J. Synchrotron Rad. (1998). **5**, 1105–1107

Studies on intracellular structures of COS cells by X-ray microscopy

Akitsugu Yamamoto,[a]* Ryuichi Masaki,[a] Peter Guttmann,[b,c] Günter Schmahl[c] and Hiroshi Kihara[d]

[a]Department of Physiology, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan, [b]Forschungseinrichtung Röntgenphysik, Georg-August-Universität Göttingen, Göttingen, Germany, [c]BESSY, Berlin, Germany, and [d]Physics Laboratory, Kansai Medical University, Hirakata 573-1136, Japan. E-mail: yamamota@takii.kmu.ac.jp

(*Received 4 August 1997; accepted 7 November 1997*)

COS-7 cells, fixed with glutaraldehyde, were studied using the transmission X-ray microscope at the electron storage ring BESSY, Berlin. The border of the cell, the nucleus, nucleoli and mitochondria of the cells were clearly visualized with the X-ray microscope. In addition, we found many X-ray dense granules preferentially located around the nucleus. Electron microscopy showed that numerous multivesicular bodies, whose structures belong to the endosome–lysosomal system, were present around the nucleus. The size and localization patterns of the X-ray dense granules were quite similar to those of multivesicular bodies. These results strongly suggest that the X-ray dense granules are multivesicular bodies.

Keywords: X-ray microscopy; X-ray dense granules; endosomes; COS cells; lysosomes; multivesicular body.

1. Introduction

X-ray microscopy provides the opportunity to image internal fine structures of cells with a higher resolution than with light microscopy under physiological conditions. As biological specimens are mostly composed of carbohydrates, proteins and lipids, they show naturally high contrasts when using X-rays in the 'water window' wavelength region (Wolter, 1952). X-ray microscopes using zone plates as imaging elements now have the highest resolving power, of less than 40 nm (Schmahl *et al.*, 1995).

Although X-ray images show contrast without staining, special labelling is often useful for X-ray microscopy, as with electron microscopy. Previously we reported that COS-1 cells fixed and stained by potassium permanganate gave a good contrast in X-ray microscopy, and that internal membrane systems, such as the endoplasmic reticulum, mitochondria, the nucleus and the border of the cell, are well visualized (Kihara *et al.*, 1996). We also showed that the X-ray microscopic images of dried COS cells significantly differed from those taken in wet conditions and were more similar to those taken by electron microscopy (Kihara *et al.*, 1996).

In the present work, COS-7 cells fixed with glutaraldehyde were observed in wet conditions with the transmission X-ray microscope at the electron storage ring BESSY, Berlin (Schmahl *et al.*, 1995) without any staining. We found many X-ray dense granules in the cytoplasm of COS-7 cells and showed that they are multivesicular bodies (MVBs) which cause the degradation of endogenous and exogenous materials as structures belonging to the endosome–lysosomal system.

2. Materials and methods

2.1. *Cell culture and fixation*

COS-7 cells, cell lines derived from the kidney of an African green monkey, were cultured on support foils (Niemann *et al.*, 1994) in Dulbecco's modified Eagle medium with 10% fetal bovine serum at 310 K in a 5% CO_2 incubator for 2 h. To ensure adhesion of the cells to the membrane of the foil, the foils were previously treated with 5 μg ml^{-1} poly-L-lysine solution. Cells were fixed with 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer at pH 7.4 for 1 h and washed three times with the same buffer. In some cases, the glutaraldehyde-fixed cells were post-fixed with 1% OsO_4 in the cacodylate buffer for 1 h and washed three times with the same buffer.

2.2. *X-ray microscopy*

The specimens prepared on the support foil were placed in small, hermetically closed envelopes filled with the buffer medium of the last preparation step and were sent by express mail to the transmission X-ray microscope at BESSY, Berlin. All investigations were performed within a week of the preparation of the specimens. Preservation of the fixed cells in the buffer for 1 week caused no changes in the ultrastructure under an electron microscope. The support foil with the specimen was placed into the environmental chamber, which was closed with a cover foil (Niemann *et al.*, 1994). The chamber was then transferred into the transmission X-ray microscope. The liquid layer thickness was then reduced to < 10 μm using a light microscope for control (Zeiss Axioskop with differential interference contrast mode), which is incorporated at the X-ray microscope station. This light microscope was also equipped with a CCD camera to obtain light

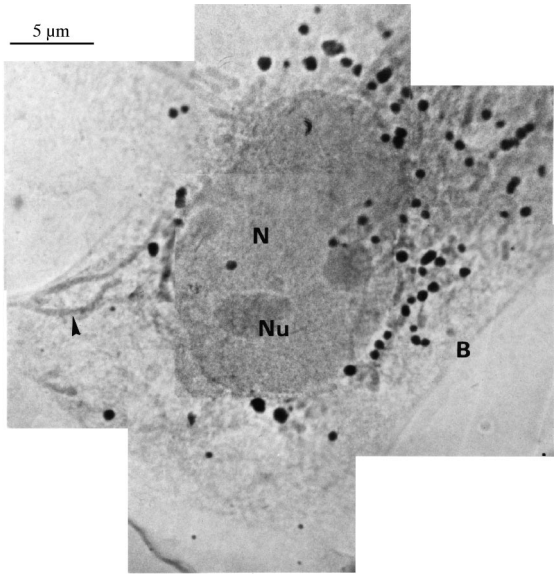
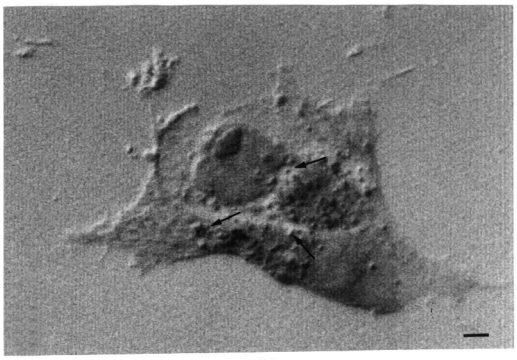


Figure 1
X-ray image of a wet fixed COS-7 cell. COS-7 cells were fixed with 2.5% glutaraldehyde, embedded in a thin layer of 0.1 *M* cacodylate buffer at pH 7.4 and observed with an X-ray microscope. The figure was obtained by combining several X-ray micrographs. Numerous X-ray dense granules are seen around the nucleus (N). An arrowhead shows a mitochondrion. Nu: nucleolus; B: border of the cell. Bar: 5 μm.

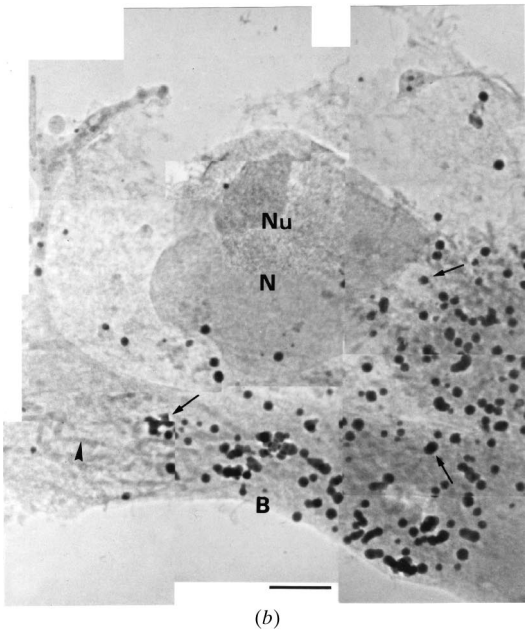
microscope images of the cell under investigation. The specimen was held under atmospheric pressure during the investigations. The micro zone plate used as the X-ray objective for imaging showed an outermost zone width of 30 nm. The X-ray images were taken at 2.4 nm wavelength using a thinned backside-illuminated CCD as a detector (Wilhein *et al.*, 1994).

2.3. *Electron microscopy*

Cells were cultured on collagen-coated plastic coverslips (cell tight C-1 cell disk, Sumitomo Bakelite Co. Ltd, Tokyo, Japan) and were fixed with 2.5% glutaraldehyde in 0.1 *M* cacodylate buffer at pH 7.4 for 1 h. The cells were washed with the cacodylate buffer and post-fixed with 1% OsO_4 in the same buffer for 1 h. The cells were then washed in distilled water, incubated with 50% ethanol for 10 min and block-stained with 2% uranyl acetate in 70% ethanol for 2 h. The cells were further dehydrated with a graded series of ethanol and embedded in epoxy resin. Ultrathin sections were doubly stained with uranyl acetate and lead citrate, and observed under a Hitachi H7000 electron microscope.



(*a*)



(*b*)

Figure 2
Light and X-ray microscopic images of a wet fixed COS-7 cell. COS-7 cells were fixed with 2.5% glutaraldehyde, embedded in a thin layer of 0.1 *M* cacodylate buffer at pH 7.4 and observed with an X-ray microscope. (*a*) Differential interference contrast microscopic image of a cell; (*b*) X-ray microscopic image of the cell. The figure was obtained by combining several X-ray micrographs. Localization of the X-ray dense granules (arrows) corresponds well to that of the granules under a light microscope. An arrowhead shows a mitochondrion. N: nucleus, Nu; nucleolus; B: border of the cell. Bar: 5 μm.

3. Results

3.1. *Studies of COS-7 cells by transmission X-ray microscopy*

Figs. 1 and 2(*b*) show X-ray images of wet COS-7 cells fixed with glutaraldehyde. Several X-ray images are placed together to obtain a larger field of view. In the figures, we can see a nucleus, one or two nucleoli, many rod-shape mitochondria and the border of the cell clearly. In addition, numerous X-ray dense granules are seen preferentially around the nucleus. These granules are spherical in shape and their average diameter was found to be 450 ± 120 nm (average ± standard deviation; 200 granules were counted). These X-ray dense structures were also observed in cells fixed with glutaraldehyde dissolved in Na phosphate buffer and in cells post-fixed with osmium (data not shown). The number of X-ray dense granules varied among cells and among experiments. As these structures show high X-ray density without any staining, the density of the structure itself is plausibly very high. In fact, these granules can be seen under a light microscope using differential interference contrast (DIC). Fig. 2(*a*) shows a DIC image of the same cell shown in Fig. 2(*b*). Localization of optically dense granules under a light microscope (arrows in Fig. 2*a*) corresponds well with that of X-ray dense granules under an X-ray microscope (arrows in Fig. 2*b*). Optically dense granules are also visible in living COS-7 cells cultured on a cover slip under a phase contrast microscope (data not shown). The presence of optically dense granules in living cells suggests that X-ray dense granules are not an artifact of the cell preparation and fixation.

3.2. *Observation of COS-7 cells by electron microscopy*

To identify the X-ray dense granules, we examined COS-7 cells by electron microscopy. Fig. 3 shows an electron micrograph of a COS-7 cell. Numerous MVBs exist; vacuoles containing internal vesicles of 40–50 nm in diameter are preferentially located around the nucleus. MVBs showed higher electron densities than other cellular components, such as the mitochondria and the nucleus. The electron densities of the MVBs were variable and some MVBs showed very high electron densities. It is generally thought that MVBs arise from endosomes, are converted to

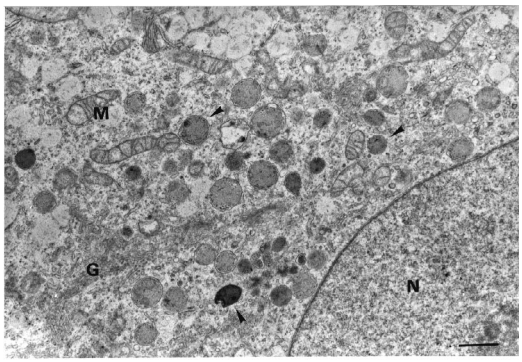


Figure 3
Electron micrograph of a COS-7 cell. COS-7 cells were fixed with glutaraldehyde and OsO_4 successively, and embedded in epon. Ultra-thin sections were cut and doubly stained with uranyl acetate and lead citrate. Numerous MVBs (arrowheads) containing internal vesicles of 40–50 nm diameter can be seen. N: nucleus; M: mitochondria; G: Golgi apparatus. Bar: 1 μm.

lysosomes by acquiring hydrolytic enzymes, and increase their electron density as they become mature lysosomes. The differences in the electron densities among the MVBs may result from the difference in the stages of the maturation process. The average diameter of the MVBs was found to be 480 ± 160 nm (average ± standard deviation; 100 MVBs were counted) and gave good agreement with that of the X-ray dense granules. The number of MVBs varied among cells and experiments, as in the case of the X-ray dense granules.

4. Discussion

In the present work, intracellular structures of COS-7 cells, such as the nucleus, nucleoli, mitochondria and the border of the cell, were well visualized under an X-ray microscope without any staining. To our surprise, very X-ray dense granules appeared around the nucleus.

Gilbert (1992) had noticed similar X-ray dense granules in cultured chick fibroblasts with the Stony Brook/NSLS scanning transmission X-ray microscope (Gilbert, 1992; Pine & Gilbert, 1992; Gilbert *et al.*, 1992). He reported that the granules showed a higher density of carbon and a lower density of oxygen than other parts of the cells, determined by elemental analysis using X-ray absorption edges, and suggested that the density of proteins in the granules was high (Gilbert, 1992). However, he did not mention what the granules are.

In the present report, we have suggested that these X-ray dense granules in COS-7 cells are MVBs by comparing with images achieved from electron microscopy. MVBs are structures belonging to the endosome–lysosomal system. They originate from endosomal structures and are thought to be converted to mature lysosomes by acquiring hydrolytic enzymes. MVBs play a role in the degradation of exogenous materials internalized by endocytosis, and also in phagocytosis and autophagy (Holtzman, 1989). Endosomes and lysosomes are ubiquitous organelles in eucaryotic cells. The different types and stages of these organelles appear according to the differentiation and functional states of the cells. In COS-7 cells, MVBs are predominant structures of the endosome–lysosomal system. Lysosomes including MVBs show, in general, very high density in electron microscopic images. In the case of electron microscopy, the electron density arises from metal adsorption to proteins and nucleic acids by uranium and lead staining. This means that the density of protein in the organelles is very high. Therefore, it is plausible that lysosomes and MVBs, in general, have high X-ray densities. The condensation mechanism is thought to be operated in lysosomes and MVBs, but the real nature and the role of condensation are still unclear (Holtzman, 1989). The number of X-ray dense granules and MVBs varied among the cells and experiments; the reason still remains unsolved.

References

Gilbert, J. R. (1992). PhD thesis, California Institute of Technology, California, USA.

Gilbert, J. R., Pine, J., Kirz, J., Jacobsen, C., Williams, S., Buckley, C. J. & Rarback, H. (1992). *X-ray Microscopy III* (Springer Series in Optical Sciences, Vol. 67), edited by A. Michette, G. Morrison & C. Buckley, pp. 388–391. Berlin/Heidelberg: Springer-Verlag.

Holtzman, E. (1989). *Lysosomes*. New York: Plenum Press.

Kihara, H., Yamamoto, A., Guttmann, P. & Schmahl, G. (1996). *J. Electron Spectrosc. Relat. Phenom.* **80**, 369–372.

Niemann, B., Schneider, G., Guttmann, P., Rudolph, D. & Schmahl, G. (1994). *X-ray Microscopy IV*, edited by V. V. Aristov & A. I. Erko, pp. 66–75. Chernogolovka, Moscow Region: Bogorodski Pechatnik.

Pine, J. & Gilbert, J. R. (1992). *X-ray Microscopy III* (Springer Series in Optical Sciences, Vol. 67), edited by A. Michette, G. Morrison & C. Buckley, pp. 384–387. Berlin/Heidelberg: Springer-Verlag.

Schmahl, G., Rudolph, D., Guttmann, P., Schneider, G., Thieme, J. & Niemann, B. (1995). *Rev. Sci. Instrum.* **66**, 1282–1286.

Wilhein, T., Rothweiler, D., Tusche, A., Scholze, F. & Meyer-Ilse, W. (1994). *X-ray Microscopy IV*, edited by V. V. Aristov & A. I. Erko, pp. 470–474. Chernogolovka, Moscow Region: Bogorodski Pechatnik.

Wolter, H. (1952). *Ann. Physik*, **10**, 94–114.