Ki-67 Antigen Retrieval in Formalin- or Ethanol-fixed, Paraffin-embedded Tissues: An Enhancement Method for Immunohistochemical Staining with Autoclave Treatment

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We developed a new immunohistochemical method to visualize Ki-67 antigen in formalin-fixed, paraffin-embedded sections of bone tumors. The method uses autoclave pretreatment of sections immersed in citrate buffer before incubation with the MIB 1 antibody, which reacts with the Ki-67 gene product. MIB 1 antibody showed strong and

discrete staining of cell nuclei in proliferating tumor cells. This method also retrieved antigen in paraffin-embedded sections of alcohol-fixed tissue. Therefore, tissues previously considered unsuitable for immunohistochemical analysis can be studied after autoclave treatment.

Key words: Immunohistochemistry, MIB1 antibody, Autoclave treatment

I. Introduction

Progress in immunohistochemical procedures has led to the development of highly specific stains of great value in diagnosis and investigation. Some antigens, however, are difficult to detect by established methods, having lost immunoreactivity because of fixation and paraffin embedding. Previous studies have tried unsuccessfully to resolve this problem using protease digestion to expose antigenic sites blocked by formalin-induced protein cross-linkages [5].

Shi *et al.* have shown that microwave-oven heating of tissue sections in the presence of metal solutions can retrieve many antigens without enzyme predigestion of tissue. Although the mechanism of microwave antigen recovery is not clear, cross-linking of proteins by formalin may be altered by microwave heating [7]. Shin previously reported that high temperature pretreatment accomplished retrieval of masked antigen [8]. In this study, we

attempted to retrieve Ki-67 antigen in giant cell tumor of bone using autoclave treatment.

II. Materials and Methods

Specimens were obtained from a giant cell tumor of bone occurring in the femur of a 20-year-old woman. This tumor had immunocytochemically revealed 14% positivity for Ki-67 antigen using fresh tumor tissue. For immunohistochemical study of paraffin-embedded tissue, the specimens were fixed with 10% buffered formalin (F) or 70% ethanol (E) for 3 days, decalcified with 5% ethylenediaminetetra-acetic acid (EDTA)-0.1 M cacodylate buffer (pH 7.4) for 7 days, and embedded in paraffin blocks. The sections were cut at a thickness of 4 μ m and stretched on glass slides coated with poly-L lysine (Sigma, St. Louis, Mo). The slides were deparaffinized and then rehydrated to water. Endogenous peroxidase was blocked with 1% H₂O₂ in methanol for 30 min. The slides were washed with distilled water for 5 min, placed in glass Coplin jars containing 10 mM citrate buffer (10 mM citric acid monohydrate, pH 6.0 adjusted with 2N NaOH), and divided into three groups. Group 1 was stained with a conventional avidin-biotin complex (ABC) method, group 2 was pretreated with autoclave heating (100°C, 5 min) and group 3 was pretreated with autoclave heating (120°C, 5 min) before incubation with MIB 1 antibody (D.B.A.

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Fig. 1-A, B

Fig. 1. Immunohistochemical staining of Ki-67 antigen in a case of human giant cell tumor of bone. The specimen was fixed with 10% buffered formalin (A, C, E) or 70% ethanol (B, D, F). A, B: Sections stained by a conventional ABC method. Neither mononuclear cells nor multinucleate tumor cells were stained with MIB 1 antibody. $\times 200$. C, D: Sections treated with autoclave heating at 100°C for 5 min. $\times 200$. E, F: Sections treated with autoclave heating at 120°C for 5 min. $\times 200$. Bar = 10 μm ; same for Fig. 1A-F.

ITALIA, Milano). After heating, the Coplin jars were allowed to cool in the autoclave until the temperature reached to 50°C, and then were removed. The Coplin jars were allowed to cool for 30 min. Slides were incubated overnight at 4°C with MIB 1 antibody at a concentration of 2.67 µg/ml. Sites of antibody-antigen reaction were visualized by treating the sections successively with

biotinylated anti-mouse IgG antibody (Dakopatts, Denmark) at 1 : 300 for 30 min, streptavidin-biotinylated peroxidase complex (Dakopatts) at 1 : 50 for 30 min, and finally 3,3'-diaminobenzidine tetrahydrochloride solution in the presence of H₂O₂, prior to counterstaining with hematoxylin.

Fig. 1-C, D

III. Results

None of multinucleated cells in any group stained for MIB 1 antibody (Fig. 1). In immunohistochemical staining of group 1, no tumor cells with single nuclei were stained with MIB 1. In group 2F, 9.4% of mononuclear tumor cells showed nuclear staining, and 10.4% of such

cells in group 2E stained with MIB 1 (Fig. 1-C, D). In group 3F, 15% of cells were stained, as were 19.7% of cells in group 3E (Fig. 1-E, F and Fig. 2). However, specimens fixed with 70% ethanol revealed vacuoles in the nuclei after autoclave treatment (Fig. 3).

Fig. 1-E, F

Fig. 2. Percentages of MIB 1-positive cells, by fixation and pretreatment.

Fig. 3. Percentages of vacuolated cells, by fixation and pretreatment.

IV. Discussion

Ki-67 is a monoclonal mouse antibody which has been shown to react with a nuclear antigen in proliferating cells [3, 4]. Numerous studies have found the Ki-67 labeling index to be prognostically relevant in human tumors [1, 2]. However, routine use of Ki-67 has only involved fresh tissue, since Ki-67 antigen is masked by fixation and paraffin embedding. Key *et al.* have raised a new monoclonal antibody, designated MIB 1, by immunizing mice with recombinant Ki-67 gene product. They reported that after paraffin sections immersed in metal salt solutions were heated in a microwave oven, MIB 1 showed strong and discrete staining of proliferating cell nuclei in

lymph nodes [6]. Shin *et al.* reported that autoclave treatment retrieved antigen clearly [8]. In this study, we attempted to retrieve Ki-67 antigen in giant cell tumor of bone using autoclave treatment.

The results we obtained indicated that Ki-67 antigen was retrieved by autoclave treatment, even in these decalcified specimens. Positivity for Ki-67 antigen was increased in group 3E and 3F over the less strongly heated group 2E and 2F (Figs. 1, 2). However, the higher temperature also produced more damage in specimens, especially when fixed with ethanol (Fig. 3). Although the mechanism concerning autoclave recovery of antigen is not clear, heat itself may be important since the higher temperature produced stronger retrieval.

The method described in this report is simple, and has the advantages of little background staining and enhanced laboratory safety since the immersion solution does not contain metal salts such as lead.

These results showed that autoclaving can expand possibilities for retrieval of masked antigen, promising to salvage tissues previously considered unsuitable for immunohistochemical analysis. This method reduces the incidence of false-negative staining, enhancing diagnostic accuracy.

V. References

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