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Crucial factor causing collapse and aggregation of cultured cells in epon resin

By

Prasarn TANGKAWATTANA[1], Mamoru YAMAGUCHI[2], Wuthichai KLOMKLEAW[3], Hua NIU[2], Jun A. MINAGUCHI[4] and *Kazushige TAKEHANA[4]

[1]Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[2]Muscle Biology Research Laboratory, Department of Veterinary Biosciences, The Ohio State University, Columbus, OH 43210, USA
[3]Department of Veterinary Anatomy, Faculty of Veterinary Science, Chulalongkorn University, Bangkok 10330, Thailand
[4]Laboratory of Microanatomy, Department of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069-8501, Japan

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Summary: Ultrastructural artifacts regarding collapse and aggregation of cultured cells have been problematic, especially when investigated apoptotic cells. The infiltration process during sample preparation is considered to be the most crucial factor for this problem. This study was conducted using two culture systems: a suspension culture system of human T-lymphocyte Jurkat cells and rabbit mature dendritic cells and a monolayer culture system of human lung macrophages, human breast cancer cells (A-546 cells) and cat bone-invasive gingival cancer cells (sccf3 cells). Fixation was conducted prior to removing or detaching the cells from the culture dishes. Initial infiltration with a 1 : 3 volume ratio of epon resin : propylene oxide was found to be the most crucial step among these cultured cells. The improved epon-resin infiltration method could eliminate the artifacts. Thus, differentiation between artifactual images and true images is highly possible.

Introduction

Aggregation and shrinkage of cultured cells are often observed under an electron microscope. These artifacts are seldom formed in electron micrograph sample preparation with tissues. Thus, some researchers perform initial fixation after removal and detachment of cells from culture dishes. In a monolayer culture, when cells have been removed or detached from dishes before fixation, cell membranes are damaged and their original structure is altered. The cells usually become round and appear as if they are normal despite their partially broken plasma membranes. To obtain accurate images, fixation must be carried out before removal and/or detachment of cells from culture dishes.

Apoptotic cells often provide images similar to those of cells with artifacts induced by sample preparation. These images make it difficult for researchers to differentiate living cells from apoptotic cell. The use of epon resin is a standard method for sample preparation when working with tissues (1, 2) and cell cultures (3–6).

Here, we discuss the importance of infiltration of epon resin into cultured cells and we described an improved method to achieve successful infiltration. When epon resin had infiltrated into the cells, no shrinkage or aggregation of cells occurred during the processing of cultured cells. Our method allowed cells that had been growing to be differentiated from aggregated apoptotic cells.

Materials and Methods

We used a routine method for pre-fixation with GA (less than 1 hr, cold/room temp.) followed by post-fixation with 1% osmic acid (less than 1 hr, cold/room temp.) and dehydration with a series of ethanol concentrations (35, 50, 75, 90, and 100% at cold temp. and then two times at 100%) at 5-min intervals. Ethanol was replaced by propylene oxide at room temperature. This was repeated three times at 5-min intervals using fresh

Corresponding author: Kazushige Takehana, Laboratory of Microanatomy, Department of Veterinary Medicine, Rakuno Gakuen University, Ebetsu 069-8501, Japan. E-mail: takechan@rakuno.ac.jp

propylene oxide.

The most important procedure was infiltration with epon resin (after a catalyst has been added). The cell must contain a sufficient amount of epon resin at the final stage of infiltration. The use of low concentration of epon is important, especially during the initial stage of resin infiltration.

We have tried many infiltration methods, including methods using various concentrations of epon resin diluted with propylene oxide, varying amounts of the catalyst, and changes in the time of infiltration. A satisfactory result was obtained only by decreasing epon resin concentration so that the volume of epon resin was less than 1/3 that of the organic solvent (propylene oxide). When the concentration of epon resin exceeded 25–30%, even prolonging the incubation time did not resolve the infiltration problem.

We also used various formulas for the epon residue mixture with low-viscosity ingredients (7). Spurr's resin (8) must be diluted with an organic solvent to achieve satisfactory infiltration. In fact, the dilution of regular epon resin with organic solvent resolves most of the infiltration problems; thus, even for plants, there is no reason to use Spurr's resin.

The following is an example of the methods that we used. We soaked cells at a 1 (epon mixture) : 3 (propylene oxide) volume ratio during the initial infiltration step for at least 5 hours at 4°C in a refrigerator, then at a 1 : 2 ratio for 3 hours, and finally at a 1 : 1 ratio for 2 hours with use of a rotary disc plate at room temperature. In the final step of centrifugation, the sample pellet was soaked in 100% epon resin overnight in a refrigerator (4°C). Epon resin was replaced with a fresh mixture after the sample had been spun down with a microfuge.

When the sample was exposed to a low concentration of epon resin, good infiltration into the cells was confirmed. Thus, initial soaking of the sample with a low concentration of resin played a key role in efficient infiltration of resin into the cells. The sample was incubated at 75°C in an oven. The sections were stained with uranyl acetate and lead citrate and observed under an electron microscope. Acetone could be substituted for propylene oxide. However, acetone often extracts lipid components; thus, use of propylene oxide is recommended with the following chemicals being required: Eponate 12 Resin (glycerol polyglycidyl ether) or Medcast, DDSA (dodecenyl succinic anhydride), NMA, methylnadic anhydride; methyl-5-norbornene-2,3-dicarboxylic anhydride; methyl bicycle (2,2,1) hept-5-ene-2,3-dicarboxylic anhydride; BDMA (benzyl dimethylamine: catalyst for Eponate 12) or DMP-30 (catalyst for Medcast); Ted Pella, Inc. USA and, as Spurr's resin, ERL 4221, DER 736, NSA, and DMAE (catalyst).

Results

Fig.1 shows images from suspension cell culture and Fig.2 shows cells from a monolayer culture system. Successful infiltration of epon resin resulted in the images shown in Fig.1a (Etoposide-induced apoptosis) with HL-60 cells (ATCC, Manassas, VA; suspension culture), Fig. 1c (human T-lymphocyte Jurkat cells; suspension culture), and Fig. 2c (sccf3, cat bone-invasive gingival cancer cells; monolayer culture).

In Fig.1a, both cells are in an apoptotic state, with a more advanced stage in the upper cell. Chromatin aggregates have adhered to the nuclear membrane, a typical step of apoptosis, and the nucleolus is starting to disperse. In the Jurkat cell samples, most of the cells resemble the bottom cell in Fig.1c, indicating that epon had successfully infiltrated into the cells. The upper cell is an example of a dead cell, due to calcium influx. Fig. 2c shows an example of perfect epon infiltration. No aggregated cells appeared in this preparation. All cells appeared with the bottom part attached to the culture dish.

An example of unsuccessful epon infiltration of mature dendritic cells is shown in Fig.1b (low magnification) and d (high magnification); the cells were obtained from rabbit monocytes under suspension culture. All steps of the sample preparation were the same as those of the successful process except for the infiltration procedure. No clear appearance of either the cytoplasm or nucleus was observed. We could not detect a clear image of membranes. There is no doubt that poor epon infiltration greatly affected the sample.

Fig.2a (human lung macrophage; monolayer culture) shows a side view of a macrophage with engulfing foreign material. The bottom corresponds to the site of attachment to the culture dish. We cannot distinguish between the cytoplasm and nucleus.

Fig.2b (human breast cancer A-549 cell) shows an example of a cell with partial epon infiltration. Although infiltration of epon was not completely successful, an organelle area in the cytoplasm can be seen partially along with the cell shape. The bottom is the site of attachment to the dish. Numerous projections can be seen along the top of the picture.

Fig.2c (cat bone-invasive gingival cancer cell) shows an example of electron micrograph of a cell after successful epon resin infiltration. Our method allows any cultured cells to be processed for EM reproducibly, as this photo shows. The bottom is the site of attachment to the dish.

Discussion

Researchers have used an electron microscope to observe images of cell collapse-aggregation in sample of various cultured cells. Even if a cell appears to be normal,

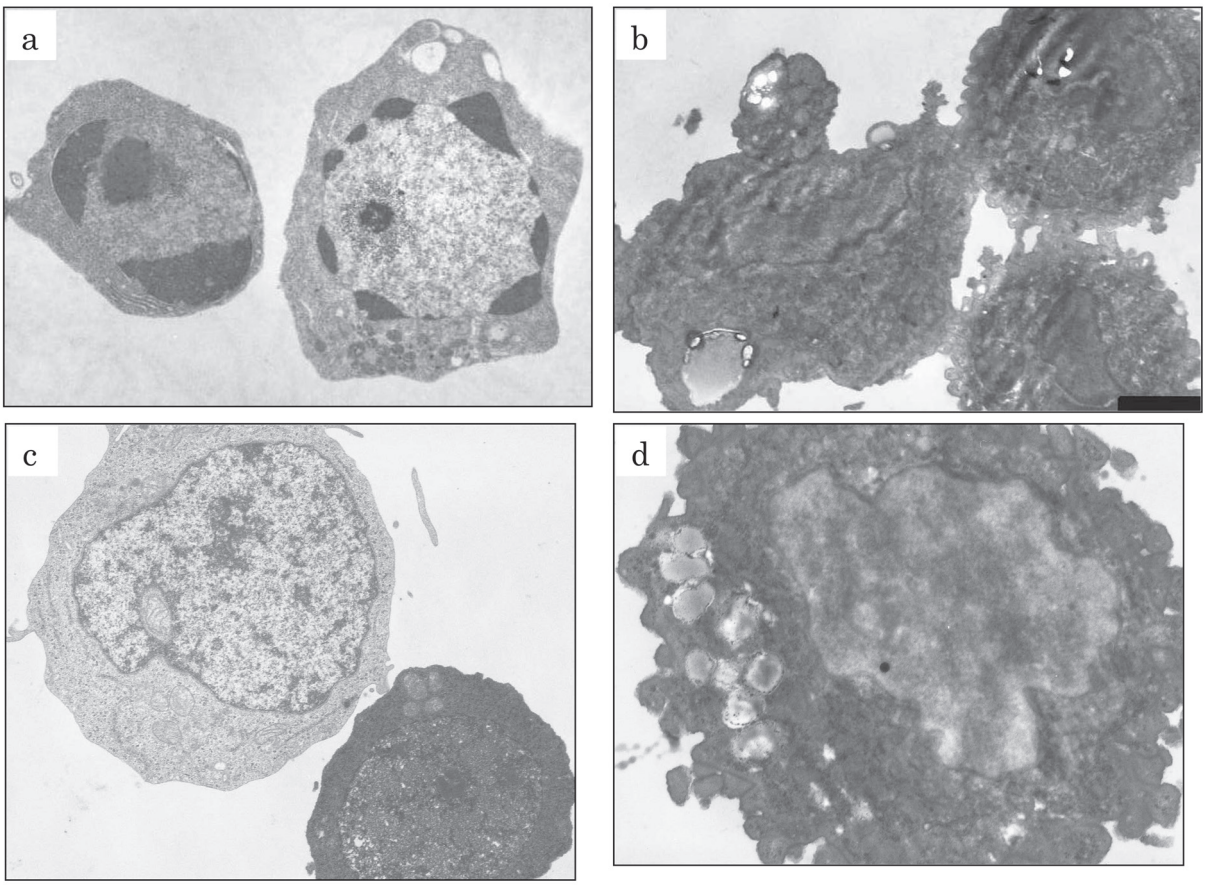

Fig. 1. Cells from suspension culture of a) HL-60 (ATCC, Manassas, VA) x 8,000, b) rabbit mature dendritic cells x 5,300, c) Human T-lymphocyte Jurkat cells x 7,000, and d) rabbit mature dendritic cells x 18,000.

there is no guarantee that a part of its cell membrane has not been damaged to allow easy infiltration of epon resin. If a portion of the cell membrane has been scraped during cell collection, epon resin can easily enter the cell. The structural arrangement and distribution of the cytoplasm would appear to be normal. However, some of the structural damage of the cytoplasm would have resulted from cell membrane damage. The fine structure of these cells might be modified even if the cells appear normal. Infiltration of epon resin must be achieved through an undamaged cell membrane. The fixation method certainly influences organelle structures depending whether the fixation was done before or after removal/detachment of cells from culture dishes. To obtain a correct image, fixation must be carried out for cells that are still attached to culture dishes, especially in the case of monolayer cell cultures. Cell damage during sample preparation leads to confusion among artifacts, apoptosis, and real cell images.

Infiltration of epon resin into the cell is crucial for obtaining good quality of EM images. Short chains of the resin polymer easily penetrate the cultured cell membrane. Infiltration problems seldom occur with tissue samples. Unlike tissues, cultured cells cannot maintain tension once they are removed from dishes even after fixation. In addition, no connective tissues are present to support a stretch in cultured cells. With no supporting elements such as connective tissues, the cells would receive direct osmotic pressure from epon resin, which would cause collapse and shrinkage. Thus, permeability of the cell membrane would decrease considerably in cultured cells, with the resulting difficulty in penetration of epon resin.

We do not know why monolayer cultured cells are more difficult to process by epon infiltration than suspension culture cells. One possibility is that once a monolayer culture loses support when detached from the dish surface, the degree of shrinkage and aggregation becomes much greater than that of cells in a floating system.

The amount of chain formation in an epon resin-propylene oxide mixture in a 1 : 3 volume ratio must be very small at the beginning of infiltration. Once the concentration of short-chain epon polymers that have entered the cell increases dramatically, the degree of polymerization

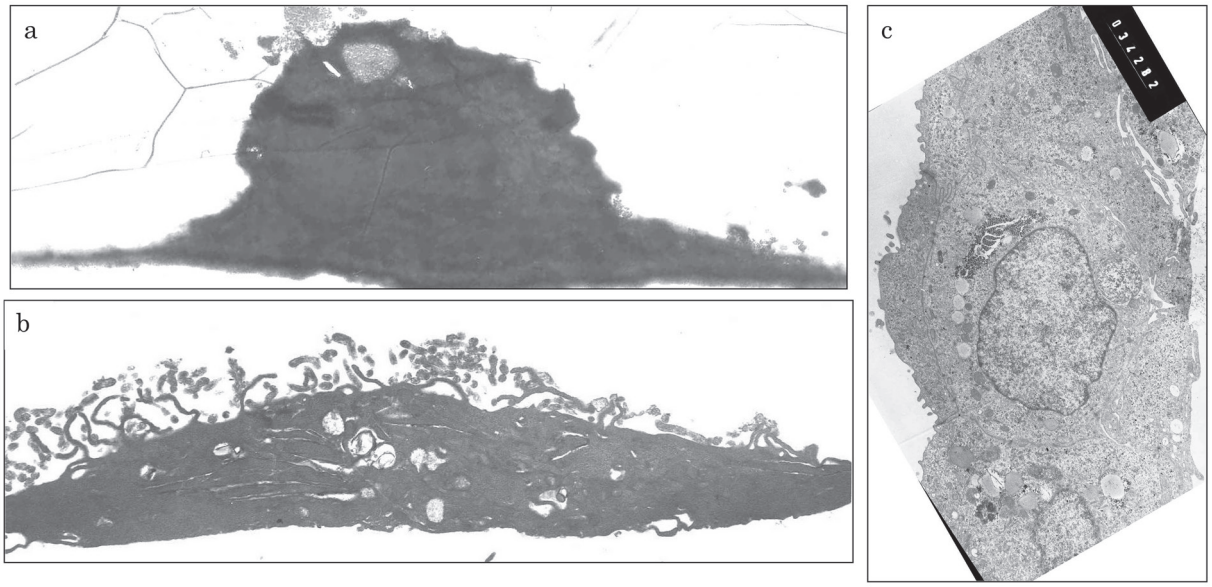

Fig. 2. Cells from monolayer culture of a) human lung macrophages x 7,900, b) human breast cancer epithelial A-549 cells x 9,000, and c) sccf3, cat bone-invasive gingival cancer cells x 5,100.

increases to give high-molecular-weight polymers. The short-chain polymers that continuingly influx into the cell immediately react with the pre-entering polymer molecules to form longer polymers. This step must be a key for the infiltration of cultured cells.

We have considerable knowledge about apoptosis from physiology and chemistry. However, we do not know the ultrastructure of cells in the initial, progressing, and advanced stages of apoptosis. Images of artifactual cells that have been growing regularly but were changed through technical error could be confused with images of truly apoptotic cells. We have developed an improved infiltration method to avoid this problem in our study of apoptosis.

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