Background: We previously observed decreased p53 immunostaining over time in paraffin-embedded sections of ductal carcinoma in situ of the breast of women; these sections had been stored on slides at room temperature. This observation suggests that slide storage adversely affects p53-immunostaining intensity and could result in spurious negative staining for p53 in patient samples. Purpose: The goals of this study were to determine the time course and factors influencing loss of p53 immunoreactivity and to investigate whether a similar loss of reactivity occurs with other antigens commonly used to study breast cancer. Methods: Serial sections cut from 12 formalin-fixed, paraffin-embedded, p53-positive invasive ductal carcinomas of the breast were stored on slides at room temperature or at 4 °C, with or without an additional paraffin coating, for 2, 4, 8, or 12 weeks. For each case, freshly cut slides from the same block (day 0) and stored slides were simultaneously stained for p53 by use of an automated immunostainer. Slides cut from formalin-fixed, paraffin-embedded breast carcinomas and stored for 12 weeks were also stained for factor VIII-related antigen (n = 12), estrogen receptor (ER) (n = 9), and Bcl-2 protein (n = 9). The staining intensity of all slides was assessed by visual microscopic examination and was also quantified by image analysis. Quantitative results were expressed as a percentage (mean ± standard error) of the staining intensity on day 0. Data were analyzed by the Friedman Repeated Measures Analysis of Variance on Ranks, with statistical significance set at two-sided P < .05. Results: The intensity of p53 staining decreased over time in nine (75%) of the 12 cases studied. In three (or 25% of all cases studied) of the nine cases that showed decreased p53 staining, slides stored for 12 weeks were scored as p53 negative. Antigen loss on slides stored at 4 °C was significantly less than that on slides stored at room temperature at all time points (all P < .05). At 12 weeks, the average staining intensity of slides stored at 4 °C was 33.2% ± 9% of that on day 0 compared with 8.4% ± 3% of that on day 0 for slides stored at room temperature (P < .001). Paraffin coating of the sections did not significantly diminish antigen loss at either room temperature or 4 °C, except for slides stored at room temperature for 12 weeks. The intensity of factor VIII staining decreased in nine of 12 cases (average staining intensity, 37.3% ± 6% of that on day 0 at 12 weeks; P = .001). The intensity of ER and Bcl-2 staining decreased in all nine cases studied at 12 weeks (average staining intensity, 14.0% ± 6% and 21.0% ± 4% of that on day 0, respectively; P = .0001 for each). Conclusions and Implications: Slide storage, particularly at room temperature, results in substantial loss of p53 reactivity, with some p53-positive cases becoming p53 negative after 12 weeks of storage. Substantial loss of immunoreactivity for factor VIII, ER, and Bcl-2 occurs on slides stored at room temperature for 12 weeks. Storage of unstained slides for up to 12 weeks may lead to false-negative immunostaining for p53 and other antigens. [J Natl Cancer Inst 1996;88:1054-9]
permits the rapid return of blocks to the originating institution, obviating the need to retain these blocks indefinitely. However, if loss of immunoreactivity is a consequence of storage of sections on glass slides at room temperature, the potential exists for spurious negative results. This outcome, in turn, could have serious implications for the results of both clinical and research laboratory studies that utilize such material.

Since we have previously shown that immunoreactivity for the p53 protein diminishes over time in stored slides of breast cancers (3), the purposes of the present study were 1) to examine in detail the time course and factors influencing p53 antigen loss on stored slides and 2) to determine if loss of reactivity on stored slides occurs for several other antigens commonly used to study breast cancers, i.e., factor VIII-related antigen, ER protein, and Bcl-2 protein.

Materials and Methods

Study Design

In the first part of this study, we evaluated the effect of storage time on p53-immunostaining intensity. We also assessed the effects of temperature (storage at room temperature compared with storage at 4 °C) and of exposure of the sections to air, singly and in combination. To evaluate the effect of exposure to air, we compared the immunostaining intensity on slides dipped in paraffin to produce an occlusive coating on the surface of the tissue sections with that on slides that were not coated with paraffin.

Twelve cases of invasive ductal breast carcinoma previously shown in our laboratory to express the p53 protein by immunohistochemistry were retrieved from the surgical pathology files of Beth Israel Hospital, Boston, MA. For each case, 5-μm tissue sections were cut from one paraffin block and stored unstained on glass slides for 2, 4, 8, or 12 weeks at room temperature or at 4 °C, with or without an additional paraffin coating of the tissue sections. Slides cut on the day of immunostaining (day 0) were stained for the p53 protein simultaneously in the same run with the stored slides from the same paraffin block by use of an automated immunostainer (Ventana Medical Systems Inc., Tucson, AZ); this procedure ensured standardization of the staining procedure. Thus, we studied a total of 17 slides for each case. Staining intensity on all slides was quantitated by image analysis in a blinded fashion, as detailed below. For each slide at each storage interval, the intensity of staining was expressed as a percentage of staining observed on day 0. In addition, slides stored at room temperature for 12 weeks and freshly cut slides from the same paraffin blocks of these 12 cases were simultaneously stained for factor VIII-related antigen on the automated immunostainer. The staining intensity of the slides stored for 12 weeks was compared with that of the slides on day 0.

We also studied three of these 12 cases known to be ER positive and an additional six cases of ER-positive invasive ductal carcinomas retrieved from our surgical pathology files to assess antigen loss for ER and for the Bcl-2 oncoprotein. Slides stored at room temperature for 12 weeks and freshly cut slides from the same paraffin blocks of these nine cases were stained for both ER and Bcl-2 protein by use of manual immunohistochemical methods, since these antibodies produced suboptimal staining results with the use of the automated immunostainer. Again, the staining intensity of the slides stored for 12 weeks was compared with that of the slides on day 0 for both ER and Bcl-2 by use of computer-assisted image analysis.

In addition to quantifying staining intensity by image analysis, two observers (T. W. Jacobs and S. J. Schmitt) visually examined all slides under the light microscope and categorized them as "negative" or "positive" for p53, ER, and Bcl-2. Cases were scored as p53 negative, ER negative, or Bcl-2 negative when virtually no tumor cells showed staining for these antigens upon routine microscopic examination. Slides stained for factor VIII were assessed as "adequate" or "inadequate" for microvessel quantification. Cases were scored as inadequate when small vessels that were clearly identifiable histologically showed no immunostaining for factor VIII.

All cases evaluated in this study had been accessioned in 1995. Tissue had been embedded in paraffin after fixation in 10% buffered formalin supplemented with 70% alcohol (Anatech Ltd., Battle Creek, MI) for up to 18 hours.

Immunohistochemical Techniques

Slides that were stored following an additional coating of paraffin were deparaffinized in four 5-minute changes of xylene. All other slides were deparaffinized in two 5-minute changes of xylene. Sections were then rehydrated through graded alcohols to distilled water. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol for 10 minutes.

p53 Immunostaining. Immunostaining for p53 was performed with the Ventana 320 automated immunostainer. Before applying the primary antibody, we subjected the sections to antigen retrieval by heating the slides in a microwave oven in citrate buffer (10 mM, pH 6) for a total of 10 minutes (i.e., two 5-minute periods with replacement of evaporated room buffer every period of heating). The primary antibody to p53 (clone PAb 1801, IgGl kappa; Zeneca Corp., London, U.K.) was used at a 1:1500 dilution in Ventana's proprietary buffer. 3,3'-Diaminobenzidine (DAB) was used as the chromogen (Ventana proprietary solution), and the sections were lightly counterstained with methyl green.

Factor VIII Immunostaining. Factor VIII immunostaining was also performed with the Ventana 320 automated immunostainer following a microwave antigen retrieval method. This method consisted of a 20-minute incubation with the use of the proprietary protease 2 reagent supplied by Ventana Medical Systems Inc. The primary polyclonal antibody to factor VIII (Dako Corp., Carpinteria, CA) was used at a 1:450 dilution (Ventana proprietary buffer). DAB was used as the chromogen (Ventana proprietary solution), and the sections were lightly counterstained with hematoxylin.

ER Immunostaining. Following the microwave antigen retrieval method as described above, we applied the primary murine monoclonal antibody to ER (clone ER1D5, IgGl kappa; Immunotech, Westbrook, ME) overnight at 4 °C at a 1:10 dilution in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA). The slides were then sequentially incubated for 30 minutes with biotinylated horse anti-mouse immunoglobulin (immunoglobulin G heavy and light chains; Vector Laboratories, Inc., Burlingame, CA) in a 1.5-mg/mL stock solution diluted 1:500 in PBS supplemented with 1% BSA and for 30 minutes with streptavidin-horseradish peroxidase (Zymed Laboratories Inc., South San Francisco, CA) diluted 1:200 in PBS supplemented with 1% BSA and for 30 minutes with streptavidin-horseradish peroxidase (Zymed Laboratories Inc., South San Francisco, CA) diluted 1:200 in PBS supplemented with 1% BSA. DAB (0.6 mg/mL; Sigma Chemical Co., St. Louis, MO) was used as the chromogen, and the sections were lightly counterstained with methyl green.

Bcl-2 Immunostaining. The immunostaining method used for Bcl-2 was similar to that used for ER. The primary murine monoclonal antibody to the Bcl-2 protein (clone 124, IgGl kappa; Dako Corp.) was applied to the sections overnight at 4 °C at a 1:100 dilution in PBS supplemented with 1% BSA. Sections were lightly counterstained with hematoxylin.

Controls. For all four antigens, positive controls were included in each staining run. They consisted of freshly cut, paraffin-embedded sections of cases known to express the antigen of interest.

Image Analysis

Staining intensity for all antigens was quantified by computer-assisted image analysis. Image analysis was performed by a single observer (T. W. Jacobs), who was blinded with regard to the status of the slides (i.e., length of storage, storage temperature, and whether or not slides had had an additional paraffin coat). Microscopic images were studied with a Zeiss Axioscope microscope (Carl Zeiss, Oberkochen, Federal Republic of Germany) equipped with a Chromoclip II video camera (Javelin Electronics, Inc., Torrance, CA) connected to a color video monitor. A SummaSketch Plus digitizing tablet (Summagraphics Corp., Fairfield, CT) was used as an interface. The BioQuant Elite image analysis software package (version 7.31.91, R & M Biometrics, Nashville, TN) was used for morphometric analysis. To determine immunostaining intensity, we evaluated one complete 10x microscopic field of each immunoperoxidase-stained section. In each case, the same 10x field was selected on all slides. A density threshold (red, green, blue, alpha) was set to quantify the positive immunoperoxidase reaction product (nuclear for p53 and ER, cytoplasmic for Bcl-2, or endothelial cell cytoplasm for factor VIII). The threshold was selected to exclude the background hematoxylin and methyl green counterstains. The same thresholds and system settings were used for all slides quantitated. The number of pixels falling within the threshold, indicating the quantity of immunoperoxidase reaction product, was recorded for each field. Staining intensity for each slide within a case was expressed as a percentage of the pixels seen on the day-0 slide from that case.
Results

Effect of Storage at Room Temperature on p53-Staining Intensity

Nine (75%) of the 12 p53-positive cases showed significant loss of p53-staining intensity following storage at room temperature. After 2 weeks of storage, p53-staining intensity had already significantly decreased to 30.5% ± 7% of that on day 0 (P < 0.05) (Figs. 1 and 2, A and B). Staining intensity was reduced further by longer storage. The p53-staining intensity at 4 weeks was 17.7% ± 5% of that on day 0; at 8 weeks, it was 10.5% ± 4% of that on day 0; and at 12 weeks, it was 8.4% ± 3% of that on day 0 (Figs. 1 and 2, A and C). In addition, on the basis of visual light microscopic examination, three of these nine lesions were scored as p53 negative on the slides stored for 12 weeks. In two of the 12 cases, there was no significant loss of intensity of p53 staining after 12 weeks of storage at room temperature (85.1% and 95.3% of staining intensity on day 0, respectively). One case stained very weakly for p53 at all time points, including day 0.

Effect of Storage at 4 °C on p53-Staining Intensity

The nine cases that showed significant loss of p53-staining intensity after 12 weeks of storage at room temperature also revealed significantly decreased staining intensity at all time points when stored at 4 °C. The p53-staining intensity on slides stored at 4 °C was reduced to 56.7% ± 9% of that on day 0 at 2 weeks, 41.9% ± 5% at 4 weeks, 28.4% ± 6% at 8 weeks, and 33.2% ± 9% at 12 weeks. At each time point, however, the staining intensity of slides stored at 4 °C was significantly greater than that of the corresponding slides stored at room temperature (all P < 0.05) (Fig. 1). In addition, on the basis of visual light microscopic examination, all nine lesions were still p53 positive on the slides stored for 12 weeks.

Effect of Paraffin Coating of Slides on p53-Staining Intensity

Coating the surface of the tissue sections with paraffin before slide storage did not significantly reduce the loss of p53-staining intensity on slides stored at either room temperature or 4 °C, except for slides stored at room temperature for 12 weeks (Fig. 1). In those slides, however, the mean staining intensity on both coated and uncoated slides was still reduced to less than 15% of that on day 0 (14.9% ± 3% versus 8.0% ± 3% for coated and uncoated slides, respectively).

Immunostaining Intensity of Factor VIII, ER, and Bcl-2 After Storage at Room Temperature

Nine of the 12 cases studied showed significant loss of staining for factor VIII after 12 weeks of storage at room temperature (37.3% ± 6% of that on day 0; P = .0001) (Fig. 3, A and B). Staining intensity in three cases did not decrease with storage. In cases showing diminished immunostaining for factor VIII, microscopic examination of the slides stored for 12 weeks showed numerous unstained microvessels. Therefore, these cases were scored as inadequate for accurate microvessel quantification.

Both immunostaining for ER and immunostaining for Bcl-2 were significantly decreased after 12 weeks of storage at room temperature in all nine cases tested (14.0% ± 6% of immunostaining intensity on day 0 for ER and 21.0% ± 4% of that on day 0 for Bcl-2; each P < 0.001) (Fig. 3, C and D, and Fig. 3, E and F, respectively). Upon visual light microscopic examination of the slides stored for 12 weeks, one ER-positive tumor was scored as ER negative. All Bcl-2-positive cases remained Bcl-2 positive after 12 weeks of storage, albeit at a substantially weaker intensity.

Loss of p53-Staining Intensity Related to Reduction of Immunostaining for ER, Bcl-2, and Factor VIII

Of three cases stained for p53, ER, and Bcl-2, two showed significantly decreased immunostaining intensity for all three antigens and one showed significant loss of staining intensity for ER and Bcl-2 but not for p53 after 12 weeks of slide storage at room temperature. Among the 12 cases stained for both p53 and factor VIII, seven showed significantly reduced immunostaining intensity for both antigens, two showed a significant reduction in staining intensity for factor VIII but not for p53, two showed significant loss of staining intensity for p53 but not for...
factor VIII, and one showed no significant reduction in staining intensity for either antigen after 12 weeks of slide storage at room temperature.

Discussion

We have shown that, in most cases, slide storage at room temperature results in rapid and substantial loss of p53 immunoreactivity in paraffin-embedded sections of breast cancer. In fact, some cases initially categorized as p53 positive were scored as p53 negative on slides stored for 12 weeks. A significant loss of staining intensity also occurs for factor VIII-related antigen, ER protein, and Bcl-2 protein after storage of slides at room temperature for 12 weeks. These findings indicate that storage of paraffin-embedded sections on glass slides may produce spurious negative immunostaining results for several antigens frequently used to study breast cancers.

The fact that antigen loss can occur in stored slides does not appear to be widely appreciated. To the contrary, the general perception is that stored, unstained paraffin-embedded sections on slides “have always been acceptable” for immunohistochemical studies (4). The results of our study, in conjunction with those of two prior smaller studies (3,5), however, call into question the conventional wisdom regarding the ability to obtain accurate immunostaining results for all antigens on stored slides. Unfortunately, the range of antigens and tissues for which this is a potential problem is currently unknown, but diminished immunoreactivity on stored slides is unlikely to be limited to the antigens studied here or to breast specimens (6).

The mechanism of antigen loss in fixed, paraffin-embedded tissue sections mounted on glass slides is not known. Our results, however, suggest that this phenomenon is at least in part temperature dependent, since storage at 4 °C resulted in less loss of p53 immunoreactivity than storage at room temperature. It is possible that slide storage at even colder temperatures might further diminish loss of immunostaining intensity. Exposure of tissue sections to the atmosphere may be another explanation for antigen loss in stored slides, since this exposure could theoretically result in oxidation of certain antigens or epitopes. In our study, however, coating the surface of the tissue sections with paraffin to diminish contact with the ambient atmosphere did not significantly reduce the loss of immunoreactivity for p53 protein. Nonetheless, it is possible that slide
Fig. 3. Invasive ductal carcinoma immunostained for factor VIII at day 0 (A) and after storage at room temperature for 12 weeks (B); for estrogen receptor at day 0 (C) and after storage at room temperature for 12 weeks (D); and for Bcl-2 at day 0 (E) and after storage at room temperature for 12 weeks (F). In these examples, staining intensity on slides stored for 12 weeks was 28.1% of that on day 0, 42.8% of that on day 0, and 8.2% of that on day 0 for factor VIII, estrogen receptor, and Bcl-2, respectively (original magnification ×200).

Storage under conditions that result in even less exposure to the atmosphere, such as in a vacuum chamber, may be more effective in this regard. Another unanswered question is whether or not the loss of immunoreactivity in stored slides is due to irreversible antigen degradation or to masking of antigens or epitopes that is reversible if a suitable antigen retrieval method is employed. Some method of antigen retrieval was used for all four antigens evaluated.
In our study, including microwave heating for p53, ER, and Bcl-2 and proteolytic enzyme treatment for factor VIII. For each antibody, both stored and unstored slides in each case were subjected to identical antigen retrieval procedures. It is possible that more aggressive antigen retrieval is necessary to achieve optimal staining in stored than in unstored slides. Excessive exposure of the tissue sections to heat or to proteolytic enzymes, however, can result in increased background staining and/or excessive tissue digestion, compromising the interpretation of the staining results. Nonetheless, an important goal is determining whether certain antigen retrieval protocols can restore immunoreactivity in stored slides.

In most clinical immunohistochemistry laboratories, the phenomenon of antigen loss on stored slides should not create a major problem, since immunostainings required for patient diagnosis are typically performed on slides only hours to days after the paraffin sections have been cut. Nonetheless, antigen loss could be a problem in stored slides used as positive controls. One of the most unsettling implications of our findings relates to the frequent use of stored slides for immunostaining in the research environment. It is common practice in many research laboratories to archive unstained paraffin sections for future use. In fact, collaborative groups involved in multi-institutional clinical trials often establish core or reference laboratories to perform immunostaining and other special studies on such material (4). Although storing slides clearly has logistic advantages over collection and storage of paraffin blocks, these advantages may be offset by the potential for spurious negative results for certain antigens when immunohistochemical staining is performed on stored slides.

References


Notes

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