchrotron Radiation in the Investigation of process of neuronal differentiation

9.1. Induction of neuronal differentiation

The method suggested by Sasay et al. was adopted to induce neuronal differentiation of mouse ES cells in this study [1]. In this method, ES cells are cocultured with the feeder layer of the mouse bone marrow-derived stromal cell line, PA6, not of the primary mouse embryonic fibroblasts [2, 1]. They call this activity for the generation of dopaminergic neurons 'stromal cell-derived inducing activity (SDIA).' After coculture with PA6 cells for 8 days, 92 % of ES cell colonies contain differentiated neurons in their experiments. In contrast to the protocol suggested by Mckay et al., this method does not require growth in serum or the selection of neural precursor cells.

The sample was prepared by fixing the mouse ES cells (129/Sv) with 20 % formalin after the coculture with the mouse PA6 feeder layers for 8 days on the Mylar films. PA6 cells were purchased from RIKEN Gene Bank, the Institute of Physical and Chemical Research. Figure 9.1 shows the optical microscopic photograph of the ES cell colony that had been cocultured with PA6 for 8 days. The dendrite-like tissue that had widely spread out of the colony can be seen and is the morphological feature of the neurons.

The sample referred as 16-3 in the previous lecture was also investigated with XANES technique as the undifferentiated control sample.



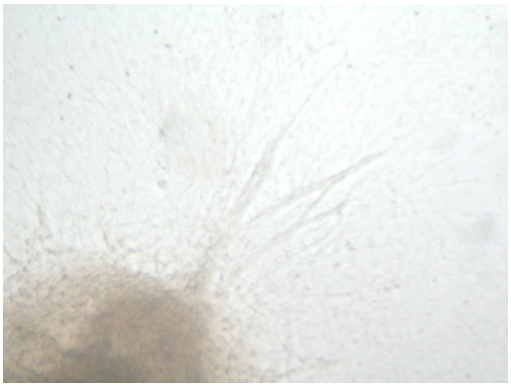


Figure 9.1. The optical microscopic photograph of the cultured mouse ES cells after coculture with the feeder layer of the bone marrow-derived stromal cell line, PA6. The dendrite-like tissue that had widely spread out of the colony can be seen and is the morphological feature of the neurons.

9.2. Experimental procedure and results

The SR-XRF elemental imaging and XANES analysis in this investigation were performed at the beam line 39XU of SPring-8. The incident x-ray energy was 8.7 keV in XRF analysis. The detailed set-up of the beam line is described in Lecture 2 and 3. The analyses were carried out in vacuum.

The elemental distribution images of the colony of the sample were obtained. The elemental images of (a) P, (b) S, (c) Cl, (d) Fe, (f) Zn and the corresponding microscopic photograph of the sample (a) are shown in figure 9.2. The scale on the right side of the images shows the count of the x-ray intensity. The measurement area was 280 × 320 μm^2 and the measurement time was 3 sec/point. The range of intensity was from 154 to 932 for P, 6 to 291 for S, 4 to 94 for Cl, 10 to 211 for Fe and 3 to 130 for Zn respectively. The beam size was 10 μm in the diameter.

From the results of the imaging, the points with the highest fluorescent intensity of Zn and Fe were selected for XANES analysis. The Zn and Fe K-edge XANES spectra obtained at the measurement points are shown in figure 9.3 (a) and (b) respectively. These spectra were measured in fluorescence mode. The Zn K-edge absorption spectrum was collected from 9.72 to 9.63 keV with an energy resolution of 0.5 eV and energy shifts of resolved absorption peaks of 0.25 eV was detected. The spectrum was collected with a total

signal averaging of 60 sec/point. The Fe K-edge absorption spectrum was collected from 7.16 to 7.10 keV with an energy resolution of 0.5 eV and the measurement time was 70 sec/point. Each spectrum represents the ratio I_f/I_0 (I_f = fluorescence counts, I_0 = photon incident flux measured by ionization chambers) as a function of photon energy. The XANES spectra from the colony in the control sample are shown in these figures 9.3a (dotted lines). The measurement time was 30 sec/point and 90 sec/point for the Zn and Fe K-edge absorption spectra respectively. The spectra obtained from reference samples (Zn, ZnO, FeO, Fe_2O_3 and Fe_3O_4) are also shown as thin color lines. The spectra of reference samples were collected in transmission mode and each spectrum represents the value of $-\exp(I/I_0)$ (I = transparence counts, I_0 = photon incident flux) as function of photon energy. The measurement times for these reference samples were 3 sec/point. All spectra are normalized with respect to both maximum and minimum intensities.

XRF spectrum of the sample cocultured with PA6 was also obtained at Photon Factory in beam line 4A and is shown as figure 9.4. The dotted line shows the spectrum obtained outside of the ES cell colony but at the PA6 feeder layer. The incident x-ray energy was 7.2 keV and the beam size was approximately $500 \times 500\ \mu m^2$. The analysis was carried out in vacuum. The spectra were collected with a total signal averaging of 200 seconds. Quantitative analysis was then applied to the spectrum obtained in ES cell colony and the calculated concentrations of S, P, Cl and Fe are shown in table 9.1. The volume of the colony was regarded as the ellipsoid (d_x, d_y = 100, d_z = 50 μm) and the density was considered as 1.0 g/cm^3.

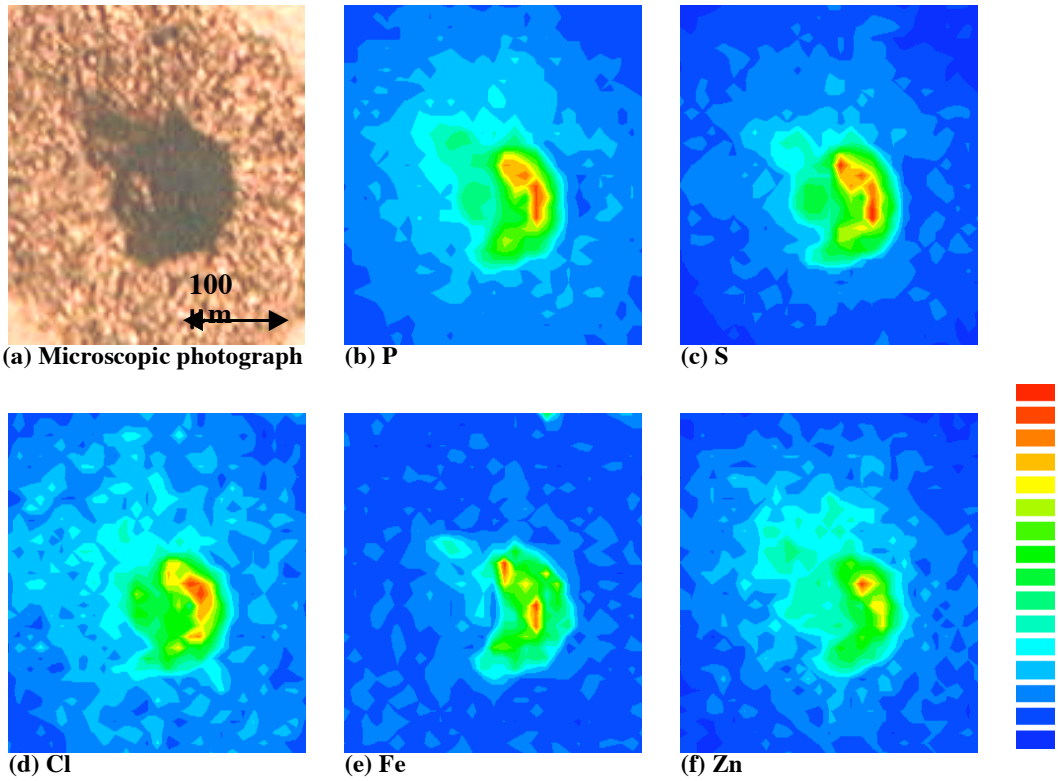


Figure 9.2. The elemental images of (b) P, (c) S, (d) Cl, (e) Fe and (f) Zn obtained in the mouse ES cell colony after coculture with PA6 for 8 days that is shown in (a) microscopic photograph. The colony contained intracellular P, S, Cl, Fe and Zn.

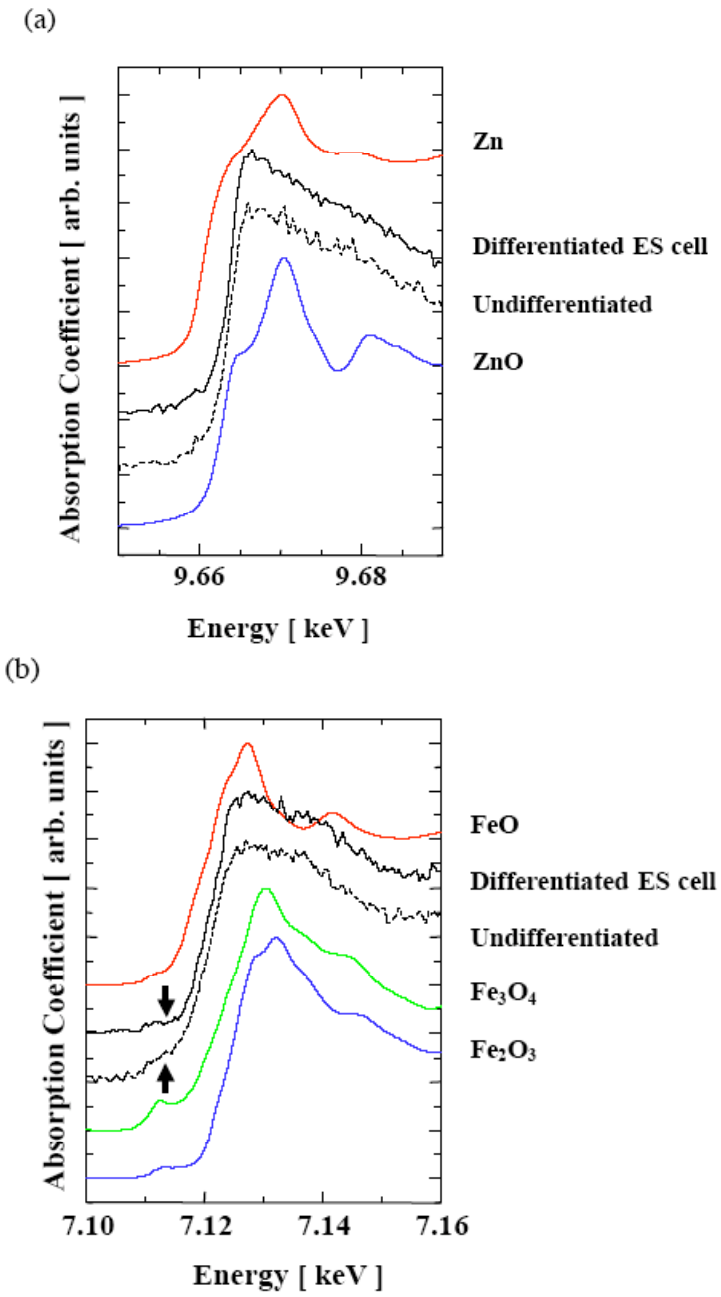


Figure 9.3. The (a) Zn and (b) Fe K-edge XANES spectra obtained at the mouse ES cell colony after coculture with PA6 for 8 days (solid line) and the colony in the undifferentiated control (dotted line). The spectra collected from reference samples (Zn, ZnO, FeO, Fe_2O_3 and Fe_3O_4) are also shown. No distinct difference can be seen between the solid and dotted spectra in (a), but at the rising edge region in (b), the dotted spectrum is

slightly higher than the solid spectrum. It can be seen that these colonies contained the elements such as P, S, Cl, Fe and Zn.

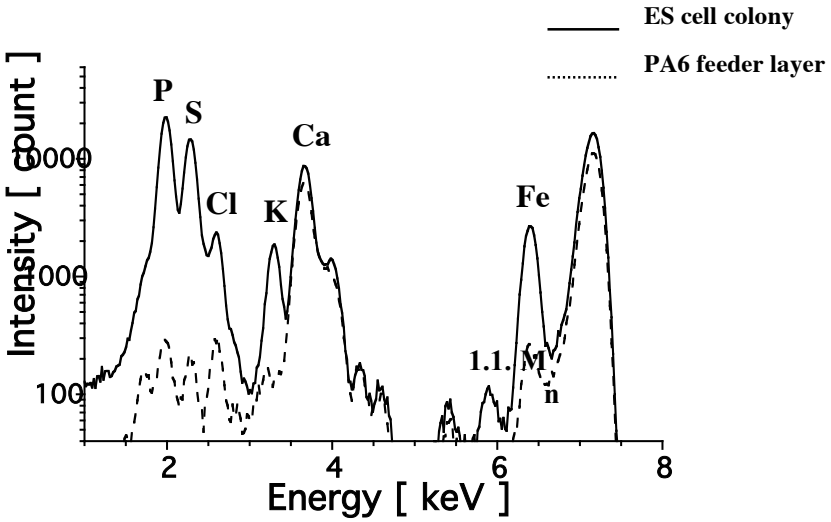


Figure 9.4. The XRF spectra obtained in the mouse ES cell colonies after the coculture with PA6 for 8 days (solid line) and at the PA6 feeder layer (dotted line). It can be seen that these colonies contained the elements such as P, S, Cl, Fe and Zn.

Table 9.1. The quantification results obtained by processing XRF spectrum of the mouse ES cell colony after the coculture with PA6 for 8 days. The concentrations of P, S, Cl, K, Mn and Fe were quantified and shown in ppm.

P	S	Cl	K	Mn	Fe
16041.0	6599.1	637.0	300.3	4.1	93.3

9.3. Discussion

Figure 9.2 shows the colony that had been cocultured with PA6 contained (b) P, (c) S, (d) Cl, (e) Fe and (f) Zn. The distribution of Fe and Zn has a minor difference from those of

other nonmetal elements while the distributions of P, S and Cl are identical. This variety of distributions suggests the heterogeneity of intracellular constituents and that the cells in this colony had differentiated into several different kinds of cells. Sasai et al. indicates the possibility of the production of less than 2 % glial or mesodermal lineages, and dopaminergic, cholinergic, and serotonergic neurons in their experiments [1]. It can be considered that the differences among neuronal functions are related to the concentrations of intracellular elements.

In Zn K-edge XANES spectra shown in figure 9.3 (a), no significant difference was seen between the spectrum obtained at the colony cocultured with PA6 cells (solid line) and that from the undifferentiated control (dotted line). This result indicates that the local structure such as inter-atomic distances and coordination number or Zn contents is similar in these samples. From the comparison of the spectra with those of reference samples, Zn was in the form of Zn^{2+} as ions and/or metalloproteins in these samples. In Fe K-edge XANES spectra shown in figure 9.3 (b), however, the spectrum obtained in the colony of undifferentiated control (dotted line) shows higher values than that from the colony cocultured with PA6 cells (solid line) at the rising edge region (7.110 – 7.115 keV). The capacity of readily exchanging electrons makes iron essential for fundamental cell functions and metalloproteins containing iron play an important role in controlling protein synthesis and cell differentiation [3, 4]. The difference in iron stereochemistry is considered to represent the difference in the functions of the proteins bound to iron. It is also possible that the free iron was contained in these ES cell colonies in different states. In both cases, the comparison of the absorption edge with reference samples shows that iron is contained as Fe^{2+} in these samples.

XRF spectrum in figure 9.4 indicates that the differentiated ES cell colony (solid line) contained P, S, Cl and Fe as well as the undifferentiated colony that was investigated in the previous experiments (figure 8.5 (a) and (b) in lecture 8). After the coculture with PA6 cells, however, the fluorescent x-ray peaks from K and Mn can be seen in the spectrum obviously. It is considered that these elements had been imported into the cell during the culture on the PA6 feeder layer. K and Mn are characteristic and essential constituents in neurons. Mn is

essential for protein synthesis, normal development and activity of the nervous tissue and prevents apoptosis induced by Fe^{2+}, amyloid beta-peptide and nitric oxide-generating agents as the active center of Mn superoxide dismutase (MnSOD) [5, 6]. K ions are widely utilized to control electric activities of neurons and are deeply related to the regulation of action potential, membrane potential and cell volume in every kind of cells. It was revealed that the acquisition of these elements had been occurred in accordance with the neuronal differentiation of ES cells.

9.4. Conclusion

In this study, the role of intracellular trace elements in the differentiation of mouse ES cells was investigated by analyzing the distribution, concentration and chemical states in the process of differentiation using micro SR-XRF and XANES analysis. The elemental information at the cell level is important to elucidate the changes in ES cells during the differentiation and reveal the optimal elemental conditions for neuronal differentiation.

Present study demonstrated that the intracellular elemental conditions such as distribution, concentration and chemical states reflect the specific biological functions. The distinct differences could be observed between the constituents of mouse ES cells with and without the induction of neuronal differentiation.

It was revealed that elements such as Cl, K, Mn, Fe and Zn behave in a unique manner and are considered to play an important role during the differentiation. This finding indicates the possibility that the differentiation of ES cells into the specific cell type can be regulated by stimulating cells with the reagents that affect the concentrations and chemical states of these intracellular elements. It is well recognized that deficiencies or excesses of essential trace elements during early development give mouse ES cells the significant effects such as structural abnormalities or embryonic death through the direct metal binding to critical membrane sites or intracellular ligands including protein and nucleic acids [7]. It is considered that modified quantity of adequate reagents has the positive and selective effect on ES cells.

The present study also confirmed that XRF spectrometry is the powerful method to investigate the multiple intracellular trace elements efficiently at the high sensitivity and resolution. XRF and XANES analysis can be applied in combination with other techniques such as staining or patch-clamp recording. These x-ray analysis methods should be utilized in the further investigation that is needed to explain the mechanism of the differentiation of embryonic stem cell.

References

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