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Molecular mechanisms of antigen retrieval: antigen retrieval reverses steric interference caused by formalin-induced crosslinks

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Abstract

The overwhelming majority of antibodies useful for formalin fixed, paraffin embedded (FFPE) tissues require antigen retrieval to reverse the effect of formalin fixation and re-establish immunoreactivity. How this reversal happens is poorly understood. We developed a new experimental model for studying the mechanism of formalin fixation and antigen retrieval. Epitope mapping studies on nine antibodies useful for FFPE tissues revealed that each consisted of a contiguous stretch of amino acids in the native protein (“linear epitope”). Small peptides representing the epitopes of antibodies to HER2, estrogen and progesterone receptors were attached covalently to glass microscope slides in a peptide array. Most peptides retained immunoreactivity after formalin fixation. Immunoreactivity was completely abrogated for all peptides, however, if an irrelevant large protein was present during formalin-induced cross-linking. We hypothesize that cross-linking the irrelevant protein to the peptide epitopes sterically blocked antibodies from bonding. Antigen retrieval dissociates irrelevant proteins and restores immunoreactivity. Because the epitopes for clinical antibodies require only primary protein structure, the fact that antigen retrieval probably denatures the secondary and tertiary structure of the protein is irrelevant. The same mechanism may occur in tissue samples subjected to formalin fixation and antigen retrieval.

Keywords

antigen retrieval; epitope; formalin fixation; immunohistochemistry; peptide; quality control

For the histotechnologist or surgical pathologist, one of the most important consequences of formalin fixation is related to the effect it has on immunohistochemistry (IHC) and in situ hybridization. Formalin is a time-honored fixative for preserving tissue integrity; however, it destroys immunoreactivity for many clinically useful monoclonal antibodies, which complicates the routine practice of IHC. This problem was solved by Shi et al. (1991), when they found that boiling tissue sections (“antigen retrieval”) restores immunoreactivity for many antibodies. Their finding sparked intense practical research to find optimal conditions

for antigen retrieval, because the ability to perform IHC on formalin fixed, paraffin embedded (FFPE) tissues provides tremendous benefit for diagnostic pathology.

Conceptually, the finding that boiling restores immunoreactivity after formalin fixation was surprising and counter-intuitive. Most of the formaldehyde in a diluted aqueous solution such as formalin is present as methylene glycol. Upon exposure to formalin, formaldehyde initially reacts with tissue proteins to create formaldehyde adducts in the form of hydroxymethyl groups. Subsequently, the hydroxymethyl groups react over a period of hours to days with other tissue proteins to form methylene bridges (protein cross-links; (Fraenkel-Conrat et al. 1947, Fraenkel-Conrat and Olcott 1948a,b, Shi et al. 1997). The specific types of cross-links depend on which amino acid side chains are involved. Previously, such protein cross-links were widely believed to result in protein denaturation and alteration of antibody epitopes, which accounted for the loss of immunoreactivity after formalin treatment. A variety of mechanisms including reversal of some formalin-induced protein cross-links have been proposed to explain why boiling tissue sections restores immunoreactivity (Gown et al. 1993, Suurmeijer and Boon 1993, Morgan et al. 1994, Shi et al. 2000). Even if protein cross-links are broken, boiling is a highly denaturing treatment in its own right. It was not clear how proteins might regain their native conformation to restore immunoreactivity after such harsh treatment as boiling for 30 min, often at temperatures above 100° C, in a pressure cooker. Studies of restoration of protein immunoreactivity after formalin fixation in vitro confirmed the expectation that a temperature that is too high during antigen retrieval can be deleterious (Rait et al. 2004a,b).

Another goal of our research was to create a technology for generating standardized formalin fixed IHC staining controls. There is a need for better standardization and reproducibility in clinical IHC laboratories (Nicholson and Leake 2000, Rhodes et al. 2000a,b, Schnitt and Jacobs 2001, Paik et al. 2002). Previous efforts have focused on process standardization of both pre-analytic and analytic variables associated with IHC (Taylor 1994, 2000, O'Leary 2001, Wolff et al. 2007). Despite the development of standard methods, there is a need for standardized controls to alert clinical laboratories when antigen retrieval or the IHC staining process is inadvertently out of range. Standardized controls also help foster inter- and intra-laboratory reproducibility by providing a quantifiable objective IHC staining end point. Standardized controls are considered essential in other clinical laboratories, but for technical reasons, have been unavailable in the clinical IHC laboratory.

We review here our findings concerning formalin fixation and antigen retrieval. Our interest in formalin fixation and antigen retrieval was in the narrowly defined practical context of how it affects immunoreactivity for IHC. We asked why antigen retrieval works, how it reverses the denaturing effect of formalin fixation, and how we can model the process experimentally. We also review a practical application of the experimental model that we used in national clinical studies for assessing laboratory proficiency.

Experimental model incorporating peptide epitopes

For most immunohistochemical stains, the antigen is a cell-associated, often multi-subunit, complex glycoprotein. Such proteins are the most relevant for clinical diagnostic work, but

often are expensive or unavailable for experimentation. To overcome this problem, we identified peptides that represent the epitope to which the clinical antibodies bond. We synthesized peptides approximately 20 amino acids long that contain the epitope and a spacer separating the epitope from the point of attachment to a solid matrix, such as a glass microscope slide. By using a peptide, we could investigate a well-defined target for formalin fixation that allows us to correlate experimental variables with immunoreactivity. Because each amino acid residue in the peptide is known, it also is possible to identify specific chemical reactions associated with formalin fixation.

Antibody epitopes can be classified as linear or conformational. The linear epitopes are described as a stretch of approximately five to seven amino acids that are contiguous. Although other amino acids may lend additional strength to the noncovalent interactions associated with antibody bonding, these five to seven amino acids are responsible for the majority of the bonding energy. Conformational (“discontinuous”) epitopes, on the other hand, are formed as a result of distantly and spatially distributed small groups of amino acids brought together by conformational folding or bonding. Most protein epitopes generally are believed to be conformational (Kuby 1994, Huebner 2004).

We used combinatorial peptide library technology to identify antibody epitopes, using a method called “phage display.” There are billions of different phage particles in a random peptide library constructed for phage display, each expressing a different random peptide on their surface. The most commonly used phage is the M13 bacteriophage, which is capable of infecting *E. coli*. The phage libraries that we used contain combinatorial libraries of peptide sequences that were inserted into the amino terminus of the pIII minor coat protein of the M13 bacteriophage. We used phage libraries with approximately 10–20 amino acid inserts. With a potential variability of 20 amino acids at most of the inserted amino acid positions, billions of possible peptide combinations result.

A small fraction of the phage particles in the library express peptides that, by chance, resemble any desired epitope (Fig. 1). An antibody bonds most avidly to peptides that resemble the native epitope (Fig. 1). We isolated and enriched the phage particles that bond to the antibody using an affinity separation method called “biopanning.” For biopanning, we first attached the antibody to paramagnetic beads. Mixing the phage library with the beads caused the phage particles expressing the peptide epitope to adhere to the beads; other phage particles did not adhere. The beads then were captured with a magnet, washed, and the adherent phage particles were eluted with acid. The eluted phage particles then were amplified by transfection into *E. coli* and used for a second round of biopanning. After three rounds of biopanning, the resulting phage particles expressed peptides that bonded strongly to the antibody’s antigen-bonding site. The dissociation constants of the resulting peptides after synthesis typically were in the nano- to micromolar range. The bonding was also antibody-specific. The peptides on the phage particles only bonded to the selecting antibody and not to irrelevant antibodies. By sequencing the DNA encoding the peptide insert, we could deduce the peptide sequence constituting the epitope. With the peptide sequence in hand, it was easy to manufacture reproducible amounts of the peptide, in any desired quantity, on a contract basis. Details of the epitope mapping procedure are described elsewhere (Sompuram et al. 2002).

Antibodies useful in FFPE tissues bond to linear epitopes

Phage display methods identify peptides that bond strongly to almost any antibody. For conformational epitopes, the peptide sequence constituting the epitope does not match the native protein sequence. The amino acids in a conformational epitope can be quite distant from each other in the primary sequence, but are brought together by the three-dimensional folding of the protein. To understand how a conformational epitope is formed, we would need information about the three-dimensional structure of a protein. By contrast, the amino acid sequence of peptides associated with linear epitopes matches a sequence in the native protein directly.

We identified the epitopes of nine clinically important antibodies directed to estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor type 2 (HER2), and Ki-67. Each peptide bonds specifically to its corresponding antibody. To test the specificity of immunoreactivity, we attached the peptides to a glass microscope slide covalently using an isocyanate glass coating chemistry (Sompuram et al. 2004a,b). Each peptide was attached covalently by spotting a 1 μ l droplet onto the glass. We then performed an IHC stain on the peptide spots, wherein each peptide was stained by each antibody in a checkerboard fashion as shown in Fig. 2. Figure 2 reveals immunoreactivity of each antibody with its cognate peptide. Each peptide constituted a linear stretch that was found in the native protein sequence, confirming that each epitope was linear. The same peptide bonded to the CB11 and Herceptest antibodies, because both recognize the same epitope. Similarly, the ER2-123 peptide bonded to two ER antibodies, ER2-123 and ER 6F11.

Because most epitopes in immune responses to proteins are believed to be conformational (Kuby 1994, Huebner 2004), it was surprising that all nine antibodies bonded to peptides representing linear epitopes. We hypothesize that antibodies useful in FFPE tissues are a select group, distinctive by virtue of the nature of the epitope to which they bond. These data suggest that antibodies chosen for their utility with FFPE tissues may be directed predominantly or exclusively to linear epitopes. To test further why linear epitopes might be important for clinical IHC, we conducted a series of experiments using a peptide array.

Formalin fixation of peptide epitopes

We used a peptide epitope array to model the effects of formalin fixation and antigen retrieval on antibody immunoreactivity. Most peptides in the array do not lose immunoreactivity after formalin fixation, because of their small size and limited number of amino acid residues. Immunoreactivity is completely abrogated, however, if an irrelevant protein is allowed to cross-link to the peptides in the peptide array during formalin fixation. Antigen retrieval restores immunoreactivity. These findings are illustrated in Fig. 3, which shows an array of peptide spots covalently attached to glass microscope slides. We used this peptide array format to determine the treatment conditions required for reproducing the effects of formalin fixation and antigen retrieval, i.e., exposure to formalin causes loss of immunoreactivity and antigen retrieval restores it.

The immunoreactivity of various antibodies to the peptides in the array was tested by IHC. Each row of Fig. 3 contains a different peptide and antibody combination, as indicated to the

left. The presence of a colored spot indicates immunoreactivity. As a baseline condition (column A), we confirmed that each peptide was capable of bonding to its respective antibody, and created a 3 mm diameter colored spot on the glass slide. The table at the top of Fig. 3 indicates that the peptides in column A were neither fixed nor exposed to an irrelevant protein prior to formalin cross-linking.

Column B, by contrast, depicts the peptide array on slides that were fixed initially in formalin before immunostaining. Formalin fixation caused complete loss of immunoreactivity of the MIB-1 antibody for its peptide and partial loss of the PR 1A6 immunoreactivity to its peptide. These two peptides are somewhat unique. For the other peptides, formalin fixation had no effect on immunoreactivity. These findings indicate that formaldehyde causes a chemical modification to the peptides that abrogates immunoreactivity. Three possible mechanisms seem likely: 1) formalin results in the formation of intermolecular cross-links, 2) formalin results in the formation of intramolecular cross-links, or 3) formalin results in the formation of formaldehyde adducts without cross-linking. Our data do not distinguish among these possibilities.

Based on the amino acid composition of the various peptides shown in Fig. 3, it is not surprising that the MIB-1 and PR636 peptides are the only ones that are sensitive to formalin fixation. The MIB-1 peptide is the only one with a lysine at the antibody epitope, which raises the likelihood that formalin fixation creates cross-links at the epsilon amino group of lysine. For example, the lysine of one peptide might crosslink to a lysine in an adjacent peptide to form intermolecular cross-links such as illustrated in Fig. 4. Such cross-linking could alter the site at which the antibody binds and change the three-dimensional structure of the epitope or render the linear sequence inaccessible to antibody bonding.

The PR 1A6 peptide also is mildly formalin-sensitive, but it lacks a lysine. It is the only peptide in this group with a tyrosine at the epitope and a nearby arginine, raising the possibility that formalin fixation creates crosslinks between adjacent peptides through a Mannich condensation reaction as previously described (Sompuram et al. 2004a). Therefore, these two peptides have the amino acid composition to facilitate the creation of formalin-induced modifications to the peptide structure.

For the remaining peptides, formalin fixation produced no change in immunoreactivity (Fig. 3, column B). We saw a markedly different result, however, when an irrelevant protein (e.g., casein) was present during formalin cross-linking. To abrogate immunoreactivity with formalin fixation, the peptide array initially was incubated in a solution of 0.2% casein to produce an initial weak noncovalent physical adsorption of casein onto the array surface. The array then was incubated overnight in formalin vapor at 37° C. The use of formalin vapor rather than liquid helped retain the casein on the array surface during the formalin cross-linking reaction. Had we dipped the slides in liquid formalin, it would have rinsed the casein off before it could be cross-linked. Covalent cross-linking of another protein onto the peptides in the array resulted in complete abrogation of immunoreactivity (Fig. 3, column C). Column C shows that fixation in the presence of an irrelevant soluble protein prevented antibody binding; none of the peptide spots were immunoreactive. The peptide array, coupled to casein, then was treated according to our usual antigen retrieval protocol and

immunoreactivity was restored (Fig. 3, Column D). Therefore, column C represented a condition analogous to fixed tissues before antigen retrieval and column D was analogous to fixed tissues after antigen retrieval.

Why antigen retrieval restores immunoreactivity

Our findings suggest why antigen retrieval, a highly denaturing procedure, regenerates antibody epitopes after formalin fixation. In tissues, formalin fixation causes cross-links between adjacent proteins, which results in steric interference to antibody bonding to its linear epitope. Antigen retrieval reverses cross-links and dissociates proteins that are sterically interfering with antibody bonding to linear epitopes. Protein re-folding to a native conformation is irrelevant in the context of linear epitopes.

Figure 5 illustrates the proposed sequence of events during IHC staining of the peptide array. Amino acids are represented as circles. Each peptide is a string of amino acids covalently anchored to the glass substrate at one end. In Fig. 5A, the antibody is immunoreactive with a peptide containing the epitope (shaded circles). The antibody recognizes a linear epitope, i.e., a contiguous series of five to seven amino acids. In a tissue section, the peptide epitope is part of a larger protein with many adjacent proteins in the normal cellular milieu.

In Fig. 5B, the peptides are represented as they might be after formalin-induced cross-linking to an irrelevant nearby protein. Cross-linking to an irrelevant protein, e.g., casein, blocks antibody access to the linear epitope, which accounts for the loss of immunoreactivity regardless of the amino acid composition. In the analogous situation for cells and tissues, formaldehyde causes protein cross-linking, which produces cross-links within the protein itself or with other adjacent proteins. This may not necessarily result in significant denaturation. A recent study of RNase A indicated that treatment with formaldehyde does not significantly alter secondary structure (Rait et al. 2004a,b). Although formalin treatment may induce subtle changes in secondary structure, alpha helices and beta pleated sheets essentially are left intact after formalin treatment.

Figure 5C illustrates the likely outcome of antigen retrieval at the protein level. Antigen retrieval breaks formalin-induced cross-links, which results in dissociation of sterically interfering proteins from the peptide epitope. Because the peptide epitope is re-exposed, immunoreactivity is restored. In a tissue section, the temperature associated with antigen retrieval (approaching 120° C if a pressure cooker is used) exceeds the denaturation temperature of most proteins. Consequently, secondary structure likely is irreversibly altered. Regardless of the loss of secondary structure, antigen retrieval facilitates antibody bonding by re-exposing the linear epitope. The linear sequence represents the primary protein structure and is not denatured by the heat associated with antigen retrieval. We believe that our in vitro peptide array model accurately represents the situation in tissue, because we tested the peptides to which the antibodies bond in the native proteins. Moreover, the kinetics of the reactions in the peptide array mirror those in tissue sections, which suggests that the chemical process is similar.

Similarity of reaction kinetics

We examined the kinetics of formalin fixation and antigen retrieval in our peptide array model (Sompuram et al. 2006a,b). We were particularly interested to determine whether the kinetics of antigen retrieval resembles the time course often associated with tissue sections. If they are similar, it suggests that a similar chemical process may be occurring in both. For these kinetics experiments (Figs. 6 and 7), we used the method of formalin fixation corresponding to column C of Fig. 3, *viz.*, peptides initially were coated with an irrelevant protein, e.g., casein, then fixed in formalin vapor.

Figure 6 illustrates the intensity of IHC staining as a function of antigen retrieval time for the peptide array (Fig. 6A) and representative tissue section staining for estrogen receptor (Fig. 6B). Figure 6B depicts the means and standard deviations of three randomly chosen breast biopsies that expressed the estrogen receptor. Both peptides and tissue sections generally showed recovery of immunoreactivity after antigen retrieval for 20 min. Variability in the kinetics of antigen retrieval for different epitopes (Fig. 6A) suggests that different kinds of cross-linking reaction may have occurred depending on which amino acids are present in the peptide epitope.

Figure 7 demonstrates the kinetics of formalin fixation of the peptide array. The arrays were fixed for varying lengths of time as indicated on the x axis. We found that the kinetics of fixation, as evidenced by a loss of immunoreactivity, varied among the peptides. After fixation for 2 h, there was considerable variability of immunoreactivity among the peptides in the array. Some peptides were mostly immunoreactive, whereas others were not. The two peptides that were directly sensitive to formalin fixation, even without an irrelevant protein coating, MIB-1 and PR 1A6, displayed rapid kinetics (Fig. 7). Both lost their immunoreactivity within 2 h. We had demonstrated earlier that the MIB-1 and PR 1A6 peptides lost immunoreactivity after formalin fixation even without an initial coating with an irrelevant protein (Fig. 3, column B). After formalin fixation for 16 h, none of the peptides were immunoreactive. This variability in kinetics again suggests that different types of formalin cross-linking reactions may occur, each at a separate rate. The specific formalin-induced cross-linking reactions depend on the amino acid composition of the peptide in the peptide array. Examples of formalin-induced protein cross-linking reactions have been reviewed elsewhere (Shi et al. 2000).

Clinical Application

The technology of modeling formalin fixed proteins using peptides offers the opportunity to develop a novel quality control for IHC. Quality control is important for all immunohistochemical tests, but the need has been especially acute for quantitative measurement, such as HER2 immunostaining. Current practice for HER2 IHC testing includes the use of cell line controls supplied by the test manufacturer or the use of archival breast carcinoma tissue characterized previously as immunoreactive to HER2. Archival material is inherently unstandardized. Cell lines, on the other hand, have garnered interest as reproducible controls that can be grown in large quantities. In addition, many different cell lines representing low, medium, and high levels of HER2 expression can be incorporated

into a single paraffin control block. Such cell lines have been used extensively for national proficiency testing in the UK as part of the NEQAS (Rhodes et al. 2002a,b). These cell lines are not available commercially, except when packaged with HER2 test kits, because they may be more expensive to manufacture reproducibly and cheaply. Peptides, on the other hand, are attached to glass microscope slides in a high volume, automated fashion. Unlike earlier methods of quality control, the availability of both fixed and unfixed peptides offers a simple method for distinguishing causes of error. By comparing the signal intensities associated with fixed and unfixed peptide controls, it is possible to determine whether suboptimal immunostaining is associated with problems with antigen retrieval (a pre-analytical component), problems with the staining reagents, or problems with the immunostaining process (an analytical component).

Figure 8 illustrates the results of a national study conducted in collaboration with the College of American Pathologists that incorporated data from 109 clinical laboratories. The details of this study are described elsewhere (Vani et al. 2008). The dotted line in Fig. 8 represents the minimum intensity threshold for adequate staining. Of 109 clinical laboratories, staining was suboptimal in 20 (18.3%) as judged by quantification of the peptide analyte controls. These results agree with other surveys that examined HER2 immunostaining performance of laboratories nationwide (Paik et al. 2002, Roche et al. 2002). Our data identified further the cause of those staining failures: 35% failed due to antigen retrieval errors, 20% were due to antibody or staining protocol problems, and the remainder were due to a combination of the two. For clinical laboratories that failed due to antigen retrieval, the unfixed controls were immunostained quite well, but little or no immunostaining of the fixed controls was observed. Laboratories that produced poor immunostaining on both fixed and unfixed controls had a problem with the analytical component of IHC, which represented a deficiency with either an antibody or the IHC staining process. By comparing the staining results of both fixed and unfixed peptide controls, laboratories can test separately distinct components of their IHC assay. In this way, the controls serve not only to alert laboratories to out-of-range assay conditions, but also provide guidance about the cause of the problem. We expect IHC controls incorporating peptides to be available commercially in the future.

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Fig. 1. Schematic representation, not drawn to scale, illustrating how an epitope can be represented in a short peptide. The amino acids constituting the epitope also can be expressed on the surface of a bacteriophage through phage display.

Fig. 2.

Immunoreactivity of various antibodies with peptides derived from phage display analysis and correlation to the native linear sequences of ER, PR, Ki-67, or Her-2. The peptide sequence was chosen to contain the deduced antibody contact site. The grid lines were superimposed after scanning the image to enhance clarity. Reproduced from Sompuram et al. (2006).

Fig. 3. Immunostained peptide arrays after fixation, protein cross-linking and antigen retrieval as indicated at the top. Each row is a different peptide that is immunoreactive for the antibody denoted to the left. Column A represents the baseline condition without treatment. Column B shows immunoreactivity of each peptide after formalin fixation overnight. Column C shows immunoreactivity after first coating the array with an irrelevant protein, casein, followed by formalin fixation overnight. Column D illustrates immunoreactivity of the peptides after the treatment for column C, then antigen retrieval. Reproduced from Sompuram et al. (2006).

Fig. 4. Schematic representation of one possible effect of formalin fixation on peptide epitopes attached to glass. Each amino acid is represented as a circle. The shaded circles represent the antibody epitope. In a minority of peptides, the amino acid side chains may form cross-links. The cross-links illustrated here are between adjacent peptides, thereby sterically blocking antibody access to the epitope.

A. Native condition

B. Formalin fixation

Epitope inaccessible to antibody bonding after formalin fixation.

C. After Antigen Retrieval

Epitope re-exposed after antigen retrieval dissociates sterically blocking proteins.

Fig. 5.

Molecular model accounting for the loss and subsequent recovery of immunoreactivity after formalin fixation and antigen retrieval, respectively. Changes to the peptide epitopes in our in vitro peptide array assay are illustrated schematically. Panel A schematically illustrates the binding of an antibody to its linear cognate epitope attached to a glass surface. In panel B, the epitope is schematically shown to be sterically blocked by an irrelevant protein that is covalently bound to the peptide. Panel C illustrates the effect of antigen retrieval. Adapted from Sompuram et al. (2006).

A

B **Fig. 6.**

Intensity of immunohistochemical staining as a function of the length of antigen retrieval time. All values represent means + the standard deviations of triplicate measurements. Staining intensity of the peptide array after 0–30 min of antigen retrieval is shown in (A). Staining intensity of an unfixed peptide array is shown at the far right of the graph as a control. In (B), three randomly chosen ER-positive breast biopsies were subjected to antigen retrieval for the indicated periods, then immunostained. For the array, staining intensity was measured as mean pixel intensity units on a 1–256 scale. For tissues, staining intensity was measured on a 0 (no staining) to 3 (strong staining) scale. Reproduced from Sompuram et al. (2006).

Fig. 7.

Immunohistochemical staining intensity as a function of the duration of formalin fixation. Immunostaining intensity was measured in triplicate. Standard deviations were nearly all less than 10% of the mean and, for purposes of clarity, are not shown. Staining intensity was measured as mean pixel intensity units on a 1–256 scale. Reproduced from Sompuram et al. (2006).

Fig. 8.

Histogram of the distribution of scores for formalin fixed peptide analyte controls. The dotted line represents our minimum cut-off for HER2 laboratory staining proficiency in this survey. Labs to the left of the cut-off produced absent or low analyte control intensity scores and absent or weak HER2 staining in a 3+ HER2 tissue section. Reproduced from Vani et al. (2008).