ANTIGEN RETRIEVAL BASED ON MICROWAVE-EXPOSURE IN IMMUNOSTAINS OF MYOSARCOMA TISSUES

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Antigen retrieval based on microwave-exposure of formalin-fixed, paraffin-embedded tissues in a metal solution of saturated lead thiocyanate or 20% zinc sulfate was investigated. Six leiomyosarcomas and 4 rhabdomyosarcomas were studied using the ABC method applying muscle-related markers. Compared with untreated control sections, vimentin and desmin showed increased staining after the exposure. However, myoglobin immunostaining was not improved. Therefore, some tissue antigens or epitopes were retrieved that were masked by formalin-fixation, but the retrieval was not universal.

Immunohistochemistry has contributed to a better understanding of cell type differentiation, tumor histogenesis, and predicting the prognosis (1, 2, 5, 14, 18, 19). Despite these important benefits, there is ample evidence that formalin-fixation, the most common fixative, has a deleterious effect on the immunostaining of several antigens (11, 12). This may be caused by epitope masking, probably because of cross-linking of proteins around the antigenic site (4). In addition, the process responsible for the masking of the antigenicity is a progressive one that is enhanced by prolonged immersion in formalin (3). Therefore, interpretation is often difficult when negative immunostaining occurs. These results cannot clarify whether the cells do not contain an antigen (or the antigen level is below the detection sensitivity of the procedure), or whether the antigenicity was lost. Although many antibodies which can recognize formalin-resistant epitopes in immunohistochemical procedures have been developed, they have not been entirely satisfactory in resolving all problems.

Predigestion of formalin-fixed tissue sections with protease enhanced the immunostaining of several antigens by unmasking antigenic sites, but false negatives or no enhancement has been observed with others (3, 8, 13). Recently, a method for antigen

retrieval in formalin-fixed, paraffin-embedded tissue sections has been described (15). It is based on a microwave-exposure of tissue sections to temperatures of up to 100°C in the presence of metal solutions. In this study, we evaluated this new method in the immunostaining of muscle-related markers in formalin-fixed, paraffin-embedded myosarcoma tissues.

MATERIALS AND METHODS

Tissue Sections

Six samples of leiomyosarcoma (LMS) and 4 of rhabdomyosarcoma (RMS) were obtained from the Kansai Medical University Hospital or the Saiseikai Nakatsu Hospital. Five LMS specimens and 1 of RMS were from surgery, and the remaining 5 were from autopsy. All tumor tissues were routinely fixed in 10% formalin within 1 week, and embedded in paraffin. All diagnoses were based upon the HE-stained sections. Serial sections of 4 µm were cut and mounted on aminopropyltriethoxysilane-coated slides (Matsunami Glass, Osaka, Japan).

Tissue Antigen Retrieval

The slightly modified protocol, originally described by Shi *et al.* (15), was as follows: The tissue sections on slides were deparaffinized, dehydrated, rinsed in deionized water (DW), and incubated in 3% H₂O₂ in DW for 10 min to block endogenous peroxidase. Subsequently, the slides were washed with DW for 5 min and placed in polycarbonate staining jars

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(Kartell, Italy) filled with either DW, or a metal solution of saturated lead thiocyanate or 20% zinc sulfate. The jars were then covered and heated in a microwave oven (Mitsubishi, Japan) at 700 W for 5 min after the solution started to boil. The fluid level in the jars was then checked, and DW was added to the initial level before a further 5 min microwave-exposure. Thereafter, the slides were immediately put into warm (60°C) DW, and allowed to cool for 15 min at room temperature. Slides were rinsed twice in DW for 5 min, and washed in 0.05 M Tris-HCl buffered saline, pH 7.6, (TBS) for 5 min. These slides and serially cut untreated sections were immunohistochemically stained.

Immunohistochemistry

Antibodies and the antiserum, their specificity, clone designation, source and dilution are listed in Table 1. Using Vectastain ABC Kits (Vector Laboratories, Burlingame, CA, USA), immunohistochemical staining proceeded according to the manufacturer's instructions. Briefly, sections were incubated in the blocking reagent (normal horse or goat

serum) for 20 min, then with optimized diluted primary antibodies, for either 60 min at 37°C or overnight at 4°C. Subsequently, the link antibody (biotinylated anti-immunoglobulins) and the labeling complex (avidin conjugated to horseradish peroxidase) were added. The reaction products were visualized with DAB (Wako Pure Chemical, Osaka, Japan). Sections were counterstained with Gill hematoxylin. In the negative controls, non-immune mouse or rabbit sera replaced the specific antibody or antiserum.

We then compared the effect of boiling time (5, 10 and 15 min), and the solutions (DW, saturated lead thiocyanate, and 20% zinc sulfate) in the immunohistochemical staining of vimentin.

RESULTS

Vimentin staining intensity was increased compared with untreated sections. Particularly, under the same boiling time, the saturated lead thiocyanate effected more intense staining than zinc sulfate or DW. Prolonged boiling for 10–15 min introduced more intense immunostaining than after 5 min (Fig. 1; Table 2). The background was reduced more when the sections were incubated in the primary antibody for 60 min at 37°C than overnight at 4°C. Sometimes, the lead thiocyanate caused non-specific nuclear staining and diffuse crystal precipitates throughout the section. Insoluble crystal precipitates were formed when the lead thiocyanate was mixed with buffer. This contamination was eliminated by washing in heated DW (60°C) just after microwave-

TABLE 1. Antibodies and antiserum used in this study

Specificity	Clone designation	Source	Dilution
Vimentin	V9	DAKO	1:500
Desmin	D33	DAKO	1:100
Myoglobin ¹		DAKO	1:1000

¹ Antiserum

FIGS. 1a–c. The influence of the boiling solutions on antigen retrieval in vimentin immunohistochemistry, using a monoclonal antibody to vimentin within formalin-fixed, paraffin-embedded LMS sections. (a) DW, (b) ZnSO₄, (c) Pb(SCN)₂, all for 10 min. Vimentin staining was significantly enhanced using the saturated lead thiocyanate (Fig. 1c). Original magnification: ×200

heating in the metal solution, followed by sufficient washing in DW then with TBS. The endogenous peroxidase activity was also reduced in this manner. Considering these results, saturated lead thiocyanate with boiling for 10 min followed by sufficient washing gave satisfactory results. Applying this method, the immunoreactivity of 6 LMS and 4 RMS samples are indicated in Table 3.

Vimentin immunostaining was significantly increased after the procedure. The vast majority of tumor cells in both LMS and RMS were positively stained, and the staining was evenly distributed throughout the tumor tissues as well as in adjacent intermingled vessels (Fig. 2). Positive desmin staining was sometimes enhanced (Fig. 3). However, the immunoreactivity of myoglobin in tumor cells was not enhanced even after boiling for 15 min, though it was

enhanced in some normal muscle cells (Fig. 4).

DISCUSSION

Formalin fixative has been routinely used due to its excellent preservation of tissue structures. However, in immunohistochemistry, it occasionally causes problems in the preservation of antigenicity. The reactive aldehyde group of formaldehyde binds to amine groups on the polypeptide backbone and amino acid side chains, leaving the potential antigenic sites masked (9). Shi *et al.* developed a method for antigen retrieval in formalin-fixed, paraffin-embedded tissue sections using metal solutions in combination with microwave oven heating. They found that heating, microwave-exposure, or the presence of heavy metal solution alone enhanced the immunohistochemical staining of a variety of tissue antigens (15). The mechanism of antigen recovery might be associated with alterations in the cross-linking of proteins caused by formaldehyde, since this treatment does not affect the alcohol-fixed tissues. Protease digestion may be based upon a similar mechanism (13). Protease digestion could improve the staining of some antibodies, such as to keratins, but did not exhibit sufficient reaction on other antigens such as vimentin. Autoclaving sections immersed in distilled water (hydrated autoclaving) recovered the immunoreactivity to anti-tau antibody, in which the heating might have induced a denaturation of formalin-fixed tissue protein (16). Microwave-exposure in DW enhanced the im-

TABLE 2. Enhancement by tissue antigen retrieval in vimentin immunohistochemistry

Solution	Time (min) ^a		
	5 min	10 min	15 min
Pb(SCN) ₂	++ ^b	+++	+++
ZnSO ₄	+	++	++
Deionized water	+	+	++

^a Time of microwave-exposure after the boiling point.

^b Slight, moderate and predominant enhancement in vimentin immunoreactivity are expressed as scales of +, ++ and +++.

TABLE 3. Comparison of immunoreactivity in tissue sections of leiomyosarcoma and rhabdomyosarcoma with or without tissue antigen retrieval¹

Case	Antigens	Vimentin		Desmin		Myoglobin	
	Treatment	(-)	(+)	(-)	(+)	(-)	(+)
LMS ² -1		-/- ⁴	+/-	-/-	+/-	-/-	-/-
LMS-2		+/-	+/+	+/+	+/+	-/-	-/-
LMS-3		-/-	+/+	+/-	+/-	-/-	-/-
LMS-4		-/-	+/+	-/-	+/-	+/-	-/-
LMS-5		-/-	+/+	+/+	+/+	+/-	+/-
LMS-6		-/-	+/+	+/+	+/+	+/-	+/-
RMS ³ -1		-/-	+/-	-/-	-/-	-/-	-/-
RMS-2		-/-	+/+	-/-	+/-	+/-	+/-
RMS-3		-/-	-/+	-/-	+/+	+/+	+/+
RMS-4		-/-	+/+	+/-	+/-	+/-	+/+

¹ Routinely prepared and deparaffinized sections were incubated in boiling Pb(SCN)₂ for 10 min after blocking endogenous peroxidase, then thoroughly washed in DW.

² LMS: Leiomyosarcoma.

³ RMS: Rhabdomyosarcoma.

⁴ Normal/tumor cells. Immunoreactivity was scored on a scale of - to ++.

FIGS. 2a, b. A monoclonal antibody to vimentin demonstrated a significant increase of the immunostaining in formalin-fixed, paraffin-embedded sections of LMS after tissue antigen retrieval (Fig. 2b). a. No treatment. b. After treatment. Original magnification: $\times 400$

FIGS. 3a, b. A monoclonal antibody to desmin showed a moderate increase of the immunostaining in formalin-fixed, paraffin-embedded sections of LMS after treatment (Fig. 3b). a. No treatment. b. After treatment. Original magnification: $\times 200$

FIGS. 4a, b. The immunoreactivity of antiserum to myoglobin was not enhanced even after treatment. a. No treatment. b. After treatment. Original magnification: $\times 20$

munoreactivity, but metal solutions were more effective. Heavy metal salts act as protein precipitating agents, and protein-precipitating fixatives frequently preserve more antigens than cross-linking aldehyde fixatives (7, 9). Cross-linking of proteins alone could not explain the mechanism of the antigen recovery. Both

heating and the presence of metal solutions might mainly affect this mechanism. Additionally, the physical effect of the boiling upon the antigenic sites might play a principal part as well as heating. The influence of various solutions under similar conditions consisting of heat and a boiling period should be

evaluated.

Formalin-fixation is used almost universally by pathologists, and there is no standard method of fixation that can be applied to tissues submitted for immunohistochemical analysis. Tissue antigen retrieval did recover and/or retrieve the epitopes masked by formalin-fixation, and was applicable to some antibodies.

An enhanced immunohistochemical procedure to detect proliferating cell nuclear antigen (PCNA) has also been reported that was similarly based on microwave-exposure in combination with a metal solution (6, 20). The antigenicity of desmin is not well preserved in formalin-fixed specimens (11). Myoglobin is a specific marker for striated muscles (10, 17). Detection of intermediate filaments as vimentin and desmin is particularly difficult in routinely fixed tissues, and the enhancement and/or antigen retrieval of immunoreactivity was not always consistent even after protease digestion.

In conclusion, striking immunostaining retrieval was obtained with a monoclonal antibody to vimentin. Tissue antigen retrieval more intensely immunostained desmin. We could not however, enhance the retrieval of myoglobin immunostaining.

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