MICROWAVE-STIMULATED FIXATION FOR HISTOCYTOCHEMISTRY: APPLICATION TO SURGICAL PATHOLOGY AND PREEMBEDDING IMMUNOELECTRON MICROSCOPY

HARUO OHTANI, HIROSHI NAGANUMA AND HIROSHI NAGURA

Department of Pathology, Tohoku University School of Medicine, Sendai 980

Microwave (MW)-stimulated fixation was applied to surgical pathology and preembedding immunoelectron microscopy. Fresh specimens were microwaved in the fixatives for 15–30 sec using a conventional MW oven (500 W) containing 200 ml of water load. The temperature of fixatives was raised to 35–45°C. After MW radiation, the specimens were kept in the same fixatives for 1–4 hr at 4°C or at room temperature as the postfixation procedure, which was important for the completion of chemical fixation of whole specimens. For light microscopical examination, buffered 10% formalin was used. Hematoxylin-eosin staining from paraffin-embedded specimens was completed within 24 hr with excellent preservation of morphology and immunoreactivity. For conventional electron microscopy, MW fixation in 2.5% glutaraldehyde-2% paraformaldehyde showed excellent preservation of cell organelles. For immunoelectron microscopy by the preembedding method, 4% paraformaldehyde solution containing 0.1% glutaraldehyde was used, followed by one hr postfixation. Simultaneous improvement of both ultrastructure and antigenicity were obtained without any significant changes of the results. Our results indicate that MW-stimulated fixation with postfixation procedure is a useful tool for various fields of morphological investigations, and its beneficial effects were most remarkable for the preembedding immunoelectron microscopy technique.

Microwave (MW) radiation has recently been used in fixation, dehydration, staining and immunohistochemical procedures (1, 4). When used in fixation, it provides good results in conventional light and electron microscopy, enzyme histochemistry and immunohistochemistry (1, 4–9). However, this fixation method has not been widely used in the field of pathology in Japan. Applications to immunoelectron microscopy with the preembedding method are also still limited in number (20) and the effectiveness of MW-stimulated fixation in these fields has not yet been understood.

Most of the previous reports emphasized the heating effect of MW radiation and the final temperature of the fixatives was 55–68°C (1, 3). In the present report we adopted MW radiation with the final temperature at about 37°C because Mizuhira emphasized the deteriorating effect of high temperature on membrane structures (9). MW radiation was performed in fixatives because MW radiation in buffer solution resulted in poor preservation of cell organelles (2, 3). We also proved that the postfixation procedure after MW radiation is mandatory for the completion of the chemical fix-

Correspondence and reprint request: H. Ohtani, M. D., Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryō-machi, Aoba-ku, Sendai 980, Japan.

ation (15). We show here the excellent effects of MW-stimulated fixation with these modifications in routine histological examination and research by immunoelectron microscopy.

MATERIALS AND METHODS

Microwave Radiation

The method modified from Login (5, 6), Boon & Kok (1) and Mizuhira (9) was used in the present study. Human tissues were sliced into the size of from $5 \times 5 \times 2$ mm to $10 \times 10 \times 3$ mm immediately after surgical resection or biopsy and they were put into 15 ml of fixatives (Table 1) and radiated with MW using a domestic MW oven (2.45 GHz, 500 W) with a 200 ml of water load for 15–30 sec. The temperature of fixatives was raised from 4°C or room temperature to 35–45°C. After MW radiation, the tissues were kept in the same fixatives for 0.5–4 hr at 4°C or room temperature as a postfixation procedure as previously described by us (15).

Light Microscopy

Fifty five cases of surgical or biopsy specimens were used including gastrointestinal carcinoma, breast tumors, lymph node lesions, and others. Most of them were obtained at Tohoku Rosai Hospital, Sendai, and all the patients were Japanese. The fixative during MW radiation was 10% buffered formalin. Postfixation was examined at 0, 1, 2, 3, 4, 6, and 24 hr. After fixation, specimens were dehydrated and embedded into paraffin with a automatic tissue processor. 3 μ m-thick sections were stained with hematoxylin-eosin stain. As the control, sliced tissues were fixed in the same fixatives for about 24 hr at room temperature in all cases. Furthermore, en-bloc fixed materials of the same case for routine histopathological examination were also used as a control.

Light Microscopic Immunohistochemistry

Thirty of the above-mentioned 55 cases were processed for immunohistochemistry. After blocking endogenous peroxidase activity with 100% methanol containing 0.3% hydrogen peroxide for 30 min, 3 μ m thick sections were incubated with

TABLE 1. *Choice of fixatives and postfixation*

	Fixatives (during MW radiation)	Time of postfixation in the same fixatives
Routine histological examination and light microscopic immuno- histochemistry	10% buffered formalin	2–4 h.
Conventional electron microscopy	2.5% GA+2% PFA/0.1 M cacodylate buffer/2 mM CaCl ₂	10 min
Immunoelectron microscopy by preembedding method	4% PFA+0.1% GA/0.1 M cacodylate buffer/2 mM CaCl ₂ +2 mM MgCl ₂	30 min–1 h

Final temperature after MW radiation=35–45° (light microscopy) or 35–40°C (immunoelectron microscopy).

GA, glutaraldehyde; PFA, paraformaldehyde

TABLE 2. Summary of the results of light microscopic immunohistochemistry from paraffin blocks

Antibody	Working Dilution	Improvement of Sensitivity
anti-human von Willebrand factor* (DAKO)	1 : 7,500	$\times 5 - \times 20$
anti-S100 protein (DAKO)	1 : 1,500	$\times 1$
anti-CEA (Mochida 010)	1 : 5,000	$\times 5 - \times 10$
anti-vimentin* (Boehringer)	1 : 20-1 : 300	$\times 10 - \times 100$
anti-cytokeratin* (BioGenex) (40, 46, 50, 52, 56.5, 58, 65-67 kd)	1 : 15-1 : 100	$\times 5 - \times 10$
LN1-3 (Technidone International)	undiluted	$\times 1 - \times 4$
UCHL-1 (Nichirei)	1 : 100	$\times 1$

*=cases pretreated with 0.1% trypsin for 15 min.

the first antibodies (Table 2) which was followed by biotinylated second antibody and streptavidin-peroxidase using Biotin-StreptAvidin System (BioGenex Lab., Sierra Lane, CA). They were then reacted with 0.03% 3, 3' diaminobenzidine (DAB) containing 0.006% hydrogen peroxide. They were counterstained with either 1% methyl green or hematoxylin.

Conventional Electron Microscopy

Tissue specimens from human full-term placenta were fixed with MW radiation in 2.5% glutaraldehyde-2% paraformaldehyde-0.1 M sodium cacodylate solution containing 2 mM CaCl_2 followed by 5-15 min postfixation. After osmification, the specimens were routinely processed.

Preembedding Immunoelectron Microscopy

The sliced specimens were microwaved in 4% paraformaldehyde-0.1% glutaraldehyde-0.1 M sodium cacodylate containing 2 mM CaCl_2 and 2 mM MgCl_2 , followed by 30 min-one hr postfixation in the same fixatives or in periodatelysine-paraformaldehyde (PLP) at 4°C. The final temperature of the fixatives was set at 35-40°C for immunoelectron microscopy. After rinsing in 10% sucrose in phosphate-buffered saline (PBS) and 20% sucrose in PBS, the specimens were embedded in O.C.T. compound and rapidly frozen with dry-ice ethanol. The immunostaining was performed on 6 μm thick cryostat sections with the indirect immunoperoxidase method. The details of this have already been described elsewhere (13). We observed eight cases of gastrointestinal carcinomas, three cases of breast carcinomas, two cases of renal carcinomas, three full-term placenta, two ovaries and one case of minor salivary gland by this method. The first antibodies used are listed in Table 3. Horseradish-peroxidase (HRP)-conjugated Fab or F(ab')_2 fragments of anti-rabbit or mouse IgG antibody were used for the second antibody. In some cases, HRP-conjugated Fab' fragments of the first antibodies were used for the direct method (Table 3). The incubation time was 16-24 hr in all incubation steps with the antibodies. After the immune reactions, the sections were fixed in 1% glutaraldehyde in PBS for 10 min. Then, they were reacted with DAB, osmified, and embedded into Epon. Ultrathin sections were stained with lead citrate for one min and viewed with a JEOL 100B electron microscope. For the control of MW-stimulated fixation, the results of our present study were compared with those of our previous studies (12, 13) in which tissues were fixed by the conventional immersion method in 4% paraformaldehyde in 0.05 M

TABLE 3. *List of antibodies and results by preembedding immunoelectron microscopy using frozen sections*

Antibodies (Source and our references of results)	Localization of positive reactivity
anti-human type III collagen* (Dr. A. Ooshima) (16)	rER of fibroblasts, extracellular matrix
anti-von Willebrand factor (DAKO)	rER, Weibel-Palade bodies, membrane of endothelial cells
anti-CEA* (Mochida 010)	membrane and rER of carcinoma cells
BMA120* (Behring) (anti-endothelial)	luminal cell membrane of endothelial cells
CD8* (cytotoxic/suppressor T)	Surface and perinuclear space of the corresponding lymphocytes
DF3* (Dr. Kufe) (17)	Cell membrane, rER, granules, cytoplasm in breast carcinoma cells
FH2, FH6*+(Dr. Y. Fukushi)	Cell membrane, cytoplasm in renal carcinoma cells
anti-aromatase (Dr. N. Harada) (11)	Smooth ER and cell membrane of syncytiotrophoblast
anti-lactoferrin** (DAKO)	rER, Golgi, granules of acinar cells of minor salivary glands
anti-human IgA** (DAKO)	membrane and basal lamina of acinar and duct cells of minor salivary glands

*=monoclonal antibodies

**=cases in which direct immunoperoxidase method was applied.

+ FH2 and FH6 recognize Le^x and sialosyl Le^x, respectively.

phosphate buffer or PLP for 6–10 hr at 4°C, and processed in the same manner as mentioned above.

RESULTS

Light microscopy

Hematoxylin-eosin stain: Preservation of tissue structure was better by MW-stimulated fixation than by the conventional fixation method (Figs. 1a, 1b). Normal and pathological tissues were equally well fixed. When specimens were processed without postfixation, only peripheral areas of the specimens were fixed. In the central areas, all erythrocytes burst and nuclei showed the ethanol effect (1). Two hr postfixation was sufficient for the chemical fixation although the chromatin pattern of nuclei was still mildly coarse (Fig. 1a). Four hr postfixation completed the chemical fixation resulting in fine chromatin pattern of nuclei. In most cases, all areas of specimens were uniformly fixed by this method. Structures of all tissue components in the sections were finely preserved, and this condition was best for the histological diagnosis (Fig. 1b). Lymph nodes needed four hr postfixation.

Immunohistochemistry: Marked increase of immunoreactivity, up to 100 times, was observed in von Willebrand factor (vWF), cytokeratin, vimentin, and CEA (Figs. 2, 3) (Table 2). Cytoplasmic immunoreactivity of CEA and vimentin was also much increased by MW-stimulated fixation method. No remarkable effects for the improvement of immunoreactivity were observed in S100 protein and UCHL-1. Decrease or

FIG. 1a

FIG. 1b

FIGS. 1a, b. Hematoxylin-eosin staining from paraffin blocks by MW-stimulated fixation. a. Fundic glands of gastric mucosa fixed with two hr of postfixation. $\times 360$ b. Chronic pancreatitis fixed with four hr of postfixation. 10% buffered formalin was used. $\times 360$

loss of immunoreactivity was not observed in the antigens in the present study. Antigenicity for vWF and vimentin was best preserved in the MW fixation and worst in en-bloc fixed specimens. Two hr postfixation was best for preservation of antigenicity, followed by four hr postfixation. Twenty four hr postfixation showed significant loss of antigenicity, and resulted in a similar decrease of immunoreactivity as those by 24 hr routine fixation.

FIG. 2a

FIG. 2b

FIGS. 2a, b. Immunohistochemistry for vimentin in gastric adenocarcinoma. a. MW-stimulated fixation. $\times 180$ b. Conventional fixation. $\times 180$. Note strong immunoreactivity in the stromal area in MW-fixed tissue. Immunohistochemical procedures were performed in the same way.

Electron Microscopy

Conventional electron microscopy: Fine structures of cell organelles and cell membrane were particularly well preserved as previously reported (figures not shown).

Preembedding Immunoelectron Microscopy: Tissues were homogeneously fixed. Preservation of ultrastructures was excellent as compared with that by the conventional fixation with PLP. Mitochondria were not swollen in any cases and the membrane of the Golgi complex was intact without dilation of the cisternae. Fig. 4 shows localization

FIG. 3a

FIG. 3b

FIGS. 3a, b. Immunohistochemistry for von Willebrand factor in a case of malignant fibrous histiocyoma. a. MW-stimulated fixation. Note intense immunolabeling in the stromal capillaries. $\times 270$ b. Conventional fixation. Immunostaining is rarely observed in this condition. $\times 270$

of von Willebrand factor (vWF) in rough endoplasmic reticulum (rER) of endothelial cells in the stroma of most colorectal carcinoma. Preservation of the cellular organelles was much better with postfixation in the same fixatives or PLP than that without postfixation (14). Membrane stabilization was also better with postfixation. Penetration of the antibody into cell organelles was constantly good. Capillaries were clearly labeled with anti-endothelial antibody (BMA120) (Fig. 5). Carcinoembryonic

FIG. 4. Immunoelectron microscopy for von Willebrand factor in a capillary in the stroma of colonic carcinoma. Immunoreaction products are localized in rough endoplasmic reticulum (*er*), round granule (*Gr*), perinuclear space (*PNS*) and diffusely in the subendothelial stroma (*str*). Fixed by MW radiation in 4% paraformaldehyde+0.1% glutaraldehyde. Time of postfixation was one hr, the same condition as in Figs. 4–8 (see the Materials and Methods Section for details). L: vascular lumen, N: nucleus. $\times 22,600$

FIG. 5. Immunoelectron microscopy with monoclonal antibody BMA120 in a capillary in the stroma of diffuse-type gastric carcinoma by MW-stimulated fixation. Luminal plasma membrane is clearly immunolabeled. L: lumen, N: nuclei. $\times 9,800$

FIG. 6. Immunoelectron microscopy with monoclonal anti-CEA antibody in colonic carcinoma. Cytoplasmic reactivity is well preserved as well as reactivity along the cell membrane. L: lumen, N: nucleus, Str: stroma. $\times 5,270$

FIG. 7. Higher magnification of the carcinoma cell in Fig. 6. CEA-immunoreactivity is localized in rough endoplasmic reticulum (*er*), Golgi apparatus (*Gol*), and along the lateral cell membrane (arrowheads). Preservation of the organelles is better than by conventional method, but still not optimal due to ischemic effect after ligation of arteries. $\times 26,600$

FIG. 8. Immunoelectron microscopy for lactoferrin in a acinar cell of a minor salivary gland (MW-stimulated fixation) (biopsy specimen). Golgi membrane (*Gol*) and granules (*Gr*) are immunolabeled. Note excellent preservation of these structures without ischemic changes of cell organelles. $\times 29,500$

antigen (CEA) was clearly localized along the luminal and basolateral plasma membranes. Immunoreactivity in rER and Golgi apparatus was also well recognized (Figs. 6, 7). Being obtained at surgical operations, effects of ischemic changes after ligation of arteries were not eliminated in these specimens. Materials obtained by biopsy were nearly free from ischemic damage as revealed by well preserved Golgi apparatus in acinar cells of minor salivary gland (Fig. 8). This figure also shows localization of immunoreactivity for lactoferrin along the membrane of Golgi apparatus and in granules budding from the *trans* surface. Results of the immunolabeling with monoclonal antibodies DF3, FH2, FH6, CD 4 and polyclonal anti-aromatase antibody are summarized in Table 3. Intensity of immunoreactivity was generally better than that with conventional fixation. However, no drastic improvement of sensitivity for immunolabeling as observed in light microscopic immunohistochemistry was gained. Except for the above-mentioned qualitative changes, no other significant differences were observed between the results by MW-stimulated and conventional fixation methods. Prolonged postfixation decreased the antigenicity of CD4.

DISCUSSION

It is important for routine surgical pathology that the fixation technique gives constant results and is easy to handle. The present study clearly showed that the MW-stimulated fixation method fulfills this condition. Boon and Kok (1) described various examples of the application of MW radiation. They recommended MW radiation in radiation media such as PBS with the final temperature set at 56°C followed by formalin fixation for two hr. In the present study we used Mizuhira's method (9) by adding the postfixation procedure because a) MW radiation in buffer solution was not good for fixation of cell organelles (2, 4) and b) MW radiation in fixatives with short-time postfixation resulted in good morphological preservation in only peripheral areas of specimens (19). We have proved in this report that the postfixation procedure after MW radiation is crucial for the completion of chemical fixation of whole specimens. In this sense, our method may be more appropriately designated as "*chemical fixation accelerated by MW radiation*". Mizuhira has recently shown the theoretical background of MW-stimulated fixation; MW radiation accelerated cross-linking of aldehyde to peptides and this process was finished in 30 min to 4 hr, and temperature over 40°C was not required (10). The heating effect of MW radiation was not as important for the mechanism of fixation as previously considered. In fact, MW radiation within low temperature (below 15°C) with postfixation for one hr is optimal for preservation of cell organelles in the preembedding immunoelectron microscopy (Masuda, personal communication).

It is well known that MW-stimulated fixation has a drastic effect on the ultrastructure and immunocytochemistry (4, 5, 7). In the present paper, we emphasized its effects on the simultaneous improvement of preservation of subcellular structures and antigenicity for preembedding immunoelectron microscopy by adopting the postfixation procedure. This result is epoch-making because it has been generally believed that the preservation of both the ultrastructure and antigenicity is difficult by chemical fixation. This effect by MW radiation is probably obtained by both rapid penetration of fixatives into the specimen during MW radiation (9) and physical effects of MW itself. Side effects of MW radiation were noted by immunoelectron microscopy without the postfixation procedure; plasma membranes of free surface were liable to the focal disruption. Postfixation after MW radiation in fixatives with CaCl_2 and MgCl_2 improved these problems. Therefore, this artifact may have occurred during long-time incubation in antibody solution (48 hr in total) and/or during dehydration process. Our future efforts will be focused on the membrane stabilization in various tissues or cells. Low temperature radiation and radiation with lower energy with larger quantity of water load may be effective.

A two-step method for MW-stimulated fixation of large materials such as brain or stomach has already been introduced (1, 4). These methods were based on MW radiation in buffer solution or without any media, depending on the stabilization effect of MW radiation by high temperature. Therefore, the preservation of ultrastructures was not guaranteed. More reliable radiation instruments are necessary for much larger tissues.

In conclusion, the MW-stimulated fixation method with postfixation in the same fixatives is effective for histological and histochemical studies. We believe that the improvement of the preembedding immunoelectron microscopy by this method will

bridge the gap between morphological investigation and cell biology.

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