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Antigen Retrieval for Light and Electron Microscopy

Shuji Yamashita

Abstract

Heat-induced antigen retrieval (HIAR) method reported by Shi et al. in 1991 has greatly contributed not only to immunohistochemistry but also to studying gene expressions using archived formalin-fixed and paraffin-embedded (FFPE) specimens. Heating cleaves crosslinks (methylene bridges) in formaldehyde-fixed proteins and extends polypeptides to expose epitopes hidden in the inner portion of antigens or covered by adjacent macromolecules. In this chapter, the following topics are described to reconsider the concept of immunohistochemistry flexibly and to apply HIAR for further immunological studies using a variety of specimens: (1) antigen-antibody interactions in tissues; (2) mechanisms of chemical fixation with formaldehyde, glutaraldehyde, and osmium tetroxide; (3) unmasking of epitopes using HIAR for specimens fixed with chemical fixatives, including highly masked epitopes; (4) a standardized fixative for immunoelectron microscopy-based HIAR; (5) HIAR for conventionally processed electron microscopy specimens embedded in epoxy resins; and (6) effects of antibody diluents on immunohistochemistry.

Keywords: heat-induced antigen retrieval, antigen-antibody reaction, mechanism of chemical fixations, mechanisms of antigen retrieval, epitope exposure, immunoelectron microscopy, standardized fixative, osmicated specimens, antibody diluents

1. Introduction

Immunohistochemistry is used for identifying the localization of cellular or tissue constituents (antigens) based on antigen-antibody interactions using labeled antibodies that can be visualized under light and electron microscopes. Therefore, the use of specific primary antibodies and tissue preparation techniques that conserve fine structures, the immobilization of antigens, and antigen-antibody interaction is essential. Until the end of the 1980s, the conservation of protein conformation was thought to be important for the immunohistochemistry of protein antigens for the following reasons. (1) The production and specificity of antibodies were mainly confirmed by immunoprecipitation using Ouchterlony double diffusion test and immunoelectrophoresis, in which native purified antigen proteins or tissue extracts were reacted with antisera to form precipitation lines at the proper antigen/antibody ratio in agar or agarose gels. (2) Strong immunoreactions were observed in tissues and cells that were fixed using formaldehyde within a short time or using other physical fixatives, such as cold acetone. (3) Frozen sections provided stronger immunostaining than paraffin sections, which denatured protein conformations during dehydration and embedding.

On the other hand, monoclonal antibodies began to be prepared in many laboratories in the 1980s, and commercially available monoclonal antibodies that recognized special cell types were being applied for pathological diagnosis. Western blotting and enzyme-linked immunosorbent assays (ELISAs) were introduced as the main techniques for detecting antigen-antibody reactions. Since these techniques involve the immobilization of both native and denatured antigens on membranes or plastic plates and antigen-antibody reactions are visualized using enzyme-labeled antibodies, these techniques have a higher sensitivity than immunoprecipitation methods independent of the antigen/antibody ratio. These new antibody preparation techniques and assay systems have gradually changed the concept that it is important to expose epitopes for immunoreactions rather than preserving the conformation of whole antigen molecules.

Histopathologists have made great efforts to use formalin-fixed and paraffin-embedded (FFPE) specimens for immunohistochemistry, since such specimens have long been used as the standard for light microscopy and are archived in many biological and clinical laboratories. In the early 1970s, treatments with enzymes such as proteases, nucleases, and hyaluronidase and with protein denaturants were introduced to enable the partial recovery of immunostaining. The development of heat-induced antigen retrieval (HIAR), as reported by Shi et al. in 1991 for FFPE specimens, completely changed the concept of immunohistochemistry [1]. Although the mechanism of HIAR was originally a mystery, several studies have elucidated that heating cleaves chemical crosslinks (methylene bridges) formed by formaldehyde and exposes epitopes in tissues [2, 3]. HIAR is now applied not only to FFPE sections but also to frozen sections, cultured cells, physically fixed materials, and plastic embedded specimens for both light and electron microscopy [4]. It is also applied to other histochemical fields, such as in situ hybridization and lectin histochemistry. Furthermore, FFPE specimens are recognized as useful resources to study protein expression, DNA aberrations, and RNA expression in normal and diseased tissues [5, 6].

In this chapter, the following topics will be described, focusing on a flexible reconsideration of the concept of immunohistochemistry: (1) antigen-antibody interactions in tissues, (2) mechanisms of chemical fixation, (3) mechanisms of HIAR in formaldehyde-fixed specimens including exposure of highly masked epitopes, (4) HIAR in immunoelectron microscopy including the use of conventionally processed specimens embedded in epoxy resins, and (5) effects of antibody diluents on immunohistochemistry.

2. Antigen-antibody reactions

Antibodies recognize epitopes, which are small areas of antigen proteins but not the entire antigen molecule. When purified proteins from tissues or recombinant proteins are injected into animals, polyclonal antibodies that react with multiple epitopes and have different affinities and immunoglobulin classes for each epitope are generated. If a synthesized linear peptide is used for the immunogens, generated antibodies with different affinities and classes and subclasses of immunoglobulins may react with a single epitope. Therefore, we should notice that a commercially available polyclonal antibody may show different immunoreactions when we use the antibody with different lot numbers. On the other hand, a monoclonal antibody reacts with a single epitope with a monovalent affinity and a single immunoglobulin class and subclass. Antibodies prepared using the phage display technique have almost the same characteristics as monoclonal antibodies.

There are two types of epitopes, i.e., linear and conformational epitopes [4]. Linear epitopes are composed of a particular stretch of 5–20 consecutive amino

acids. Linear epitopes located on the surfaces of protein molecules can react with antibodies independent of whether the protein is in its native or denatured and extended states. Meanwhile, linear epitopes located on surfaces in contact with other proteins or the subunits and internal linear epitopes cannot bind to antibodies when the protein is in its native state and can only bind to antibodies when the polypeptides have been extended by heating or treatment with protein denaturants, such as sodium dodecyl sulfate (SDS) and urea. A few linear internal epitopes are rarely in contact with antibodies because the antigen proteins contain multiple disulfide bonds and the protein remains stable even after heating. The reduction of these disulfide bonds may be necessary for the exposure of such epitopes, as described later. Conformational epitopes are composed of amino acids that are located far apart in their linear sequence but become juxtaposed when the protein is folded into its native shape. Some conformational epitopes are stabilized by disulfide bonds and resistant to denaturation by heating and protein denaturants, but others associated with noncovalent forces may be sensitive to denaturation.

In immunohistochemistry, fixation is essential to conserve tissue structures and to prevent the diffusion of antigens. Chemical fixatives directly modify epitopes and crosslink proteins and nucleic acids to form gel-like structures that inhibit antigen-antibody interactions. The antigen-antibody reactions can be interfered with in special regions, such as cell organelles with intact membrane and secretory granules and deposits of protein fibrils that contain highly concentrated proteins, as well as in nuclei and extracellular matrices with highly negative electrostatic charges. Therefore, immunohistochemistry must be performed under conditions that promote the epitope-antibody association in the target tissues through the use of tissue-processing procedures, i.e., fixation, embedding, antigen retrieval, and the selection of suitable diluents for antibodies.

3. Mechanisms of chemical fixation

Chemical fixatives used for immunohistochemistry are limited to formaldehyde and glutaraldehyde. Formaldehyde is used for tissue fixation in both light and electron microscopy, while glutaraldehyde is used as a fixative only in electron microscopy. Although formaldehyde and glutaraldehyde are popular fixatives for histology and pathology, the characteristics and fixation mechanisms are assumed to be quite different. Since formalin is composed of about 35% formaldehyde aqueous solution containing about 10% methanol to prevent the polymerization of formaldehyde and is usually diluted 10-fold as 10% formalin, its fixation mechanisms should be the same as those for 4% formaldehyde.

3.1 Formaldehyde

The mechanism of fixation using formaldehyde is thought to be as follows. Formaldehyde forms an adduction of hydroxymethyl/methylol (CH_2OH) to functional groups of amino acids (such as lysine, arginine, and cysteine) (**Figure 1a, d**, and **f**), the N-terminus of polypeptides, and bases of RNA and single strand DNA [7, 8]. A part of the methylol group of lysine and arginine forms imines (Schiff base) through the removal of H_2O (**Figure 1a** and **d**), and the imines of lysine then combine with the side chains of amino acids, such as tyrosine (**Figure 1b**), tryptophan (**Figure 1c**), asparagine, glutamine, and histidine, and the imine of arginine (**Figure 1e**) to form approximately 0.25-nm methylene bridges (-CH_2-). Although lysines are reported to be major reactive residues for formaldehyde in native proteins, only lysines located on the surface area are modified by formaldehyde [9]. Methylols of cysteine form methylene
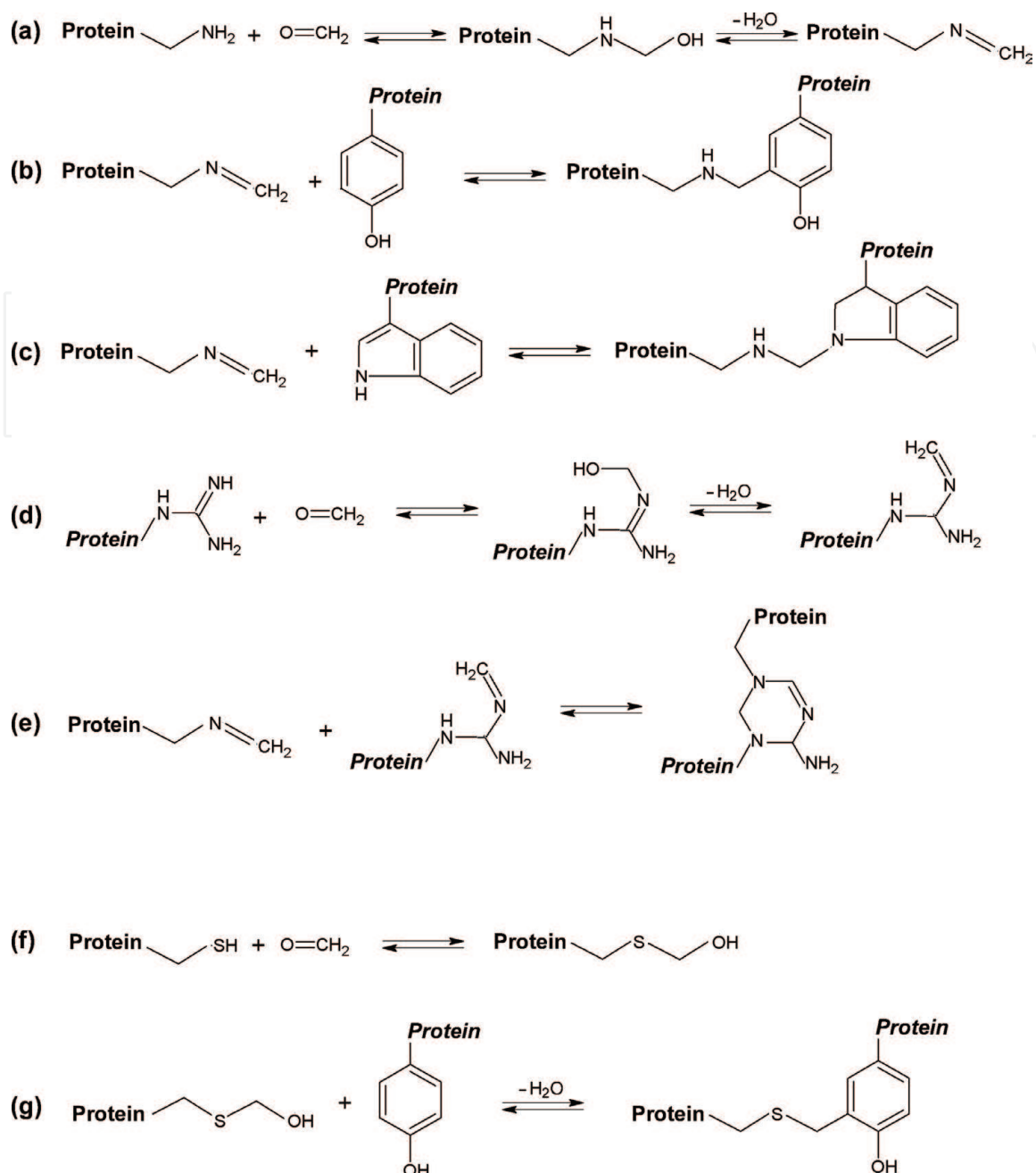
Figure 1.
Reaction of formaldehyde with proteins.

bridges with tyrosine (**Figure 1g**), arginine, and the N-terminus of peptides. These adductions, imine formations, and crosslinks progress in a time-dependent and temperature-dependent manner. Although formaldehyde is rapidly and freely permeable into cells and tissues blocks, the chemical reactions are relatively slow. Fox et al. reported that the binding of ^{14}C formaldehyde to 16 μm of fresh frozen sections only reached a plateau after 24 h at 25°C [10].

Although formaldehyde forms intra- and intermolecular crosslinks in proteins, the tertiary structures of the proteins are almost completely preserved [9, 11]. The methylene bridges between lysine and the phenyl residue of tyrosine are stable but most methylene bridges are unstable and reversible. Since basic residues of amino acids are modified with formaldehyde and the isoelectric point of proteins shifts to acidic, basic proteins should be precipitated at around the pH of the buffer (pH 7.2–7.4) used to dissolve the formaldehyde, based on the principle of isoelectric precipitation. Formaldehyde may first produce crosslinks among proteins in relatively stable core complexes, such as cell organelles, filament proteins in the cytoplasm

and extracellular matrix, and chromatin, and then soluble proteins attach to these complexes to form a gel-like structure. Thus, these crosslinks interfere with the access of antibodies to antigens even if the epitopes do not have functional groups of amino acids that are directly modified by formaldehyde, as demonstrated in the model system by Sompran et al. using peptide epitopes coupled to glass slides [12].

FFPE specimens are assumed to be highly cross-linked, compared with formalin-fixed frozen sections. Since ethanol accelerates the imine formation of methylol groups and causes the rearrangement of the β-sheet of polypeptides and the exposure of hydrophobic amino acids, which are hidden in aqueous solutions, both intra- and intermolecular crosslinking should advance during dehydration and clearing with ethanol and xylene [13], and immersion in paraffin at around 60°C facilitates further crosslinking. On the other hand, some antigens in cell organelles might come in contact with antibodies more easily than those in frozen sections because membrane lipids are extracted and barriers are destroyed during dehydration.

3.2 Glutaraldehyde

Glutaraldehyde has been widely used as the standard primary fixative for electron microscopy specimens since introduced by Sabatini et al. in 1963 [14]. A mixture of glutaraldehyde and formaldehyde is also a popular fixative for cytology, enzyme cytochemistry, and immunoelectron microscopy. Glutaraldehyde (**Figure 2a**) has two aldehydes that can directly crosslink with the ε-amino residues of lysine and the N-terminus of polypeptides by forming a Schiff base. However, most investigators think that the rapid and extremely stable crosslinks formed by glutaraldehyde are based on the oligomeric form of glutaraldehyde. Kawahara et al. demonstrated that protein crosslinkage by forming the Schiff base and the aldol condensation of glutaraldehyde monomers occur almost in parallel and result in the formation of a linear glutaraldehyde oligomer with several Schiff base linkages branching off forming $(-CH=CH-CH=N-R)_n$ (**Figure 2b**), since glutaraldehyde solution showed no absorbance at 235 nm caused by α,β-unsaturated bonds in the absence of amines [15]. The resulting resonance structures are extremely stable to both heat and acid treatments [16].
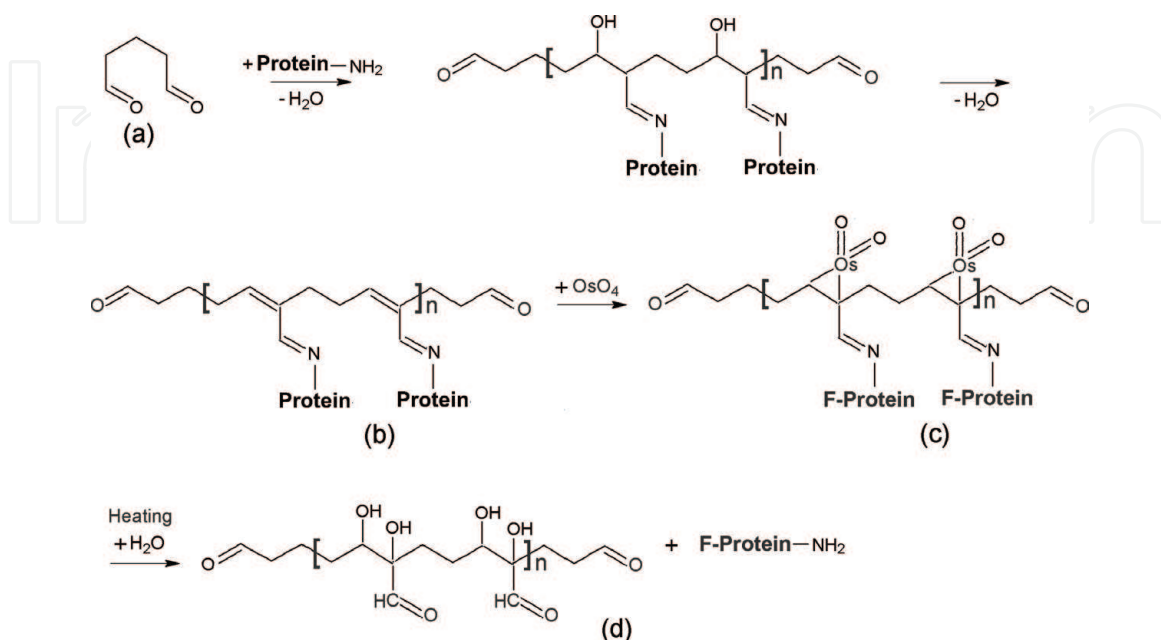

Figure 2.
Reaction of glutaraldehyde and osmium tetroxide with proteins and effect of heating. F-protein, fragmented protein by osmium tetroxide post-fixation.

Since the cross-linked proteins rapidly form harder gel-like structures, compared with those created by formaldehyde, only thin layers of tissues can be fixed well using immersion fixation. Since aldehyde residues remain in the tissues fixed with glutaraldehyde, the aldehyde should be quenched using amides, such as glycine, ammonium chloride, and tris(hydroxymethyl) aminomethane, or reduced to alcohols using sodium borohydride prior to immunostaining.

3.3 Osmium tetroxide

Since osmium tetroxide binds to the unsaturated bonds of fatty acids and fixes membrane lipids, providing contrast by scattering electron beams, it is used as the post-fixing reagent after glutaraldehyde fixation in electron microscopy. Osmium tetroxide should also bind to the carbon-carbon double bonds formed by glutaraldehyde fixation (**Figure 2c**). However, since osmium tetroxide cleaves polypeptides in tryptophan residues and oxidizes methionine to methionine sulfone and cysteine to cysteic acid [17], osmium tetroxide significantly inhibits immunoreactions.

4. Mechanisms of HIAR

After the first report for HIAR by Shi et al. [1], investigators have tried to select the most suitable heating conditions (heating devices, temperatures, kinds of solutions, solution pH, and additives). However, the total amount of applied heat energy is now recognized as being more important than the type of heating devices. In this section, the effects of pH and the ionic strength of retrieval solutions for HIAR will be reviewed, and the mechanisms of HIAR will be described.

4.1 Effects of pH on proteins treated with formaldehyde

When purified proteins are treated with formaldehyde and analyzed using SDS-PAGE (polyacrylamide gel electrophoresis), protein oligomers formed by intermolecular crosslinks were recognized. Monomer and oligomers treated with formaldehyde showed smaller apparent molecular weight compared with those of unmodified native proteins, since intramolecular crosslinks prevented the complete unfolding of proteins in the SDS solution [2, 18, 19]. The cleaving efficiency of the crosslinks was almost the same when the formaldehyde-treated proteins were heated for 5 min at 100°C in 10 mM Tris-HCl at pH 3.0, pH 6.0, pH 7.5, or pH 9.0 while analyzed with SDS-PAGE. When the proteins were drastically heated by autoclaving for 10 min at 120°C at a pH close to their respective isoelectric points, the proteins tended to produce insoluble protein precipitates [2]. However, many investigators have demonstrated that the efficiency of HIAR for immunohistochemistry is highly dependent on the pH of the retrieval solution.

4.2 Effect of pH of HIAR solutions on immunohistochemistry

Shi et al. systematically studied the effects of the pH of antigen retrieval solution on HIAR [20]. They classified the pH-influenced HIAR immunostaining patterns as follows: type A, in which staining was almost the same at any pH, with a slight decrease in intensity between pH 3.0 and pH 6.0; type B, in which a dramatic increase in immunostaining was observed at acidic and basic pH; and type C, in which the immunostaining intensity increased at basic pH. We re-examined the pH dependency of HIAR using 17 different antibodies and observed two immunostaining patterns for the pH dependency of HIAR [3]. The majority of the antibodies produced the first immunostaining pattern; that is, they yielded a positive

immunoreaction when heated in buffers that had either an acidic pH or a basic pH. This HIAR pattern may correspond to the type-B pattern of the classification by Shi et al. If highly diluted antibodies had been used in the immunohistochemical studies, the type-A pattern described by Shi et al. might have become nearly equivalent to the type-B pattern. The second immunostaining pattern that we observed was a strong immunostaining reaction when heated in basic buffer, corresponding to the type-C pattern described by Shi et al. Pileri et al. and Kim et al. have also reported that a basic buffer is effective for HIAR for most antigens [21, 22]. On the other hand, Kajiya et al. reported that heating at an acidic pH (pH 3.0 or pH 6.0) frequently enabled excellent immunostaining for the detection of basic proteins or epitopes composed of basic amino acids [23].

4.3 pH-dependent reversibility of HIAR efficiency

Yamashita and Okada demonstrated that the intensities of the immunoreactions obtained by heating in a buffer are reversibly altered by successive heating in another buffer with a different pH [2]. For example, when the first heating in a buffer (pH 6.0) yielded a weak immunostaining in FFEP sections, a second heating at pH 9.0 significantly increased the immunostaining; however, the third heating in the acidic buffer weakened the immunostaining. These results indicate that the degradation or extraction of antigens is not a major factor in the pH dependency of HIAR and that the pH of the solution is a critical factor for the exposure of tissue epitopes in HIAR.

4.4 Effect of ionic strength of HIAR solution

We studied the effects of ionic strength on HIAR using 10 antibodies. Three buffer systems with different pH values were examined. When FFPE specimens were autoclaved for 10 min at 120°C in 20 mM Tris-HCl buffer (pH 9.0), 50 mM citraconic anhydride aqueous solution (pH 7.4), or 10 mM citrate buffer (pH 6.0) containing 0, 50, 100, or 200 mM NaCl, all the antibodies showed the strongest immunostaining while the sections were autoclaved in the NaCl-free solutions. The staining intensity decreased as the NaCl concentration increased in all antibodies examined [24]. These results demonstrated that the ionic strength of the solution is a critical factor for HIAR and that a high concentration of salt inhibits the exposure of epitopes.

4.5 Mechanisms of HIAR in FFPE sections

The results described above demonstrate that the fundamental mechanism of HIAR is based on the cleavage of protein-protein crosslinks and the exposure of epitopes. Heating destroys the gel-like structure formed by formaldehyde-fixation and partially extracts the macromolecules, enabling the antibodies to penetrate tissues easily; this process is similar to the effects of enzyme digestion. Western blot analyses have demonstrated that soluble, nuclear, and membrane proteins are extracted from FFPE specimens after heating but not from those without heat treatment [2]. Recent proteomics studies using a mass spectrum technique have also revealed that heating facilitates protein extraction from archived FFPE specimens [6]. Furthermore, heat-induced cleaving of the shortest crosslinks induced by formaldehyde can be applied to chromatin immunoprecipitation assays and to the crosslinks of adjacent proteins to investigate temporal interactions [8, 25, 26].

The second mechanism is assumed to be as follows based on the pH-dependent and ionic strength-dependent phenomena described above [3, 4]. When the methylene bridges are cleaved by heating, the higher order structure of the protein is destroyed and the polypeptide chains are extended, exposing both hydrophobic

and hydrophilic regions and epitopes. The polypeptide chains then rapidly refold during the cooling process. In tissues, many kinds of proteins with different isoelectric points and molecular weights are tightly packed, and neighboring polypeptides can come in contact with each other. Therefore, epitopes should be concealed during the refolding of the proteins at around a neutral pH because a strong hydrophobic attractive force would randomly entangle the neighboring polypeptides: an electrostatic force may act locally as either an attractive or a repulsive force. At basic or acidic pH values, however, the majority of the extended polypeptides would be charged negatively or positively, and the electrostatic repulsive force would act to prevent random aggregation and entanglement with neighboring polypeptides caused by the hydrophobic force, thereby maintaining a suitable extend conformation for antigen-antibody interactions. When salt is added to the retrieval solutions, the electrostatic force between neighboring polypeptides is canceled, and the hydrophobic attractive force may cause the antigen proteins and neighboring proteins to aggregate, masking the epitopes. Namimatsu et al. reported that heating in citraconic anhydride solution at a neutral pH was useful as a universal antigen retrieval method [27]. Since citraconic anhydride binds to the ε-amino groups of lysine residues re-exposed after heating and places numerous negative charges on proteins, an electric repulsive force may help to keep polypeptides in an unfolded state.

On the other hand, strong heating at around the isoelectric points of proteins induces their coagulation [2], and increasing the ionic strength also promotes isoelectric precipitation. Therefore, many proteins with neutral isoelectric points can be precipitated in a solution with a neutral pH. The finding that an acidic buffer is effective for some basic antigens probably indicates that these antigens are precipitated by heating in basic buffers [23, 28]. Since heating may destroy the protein conformation, most conformational epitopes associated with noncovalent forces should lose their reactivity to antibodies. On the other hand, HIAR may be effective for conformational epitopes that have been stabilized by disulfide bonds.

Basic or acidic solutions are effective for HIAR as described above, whereas citrate buffer (pH 6.0) is frequently used in pathological studies. Citrate buffer may be suitable for examining detailed nuclear structures, since heating in basic solutions cleaves and extracts RNAs and reduces the nuclear stainability with hematoxylin. In practice, at least three antigen retrieval solutions at pH 3.0, pH 6.0, and pH 9.0 should be examined when studying the localization of unknown antigens for the first time.

4.6 HIAR in frozen sections

Fresh frozen sections are widely used for immunohistochemical studies, because (1) they preserve antigenicity well, (2) they provide reproducible results because the fixation time is precisely controlled and the fixation is uniform throughout the sections, and (3) they allow antigen localization within a short time for pathological diagnosis. We introduced new fixative, 10% formalin containing 25 mM $CaCl_2$ in 0.1 M HEPES-NaOH buffer (pH 7.4) that is more appropriate for the fixation of fresh frozen sections compared with buffered 10% formalin, because it has a stronger fixation ability and can crosslink proteins more rapidly and stabilize membranes, the extraction of antigens during fixation is minimized, and an excellent tissue structure is maintained after HIAR [29]. After heating in 20 mM Tris-HCl (pH 9.0) for 30 min or in 20 mM citrate buffer (pH 3.0), antigens in the nuclei, other cell organelles, cytoplasm, membranes, and extracellular matrix were clearly visible, even if they showed no immunoreactions without heating.
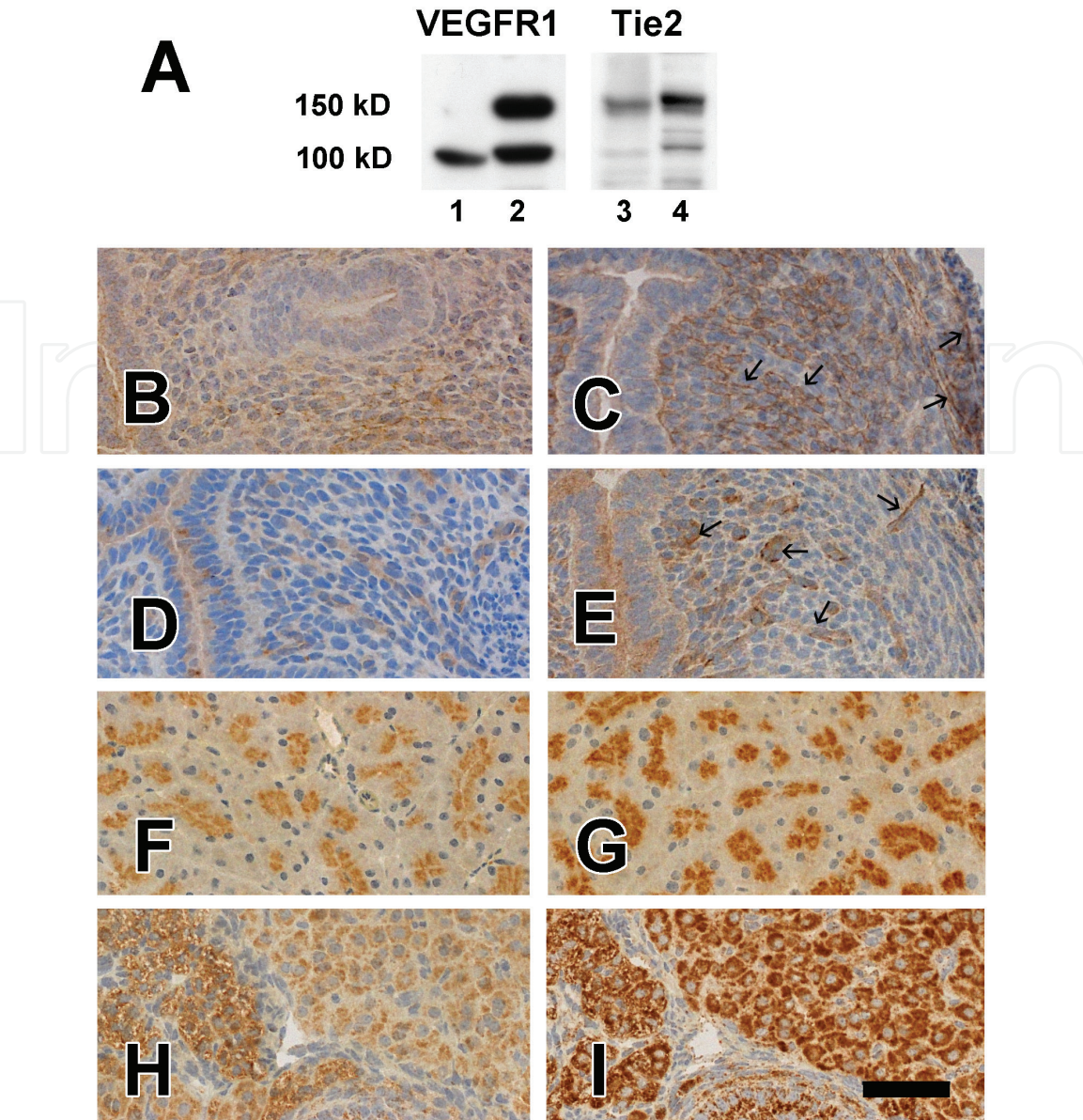
Figure 3.
Antigen retrieval for highly masked epitopes with disulfide bonds in paraffin and frozen sections. A. VEGFR1 and Tie 2 expressions in the uteri of 10-day-old mice were demonstrated using Western blotting. VEGFR1 and Tie 2 proteins were analyzed after treatment with 2-mercaptoethanol (lines 2 and 4) or without the treatment (lines 1 and 3), respectively. Membrane bound VEGFR1 (150 kD) shows no reaction to the antibody without reduction of disulfide bonds, whereas soluble VEGFR1 (100 kD) binds to the antibody independence of reduction (lines 1 and 2). Tie 2 antibody shows strong immunoreaction to Tie 2 protein-reduced disulfide bonds (lines 3 and 4). B–E. Immunostaining of VEGFR1 (B and C) and Tie 2 (D and E) in the frozen sections of 10-day-old mouse uteri. Fresh frozen sections were fixed with 10% formalin containing 25 mM CaCl$_2$ in 0.1 M HEPES buffer (pH 7.4) for 5 min, treated with 0.1 M dithiothreitol (DTT)/20 mM Tris-HCl (pH 9.0) for 1 h and then with 0.1 M iodoacetamide/0.1 M Tris-HCl (pH 9.0) for 15 min (C and E); B and D, sections without reduction. The sections were then heated in 20 mM Tris-HCl (pH 9.0) for 30 min at 95°C and immunostained with the antibodies. The stroma shows diffuse VEGFR1 immunostaining which may be based on the soluble VEGFR1 in non-reduced section (B), while vasculatures (arrows) and basal membrane of epithelial cells exhibit positive VEGFR1-immunostaining which may be corresponded to the membrane-bound VEGFR1 (C). Clear Tie 2-immunostaining is recognized in the endothelial cells of vasculatures (arrows) in the reduced section (E) but not in the non-reduced section (D). F–I. Immunohistochemistry in paraffin sections. Clathrin was detected in the mouse pancreas after reduction with DTT (G) or without reduction (F) followed by heating in 20 mM Tris-HCl (pH 9.0). Tom 20 was immunostained in the mouse ovary after reduction with DTT (I) or without reduction (H) followed by heating. Bar = 50 μm.

4.7 Antigen retrieval for highly masked epitopes with disulfide bonds

Few antigens that reduced disulfide bonds react with antibodies when they are analyzed using Western blotting (**Figure 3A**), whereas they reveal negative immunostaining even if the tissues were fixed with formaldehyde or other physical

fixatives followed by heating (**Figure 3B** and **D**). In such cases, the epitopes should be located in a highly folded portion of the antigen protein that may be stabilized with disulfide bonds to form stable secondary and tertiary structures. The reduction of disulfide bonds using dithiothreitol or 2-mercaptoethanol prior to heat treatment yields strong immunoreactions (**Figure 3C** and **E**) [28, 30]. In addition, when epitopes are covered by neighboring heat-resistant polypeptides, the accessibility of antibodies to the epitopes is also inhibited. When immunostaining is performed using old FFEP or using sections on a slide glass stored for a long time, the samples should be oxidized to produce many disulfide bonds in the tissues. The reduction and cleavage of disulfide bonds may be effective for such specimens (**Figure 3F–I**).

5. HIAR in immunoelectron microscopy

Immunoelectron microscopy is a powerful technique for observing the localization of antigens in cell organelles and for studying the relationship between antigens and other macromolecules. Three main immunoelectron microscopy methods have been used to localize antigens: the pre-embedding method, the post-embedding method, and cryoultramicrotomy. Although fixation is one of the most important aspects of sample preparation for all three methods, the choices of fixatives and tissue processing procedures are limited unlike light microscopy, because of the need to satisfy compatible requirements, such as conservation of fine structure, immobilization of antigen minimizing the diffusion, and conservation of immunoreaction. In general, fixatives that allow good morphological findings and precise antigen localization through the rapid and tight crosslinking of macromolecules also severely inhibit antigen-antibody interactions.

Fixatives containing formaldehyde as the main crosslinking regent are popular for immunoelectron microscopy using the pre-embedding method, since antibody penetration into the cells is an important factor for this method; formaldehyde solution, PLP (periodate-lysine-paraformaldehyde) [31], and a mixture of formaldehyde and a low concentration (0.05–0.5%) of glutaraldehyde. For the post-embedding method, a mixture of formaldehyde and a low concentration of glutaraldehyde is frequently used to preserve the fine structure and membrane structures, since dehydration and resin embedding are performed without osmium tetroxide post-fixation. A short period of perfusion fixation with glutaraldehyde is also applied for the post-embedding method. The suitable fixatives, fixing periods, and temperatures of fixatives have been determined by trial and error for each antigen independent of the staining methods.

In this section, a standardized fixation method that yields positive immunoreactions for the pre-embedding and the post-embedding methods after HIAR will be described and discussed how HIAR is also effective for some routinely processed materials for the electron microscopy that are fixed with glutaraldehyde and osmium tetroxide and embedded in epoxy resins.

5.1 Standardized fixative

We introduced a standardized fixative that can yield positive immunoreactions for many antigens in electron microscopy after HIAR [32]. Tissues were fixed with 4% formaldehyde containing 2.5 mM $CaCl_2$ and 1.25 mM $MgCl_2$ in 0.1 M HEPES-NaOH buffer (pH 7.4) for 2 h at room temperature and then with the same fixative composition in 0.1 M HEPES buffer-NaOH buffer (pH 8.5) overnight at room temperature. The vehicle osmolarity of the fixatives was adjusted to 300–330 mOsm by adding sucrose or glucose. Formaldehyde containing $CaCl_2$ and $MgCl_2$ was shown to

be the best fixative, producing a rapid and complete fixation that minimizes diffusion artifacts (the divalent cations are well known to act as stabilizers of membrane structures). In addition, tissues were fixed using two steps: first at pH 7.4 and then at pH 8.5. This method preserves the cellular fine structures because the crosslinking reaction produced by formaldehyde progresses rapidly at a basic pH. Fixation was then performed at room temperature to enable a faster reaction than that possible at 4°C.

5.2 Pre-embedding method

Although the pre-embedding method is the most popular and the simplest method for immunoelectron microscopy, HIAR has only been applied for the detection of a few antigens. Frozen sections or vibratome sections from specimens fixed with formaldehyde or a mixture of formaldehyde and glutaraldehyde were heated in various solutions such as citrate buffer (pH 6.0), Tris-HCl buffer (pH 9.0 or pH 10.0), or citraconic anhydride solution (pH 7.4) for different periods for each antigen [33–36]. Yamashita reported that 4% formaldehyde containing 25 mM $CaCl_2$ in 0.1 M cacodylate buffer (pH 7.4) was a suitable fixative for the pre-embedding method by applying HIAR for several antigens [4].

We applied the pre-embedding method to tissues fixed using the standardized fixative described above. Frozen sections (about 15 μm) were mounted on a slide glass and then heated in 20 mM Tris-HCl (pH 9.0) containing 10% sucrose for 2–4 h at 70°C. Immunostaining was performed using (horseradish peroxidase) HRP-labeled antibodies and antigen localization was visualized with 3,3′diaminobenzidine (DAB). Most of the antigens that were examined showed negative immunoreactions without heat treatment, but they produced strong immunoreaction after heating (**Figure 4**). Since endogenous immunoglobulins are inactivated after heat treatment (**Figure 4A** and **B**), the immunoreactions can be clearly detected even in the mouse tissues using mouse monoclonal antibodies. Tris-HCl buffer (pH 9.0) is effective for most antigens but citrate buffer (pH 6.0 or pH 3.0) yields strong reaction for a few antigens with basic isoelectric points, such as vascular endothelial cell growth factor (VEGF). Therefore, the selection of suitable solutions for each antigen should be examined using FFEP sections or frozen sections on light microscopy.

The positively immunostained sections were then post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 30 min, dehydrated with ethanol, and then embedded in epoxy resin. All antigens detected in frozen sections on light microscopy were localized using the pre-embedding method preserving fine structures (**Figure 5**).

5.3 Post-embedding method

The post-embedding method provides more reproducible and reliable immunostaining results than the pre-embedding method, which produces a limited and heterogeneous penetration of antibodies into the tissues, since immunoreactions occur on the surfaces of ultrathin sections. Furthermore, immunoreactions on ultrathin sections permit the counting of immunogold particles, enabling semi-quantitative analyses, and the simultaneous staining of multiple antigens. Although the post-embedding method has these advantages, it has only been used for a limited number of antigens because antigenicity is frequently lost during the dehydration and embedding procedures. Specimens embedded in acrylic resins without osmication show a disrupted membrane structure and poorly contrasted cell organelles.
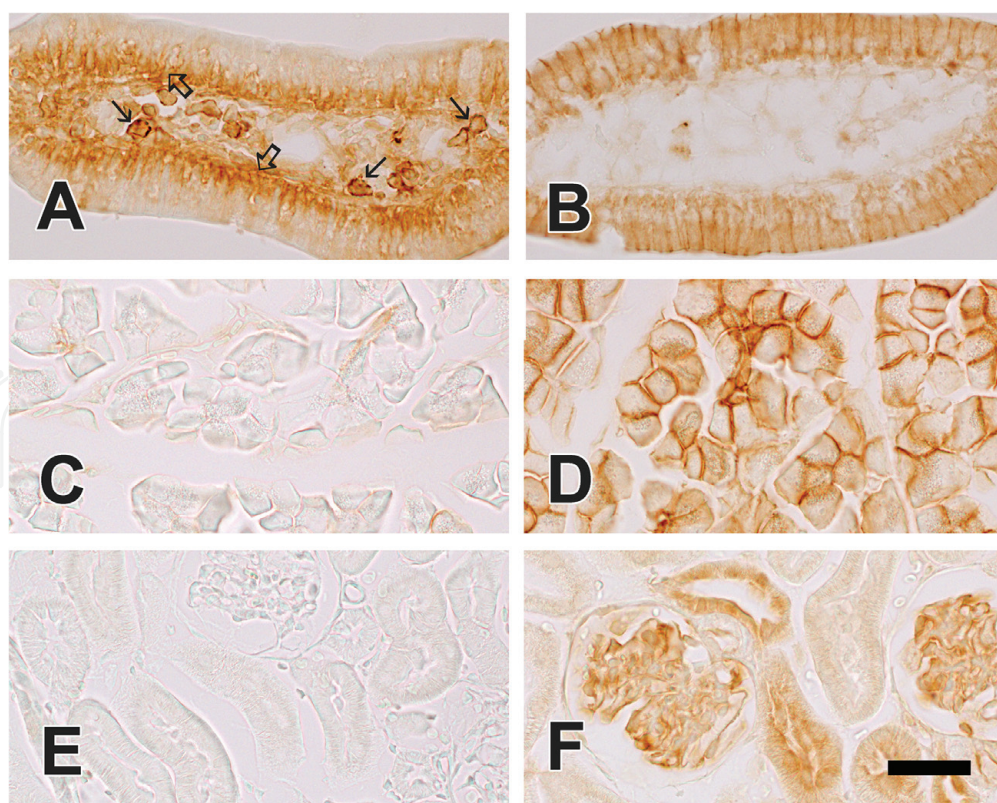
Figure 4.
HIAR in frozen sections form specimens fixed with the standardized fixative. Mouse tissues were fixed with the standardized fixative and immersed in 10, 15, and 20% sucrose and then frozen. Frozen sections (15 µm) were immunostained with anti-E-cadherin monoclonal antibody in the small intestine (A and B), anti-β-catenin monoclonal antibody in the pancreas (C and D), and anti-claudin 5 polyclonal antibody in the kidney (E and F) after heating in 10 mM Tris-HCl buffer (pH 9.0) containing 10% sucrose for 3 h at 70°C (B, D, and F) or without heating (A, C, and E). Endogenous mouse immunoglobulins are seen in the lamina propria mucosa (arrows) and plasma cells (outline arrows), but E-cadherin immunoreactions is not detected without heating (A). However, the endogenous immunoglobulin immunostaining is negative and E-cadherin is clearly localized along the lateral membrane of intestinal epithelium after heating; the junctional complexes show strong immunoreactions (B). β-Catenin immunoreaction is present along the lateral membrane of pancreatic acinar cells (D) but not in the section without heating (C). After heating, claudin-5 is localized in the glomeruli and distal tubules in the kidney (F). Bar = 50 µm.

Several investigators have applied HIAR to the post-embedding method and have reported its usefulness [37–39]. Tissues were fixed with a mixture of formaldehyde and glutaraldehyde or formaldehyde alone and embedded in acryl resins; then, ultrathin sections were heated in various solutions. We attempted to establish a standardized method for immunoelectron microscopy that would satisfy the following requirements: (1) the preservation of fine cell structures with good image contrast in tissues embedded in acryl resins without OsO_4 post-fixation, (2) the application of HIAR to obtain a high labeling density, and (3) a simple and reproducible method that does not require special equipment [32, 39].

Tissues were fixed with the standardized fixative and dehydrated with dimethylformamide (DMF) on ice and embedded in the LR-White resin, since DMF may reduce abrupt osmotic pressure changes in the tissues and the extraction of membrane lipids. The resin was polymerized for 24 h at 55°C. Ultrathin sections mounted on a nickel grid were heated in 0.5 M Tris-HCl buffer (pH 9.0) for 1–2 h at 95°C. After immunogold labeling, the sections were treated with 2% glutaraldehyde containing 0.05% tannic acid in 0.1 M phosphate buffer (pH 5.5) for 5 min and with 1% OsO_4/0.1 M phosphate buffer (pH 7.4) for 5 min and then double stained with uranyl acetate and lead citrate. This method yielded strong and reproducible immunoreactions for many soluble, membrane bound, and filamentous proteins (**Figure 6**), [32, 39]. Furthermore, tannic
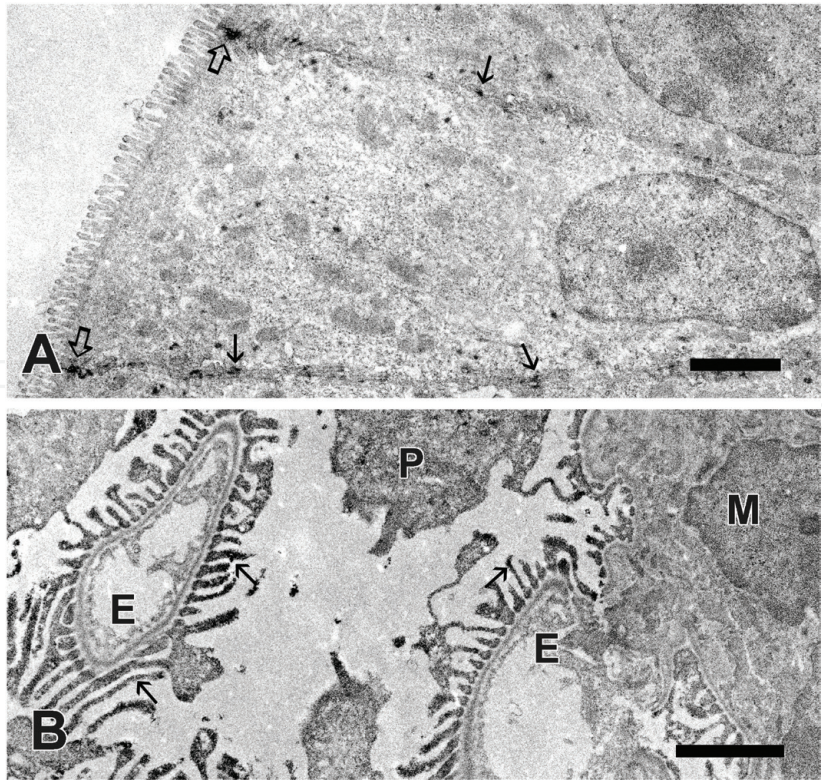
Figure 5.
*HIAR for immunoelectron microscopy with the pre-embedding method. Frozen sections immunostained with anti-E-cadherin antibody (A) and anti-claudin-5 antibody (B) demonstrated in **Figure 4** were post-fixed with osmium tetroxide, dehydrated in ethanol, and then embedded in the epoxy resin. A. Immunoreaction for E-cadherin is seen in the adherence junction (outline arrows) and spotty reaction (arrows) is present in the lateral membrane of intestinal epithelium. B. Claudin-5 is localized in the foot processes of podocyte (arrows) in the renal glomerulus: P, podocyte; E, endothelial cells; and M, mesangial cell. Bar = 2 μm.*
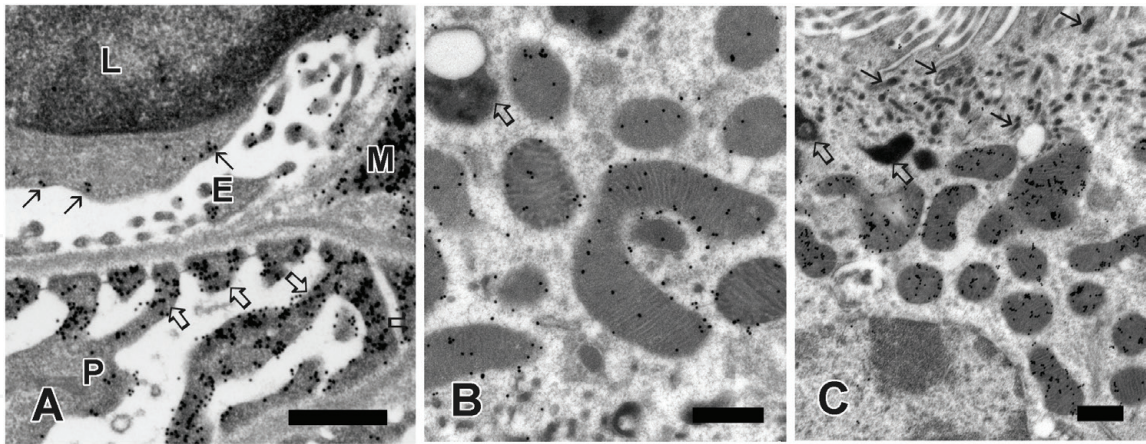

Figure 6.
HIAR for immunoelectron microscopy with the post-embedding method. Mouse kidney was fixed with the standardized fixative and then embedded in LR-White resin. Ultrathin sections were heated in 0.5 M Tris-HCl (pH 9.0) for 1 h at 95°C and treated with anti-β-actin/TBS (A), Tom 20/10 mM Tris-HCl (pH 7.4) containing 50 mM NaCl (B), and anti-mortalin (mitochondrial 70 kD heat shock protein)/TBS (C) and then treated with colloidal gold-labeled secondary antibodies/TBS. A. Strong β-actin immunoreactions are seen in the foot processes of podocyte (P) (outline arrows) and in the cytoplasm of mesangial cell (M). Lymphocyte (L) shows potty reactions beneath the cell membrane (arrows). B. Tom 20 is localized along the mitochondria but not recognized in the lysosome (outline arrow). C. Mitochondria show mortalin immunoreaction, whereas lysosomes (outline arrows), apical canaliculi (arrows), and nucleus are negative for staining. Bars = 500 nm.

acid treatment followed by osmium tetroxide treatment produced good contrasted images. The cellular membranes produced a positive image and the cell organelles, such as mitochondria, the Golgi complexes, secretory granules, and lysosomes, were well

contrasted. Nucleic acids (chromatins, nucleoli, and ribosomes), intracellular filaments (actin filaments, 10 nm filaments, and microtubules), and collagen fibers were well visualized.

5.4 Osmicated and epon-embedded specimens

Archives of materials embedded in epoxy resins are collected in many histology and pathology laboratories and in hospitals for morphological analyses, and these archives are expected to provide valuable data if they can be used for immunohistochemical studies. However, conventionally processed specimens for transmission electron microscopy have been regarded as unsuitable for immunoelectron microscopy using post-embedding methods for the following reasons: (1) Glutaraldehyde significantly suppresses antigen-antibody interactions and HIAR is ineffective for most antigens. (2) Osmium tetroxide severely inhibits immunoreactions by cleaving polypeptides and oxidizing methionine and cysteine [17]. (3) Epoxy resins produce tight three-dimensional crosslinks that suppress antigen-antibody interactions.

A limited number of antigens can be successfully detected on sections from conventionally processed materials. One method involves the oxidation and removal of osmium by treating ultrathin sections with sodium metaperiodate aqueous solution [40]. Another method involves the partial removal of epoxy resins by treating the sections with hydrogen peroxide or sodium/potassium ethoxide [41, 42]. The combined use of partial deresination and heat treatment has also been examined. Borson and Skjorten polymerized the epoxy resin to reduce copolymerization between the proteins and the resin and to make a porous polymer for applying HIAR to glutaraldehyde-fixed and epon-embedded materials [43]. We have also demonstrated that HIAR is effective for post-embedding immunoelectron microscopy using conventionally processed epon blocks, contrary to presumptions that antigen detection would be a special case in these specimens [44].

5.4.1 Frozen sections fixed with glutaraldehyde and osmium tetroxide

The effectiveness of HIAR in the post-embedding method using conventionally processed epon-embedded specimens was systematically examined for 18 antibodies. Frozen sections fixed with 2% glutaraldehyde for 30 min at room temperature or with 2% glutaraldehyde for 30 min followed by 1% osmium tetroxide for 30 min at room temperature were dehydrated with ethanol and then rehydrated to elucidate the validity of HIAR for avoiding the effects of epoxy resin embedment. In another experiment, frozen sections fixed with glutaraldehyde and osmium tetroxide were treated with sodium metaperiodate, since this reagent has been reported to be effective for osmicated materials, as described above.

After autoclaving in Tris-HCl buffer (pH 9.0), 7 of the 18 antibodies exhibited a strong immunoreaction in frozen sections fixed with glutaraldehyde and osmium tetroxide, whereas heating revealed almost no effect on the sections fixed with glutaraldehyde alone (**Table 1**; **Figure 7**). Treatment with sodium metaperiodate was ineffective for the antigen retrieval of all the antibodies (**Table 1**). The mechanisms of HIAR in the frozen sections fixed with glutaraldehyde and osmium tetroxide were assumed to be as follows [44]. Osmium tetroxide binds to ethylene bonds formed by glutaraldehyde fixation, i.e., -CH=CH-CH=N-R (**Figure 2c**). Heat treatment removes the osmium tetroxide additives and forms 1, 2-diols (**Figure 2d**), since the black color fades in frozen sections fixed with glutaraldehyde and osmium tetroxide after autoclaving. The cleaving of the double bonds should extend the antigen polypeptides and increase the flexibility of the polypeptides. However, the morphology in the frozen tissues fixed with glutaraldehyde and osmium

tetroxide was more disrupted after autoclaving, compared with those fixed with glutaraldehyde alone or 4% formaldehyde containing 25 mM $CaCl_2$. The reason may be as follows. Osmium tetroxide treatment induces polypeptide fragmentation (**Figure 2c** and **d**), and heating extracts the fragments after crosslink cleavage by glutaraldehyde.

5.4.2 HIAR in epon-embedded materials

Partial deresination with sodium ethoxide was required for light microscopy using semi-thin epon sections. After autoclaving in 100 m Tris-HCl (pH 9.0) for 10 min, six of the seven antibodies that showed positive immunoreactions in the frozen sections exhibited clear localizations in the semi-thin sections. α-Amylase (**Figure 8A** and **B**), clathrin (**Figure 8C**), and claudin-5 (**Figure 8D**), which all showed positive immunoreactions in the semi-thin sections, were localized on ultrathin sections using colloidal gold-labeled antibodies. For HIAR, ultrathin sections were heated in 500 mM Tris-HCl buffer (pH 9.0) for 1–3 h at 95°C. Although heat treatment was essential for the detection of antigens on ultrathin sections, heat treatment reduced the electron density of ribosomes, chromatins,

| | GA-EtOH | | GA-OsO$_4$-EtOH | | |
	Non	Autoclave	Non	NaIO$_4$	Autoclave
PCNA	0	1	0	0	2
Clathrin	0	0–1	0	0	3
GFAP	0	0–1	0	0	3
Occludin	0	0–1	0	0	3
Tom 20	0	0–1	0	0	3
Claudin-5	0	0	0	0	2
α-Amylase	0–1	1	0	0–1	2
NRP1	0	0	0		1
β-Catenin	0–1	0	0		0
E-Cadherin	0	0–1	0		0
Desmin	0	0–1	0		0
Caveolin	0	0	0		0
VEGFR2	0–1	0	0		0
ERα	0	1–2	0		0–1
AnR	0	0	0		0
β-Actin	1	1	0		0
γ-GTP	1	1	0		0

Fresh frozen sections were fixed with 2% glutaraldehyde/0.1 M phosphate buffer (pH 7.4) for 30 min (GA-EtOH) or fixed with 2% glutaraldehyde/0.1 M phosphate buffer (pH 7.4) for 30 min and further fixed with 1% osmium tetroxide/0.1 M phosphate buffer (pH 7.4) for 30 min (GA-OsO$_4$-EtOH). Some sections fixed with glutaraldehyde and osmium tetroxide were further treated with 1% sodium metaperiodate aqueous solution for 5 min (NaIO$_4$): all sections were hydrated with ethanol and then rehydrated after fixation. The fixed sections were immunostained after autoclaving in 20 mM Tris-HCl (pH 9.0) at 120°C for 10 min (autoclave) or without heat treatment (Non). Immunostaining was scored as followed: 3, strong; 2, moderate; 1, weak; 0–1, faint; and 0, negative.

Table 1.
HIAR in frozen sections prepared from specimens fixed with glutaraldehyde or glutaraldehyde and osmium tetroxide.
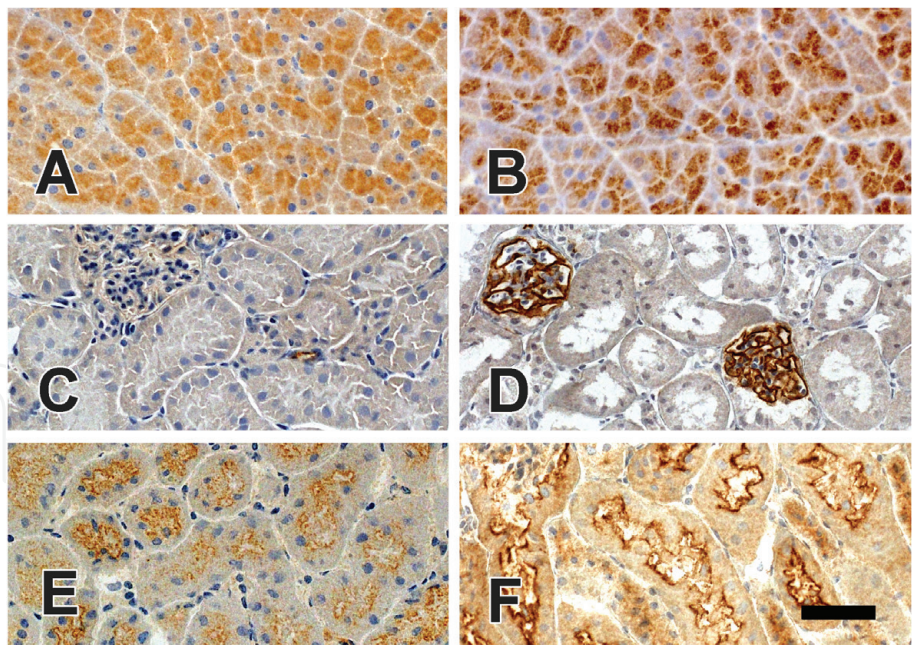
Figure 7.
HIAR in frozen sections fixed with glutaraldehyde and osmium tetroxide. Fresh frozen sections (6 μm) from mouse tissues were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature (A, C, and E) and successively with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) for 30 min at room temperature (B, D, and F). The sections were autoclaved in 20 mM Tris-HCl (pH 9.0) for 10 min at 120°C. They were then immunostained with anti-α-amylase antibody in the pancreas (A and B), anti-claudin-5 antibody in the kidney (C and D), and anti-clathrin antibody in the kidney (E and F). Although negative or weak immunostaining is seen in the sections fixed with glutaraldehyde after autoclave (A, C, and E), strong α-amylase immunoreactions are recognized in the apical cytoplasm of pancreatic acinar cells (B) and clear claudin-5 and clathrin immunoreactions are observed in the glomeruli (D) and in the apical cytoplasm of proximal tubular cells in the kidney (F), respectively. Bar = 50 μm.
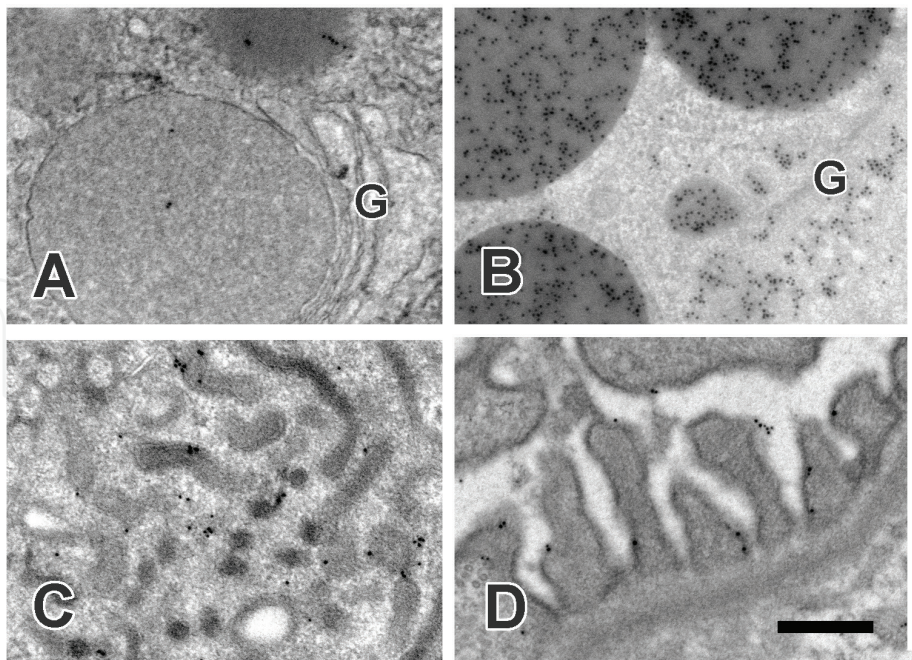

Figure 8.
HIAR for the specimens fixed with glutaraldehyde and osmium tetroxide, embedded in epoxy resin. Mouse tissues were fixed with 2% glutaraldehyde/0.1 M phosphate buffer (pH 7.4) for 3 h at 4°C and post-fixed with 1% osmium tetroxide/0.1 M phosphate buffer (pH 7.4) for 1 h and then embedded in the epoxy resin. Ultrathin sections were heated in 0.5 M Tris-HCl (pH 9.0) for 2 h at 95°C. α-Amylase is localized in the Golgi apparatus (G), condensing vacuole and secretory granules in the exocrine pancreas after heat treatment (B), whereas no reaction is seen in the sections without heating (A). Immunoreaction for clathrin is recognized in the apical canaliculi of renal proximal tubular cell (C). Claudin-5 is localized along the membrane of podocyte foot processes in the glomerulus (D). Bar = 500 nm.

intracellular membranes, and secretory granules in the exocrine pancreas. The partial removal of epoxy resins with sodium ethoxide followed by autoclaving revealed the disruption of the fine structure and no reproducible immunolabeling.

These results indicated that archived epon-embedded specimens could be a useful resource for immunohistochemical studies at both the light and electron microscopy levels, since they provide excellent morphology and detailed antigen localization compared with paraffin-embedded materials.

6. Effects of diluents on antibodies in immunohistochemistry

The relationship between epitopes and paratopes of antibodies is thought to be similar to that between keys and keyholes. However, since these structures change their conformations to form a final specific and tight binding after antigen-antibody association, conservation of the flexibility of their polypeptide chains should be important. Although hydrogen bonds, hydrophobic forces, electrostatic forces, and van der Waals forces all participate in the final tight binding, electrostatic forces are important for the initial contact and association of antigen and antibody molecules (i.e., the net charges of each molecule and the neighboring charges of antigens). Buffer type, ionic strength, pH, and the presence of detergents in solutions are likely to exert strong influences on the antigen-antibody reaction. Although many kinds of diluents are commercially available for immunohistochemistry and Western blotting and yield good results with low background staining and a high sensitivity for some antigens, systematic studies of antibody diluents for immunohistochemistry have not been performed. In this section, the effects of dilution solutions for primary antibodies on immunostaining for light and electron microscopy are described.

6.1 Kind of dilution buffers

Fifteen monoclonal antibodies were diluted in 10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl (PBS), 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl (TBS), or 10 mM FEPES-NaOH buffer (pH 7.4) containing 150 mM NaCl (HBS); 1% bovine serum albumin (BSA) (final concentration) was added to each solution. Paraffin sections from mouse tissues fixed with formaldehyde were immunostained after autoclaving in 20 mM Tris-HCl buffer (pH 9.0) for 10 min. The sections were treated with the primary antibodies diluted with the solutions overnight at 4°C and successively with Envision HRR (Dakocytomation) for 1 h at room temperature. As shown in **Table 2**, all the antibodies diluted with TBS showed stronger immunoreactions than those diluted with PBS or HBS [45]. Although the reasons are unclear, the binding of phosphate ion (a larger ion) to positively charged regions of epitopes and paratopes may reduce the flexibility of peptide chains.

6.2 Ionic strength of dilution solution

Fifteen monoclonal antibodies were diluted in 1% BSA/10 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl, 150 mM NaCl, or 300 mM NaCl. After autoclaving, the paraffin sections were treated with the primary antibodies overnight and then with Envision HRR for 1 h at room temperature. The results are shown in **Table 2**. Most of the antibodies showed strong immunostaining when they were diluted with a buffer containing 50 mM NaCl [45]. However, monoclonal antibodies to proliferating cell nuclear antigen (PCNA) showed the strongest immunostaining when diluted with a buffer containing 300 mM NaCl, and monoclonal antibodies to glial fibrillary

Antibodies	Clones and subclasses	Dilution buffer type			mM NaCl/10 mM TB		
		PBS	TBS	HBS	50	150	300
PCNA	PC10; IgG2a	1	2	2	1	2	2–3
ERα	D12; IgG2a	3	2	2	2	2	2
ERα	ID5; IgG1	1	1–2	1	2	1-2	1
S-100	M2A10; IgG1	1	2	1–2	3	2	1
Mortalin	JG1; IgG3	3	3	3	3	3	3
HSP 70	sc-27; IgG2a	1	2	2	3	2	1–2
α-Synuclein	42; IgG1	2	2	2	2	2	2
GFAP	6F2; IgG1	2	2	1	1	2	2
Desmin	D33; IgG1	1	2	1	1	2	2
β-Actin	AC-74; IgG2a	2	2	2	1	2	1–2
Clathrin	X22; IgG1	1	2	2–3	3	2	1
E-Cadherin	36B5; Ig1	1	2	2	3	2	1
β-Catenin	sc-763; IgG1	1	2	1	3	2	1
γ-GTP	5B9; IgG1	1	2	2	3	2	1
CASGM	170-5; IgG1	2	2	2	3	2	1

FFPE sections (6 μm) of mouse tissues were autoclaved in 20 mM Tris-HCl (pH 9.0) for 10 min and then immunostained with monoclonal antibodies. Antibodies were diluted with 150 mM NaCl/10 mM phosphate buffer (pH 7.4) (PBS), 150 mM NaCl/10 mM Tris-HCl (pH 7.4) (TBS), or 150 mM NaCl/10 mM HEPES buffer (pH 7.4) (HBS). The antibodies were also diluted with 10 mM Tris-HCl (pH 7.4) containing 50 mM, 150 mM, or 300 mM NaCl. Immunostaining was scored as followed: 3, strong; 2, moderate; and 1, weak.

Table 2.
Effects of diluents for monoclonal antibodies on immunohistochemistry.

acidic protein (GFAP) and β-actin diluted with 150 mM yielded the strongest immunostaining. Polyclonal antibodies to nuclear transcription factors such as estrogen receptor (ER)α, androgen receptor (AnR), glucocorticoid receptor, and p300 yielded stronger immunostaining when diluted with a buffer containing 150 or 300 mM NaCl than that using a buffer containing 50 mM NaCl (not shown). These results suggest that the net charges of antibodies and antigens influence the contact of these proteins, and the net neighboring charges of antigens also affect the interactions. Nuclear antigens are associated with highly acidic nucleic acid, and β-actin and GFAP bundles are composed of β-actin and GFAP proteins with acidic isoelectric points. Dilution solution with a high ionic strength may reduce the net charges around the antigens and antigen molecules, allowing antibodies to come in contact with their respective antigens.

6.3 Immunoelectron microscopy

In immunohistochemistry using HRP-labeled antibodies, the staining intensity can be increased using highly sensitive reaction solutions and a longer enzyme reaction time. However, the intensification of immunoreactions is difficult in the post-embedding method and ultracryotomy using colloidal gold-labeled secondary antibody. Instead, the selection of diluents for the antibodies may be more important for obtaining a high labeling density with a low background staining compared

with light microscopy, whereas almost no studies have been performed for immunoelectron microscopy.

We examined the effect of diluents of several antibodies for the post-embedding method using specimens fixed with the standardized fixative and embedded in LR-White resin [39, 41]. Glutaraldehyde and osmium tetroxide fixed and epon-embedded specimens were also used for a few antibodies. After HIAR, ultrathin sections were immunostained using antibodies diluted in the following solutions: PBS, TBS, 10 mM Tris-HCl (pH 7.4) containing 50 mM NaCl, Can Get Signal A (Toyobo Co.), and Can Get Signal B (Toyobo Co.); 1% BSA (final concentration) was then added to the diluents. In general, diluents that produced strong immunoreactions on light microscopy also produced a high labeling density of colloidal gold-labeled antibody. Anti-claudin-5 polyclonal antibody showed the strongest immunoreaction when it was diluted with Can Get Signal A for both LR-White-embedded materials and epon-embedded materials (**Figure 8D**). Can Get Signal A also showed the strongest immunoreactions when used as the diluent for E-cadherin monoclonal antibody. Monoclonal antibodies to β-catenin, β-actin, and clathrin showed a high labeling density when diluted in TBS. Polyclonal antibody to Tom 20 diluted with 50 mM NaCl/10 mM Tris-HCl (**Figure 6B**) or Can Get Signal B showed strong immunostaining. TBS was a better diluent for colloidal gold-labeled secondary antibodies than PBS.

7. Concluding remarks

The main mechanisms of HIAR are cleavage of chemical crosslinks formed by formaldehyde and extend of polypeptides chains to expose epitopes. Highly masked epitopes in heat-stable proteins can be exposed with reduction of disulfide bond followed by heating. Heating also cleaves crosslinks formed by double fixation with glutaraldehyde and osmium tetroxide. The principle of HIAR is applicable for immunoelectron microscopy using the post-embedding and pre-embedding methods, in which tissues are fixed with a standardized fixative described in this text. The association of exposed epitopes and antibodies under a suitable solution are also important for each immunohistochemical reaction.

Abbreviations

AnR	androgen receptor
BSA	bovine serum albumin
CAGSM	common antigen of secretory granule membrane
DAB	3,3′ diaminobenzidine
ERα	estrogen receptor α
FFPE	formalin-fixed and paraffin embedded
GFAP	glial fibrillary acidic protein
γ-GTP	γ-glutamyl transpeptidase
HIAR	heat-induced antigen retrieval
HRP	horseradish peroxidase
HSP	heat shock protein
NRP	neuropilin
PBS	phosphate-buffered saline (10 mM phosphate buffer (pH 7.4) containing 150 mM NaCl)
PCNA	proliferating cell nuclear antigen

Tom	translocase of outer mitochondrial protein
VEGF	vascular endothelial cell growth factor
VEGFR	VEGF receptor
TBS	Tris-buffered saline (10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl)

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