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Variability of laminin immunoreactivity in human autopsy brain

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Summary. Laminin immunoreactivity is thought to be masked in formalin-fixed sections since proteolytic treatment is required to unmask it. We analyzed this masking with frozen and formalin-fixed human autopsy brains obtained at various postmortem periods. In unfixed, frozen sections, intense immunoreactivity was invariably detected in vascular walls of entire sections. When such sections were postfixed in formalin, immunoreactivity was not diminished even after prolonged fixation. In vibratome sections of brain fixed in formalin in situ, immunoreactivity varied with postmortem delay: in most cases, immunoreactivity was weak and restricted to superficial cortical layers. However, the extent of immunoreactivity increased with postmortem delay. Two cases fixed after prolonged postmortem periods revealed moderate immunoreactivity throughout the sections. We also investigated rat brains processed without postmortem delay. In unfixed frozen sections, immunoreactivity again was observed throughout the sections, independent of the length of any postfixation. In vibratome sections of fixed rat brain, immunoreactivity was restricted to the cutting margins of the brain blocks and around a trauma-induced cortical lesion, regardless of how long the blocks had been kept in fixative. Our data suggest that postmortem proteolysis accomplishes similar unmasking of laminin antigen as digestion on paraffin sections and that such unmasking can also be effected by proteolysis induced by damaging tissue during cryostat sectioning of fresh tissue.

Introduction

Laminin, a large glycoprotein specific for basement membranes (Timple et al. 1979) can be readily localized by immunocytochemistry in unfixed frozen sections (Katz et al. 1976; Foidart et al. 1980; Ekblom et al. 1982). Material fixed in formalin requires protease pretreatment of sections whether or not blocks have been embedded in paraffin (Burns et al. 1980; Ekblom et al. 1982). For this reason, laminin immunoreactivity is considered to be masked as a result of fixation or embedding. We have now analyzed the conditions of laminin epitope masking in human autopsy brains obtained at various postmortem intervals as well as in rat brains obtained without significant postmortem interval.

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Materials and methods

Twenty-six human brains of both sexes (24 to 92 years of age) were obtained 5 to 40 h after death. Some himispheres of fresh brains were frozen immediately by carbon dioxide and stored at −85° C. Small blocks of the frontal lobe were cut at 20 μm-thickness on a cryostat and mounted on gelatin-coated slides. These sections were then fixed in 10% formalin in 0.1 *M* phosphate buffer, pH 7.4, for 0, 0.5, and 48 h, respectively.

In addition, brain slices of all cases, 0.5 cm in thickness, were immersed in changes of 10% neutral formalin for 7 days and infiltrated in graded mixtures of equal portions of ethylene glycol and glycerol to final concentrations of 30% of each and stored at −20° C. Blocks of frontal lobe were cut on a vibratome at 40 μm thickness.

Under sodium pentobarbital anesthesia, two brains of 3-months-old, male Lewis rats were removed from the skulls immediately after decapitation. One normal brain was processed for frozen sections and the other, in which a small cerebral cortical lesion had been inflicted with a scalpel blade just after removal from the skull, for vibratome sections. The former received the same treatment as human frozen sections and the latter was dissected into 3 small blocks and then immersion-fixed in 10% formalin in 0.1 *M* phosphate buffer, pH 7.4, for 24, 48 and 72 h, respectively. Forty μm-thick vibratome sections including the faces of the blocks were prepared.

To unmask antigen, some formalin-fixed sections were exposed to a solution of pepsin (4 mg/ml) in 0.01 N HCl for 2 h at 37° C prior to immunocytochemical staining (Burns et al. 1980).

Immunocytochemical staining was performed by the following sequence: 1) 0.5% hydrogen peroxide in methanol for 30 min; 2) 2% milk solids/0.05 *M Tris* hydrochloride, pH 7.61/0.3 *M* sodium chloride for 30 min; 3) Rabbit antilaminin antibody (Gibco, Gaithersburg, Md., USA) diluted 1:800 or 1:1000, overnight; 4) Sheep antirabbit IgG serum preabsorbed with normal human or rat serum

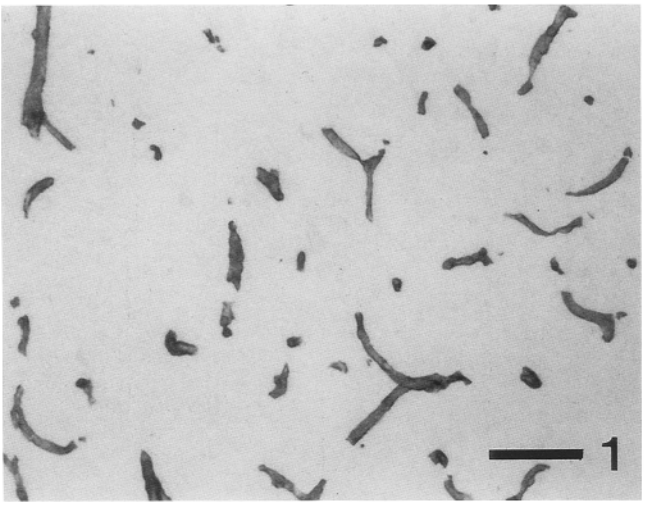

Fig. 1. Frontal cortex, human, frozen brain, no formalin postfixation. Antilaminin, 1:1000. Immunoreactivity is restricted to blood vessels; both capillary and larger vessels are intensely visualized. PAP. *Bar* = 100 μ, × 125

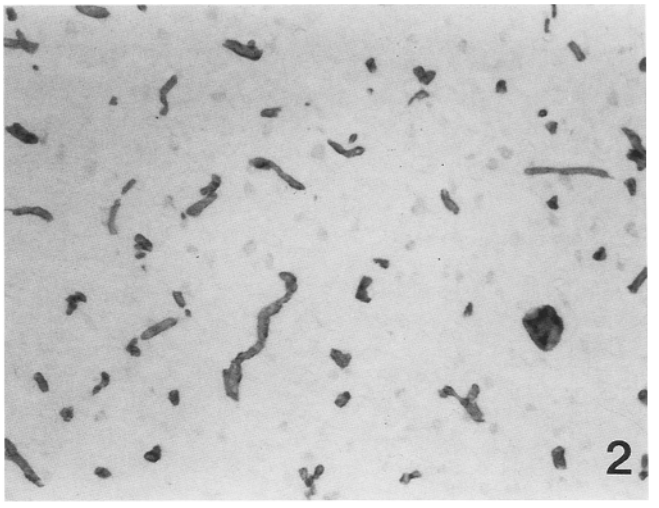

Fig. 2. Same section as Fig. 1, but postfixed with 10% buffered formalin for 48 h. Antilaminin, 1:1000. Both capillary and larger vessels are visualized equally well, and there is no significant reduction of the immunoreactivity compared to that in Fig. 1. PAP. × 125

diluted 1:40, for 30 min; 5) Rabbit peroxidase-antiperoxidase complex diluted 1:100, for 30 min; 6) 0.05% diaminobenzidine tetrahydrochloride/0.01% hydrogen peroxide/0.05 *M Tris* hydrochloride, pH 7.6/0.3 *M* sodium chloride for 8 min (Sternberger 1986). Specificity of the antibody was established by absence of immunoreactivity with preabsorbed antibody.

Result

Human, frozen brains, frozen sections

In unfixed frozen sections and in the absence of formalin postfixation, laminin immunoreactivity was invariably detected in the basement membrane of vascular walls throughout the sections (Fig. 1). There was no difference in the intensity of reactivity between capillaries and larger vessels. After 0.5, 24 and 48 h fixation of the frozen sections, no significant decrease of immunoreactivity and no change in staining pattern was observed (Fig. 2). In general, it was difficult to handle unfixed frozen section without postfixation due to their fragility and detachment, but as little as 30 min fixation made them well-adherent to slides and easy to process (Figs. 1–2).

Human, formalin-fixed brains, vibratome sections

In most undigested vibratome sections of formalin-fixed brains, only weak immunoreactivity was observed and this was restricted to the superficial cortical layer where both capillaries and larger vessels were visualized (Fig. 3). After pepsin digestion of these sections, laminin was visualized throughout the entire section; the staining pattern was the same as that in the unfixed frozen sections, but the intensity of staining was generally low and the reactivity was variable from case to case (Fig. 4). In untreated sections, the areas possessing immunoreactivity tended to be larger as the postmortem delay was

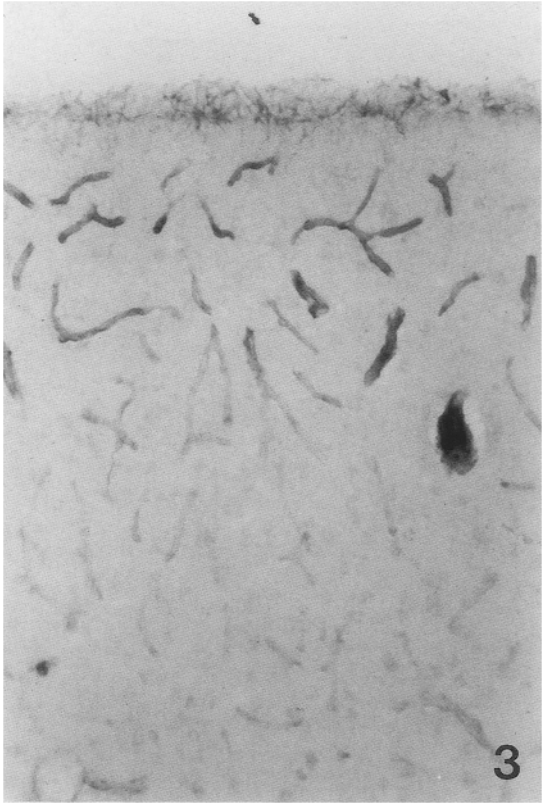

Fig. 3. Superficial layer of frontal cortex, human, formalin-fixed, vibratome section. Antilaminin, 1:800. Immunoreactive vessels are mostly observed in the subpial cortex. PAP. × 125

prolonged. This was especially notable in 2 cases fixed after prolonged postmortem periods (21 to 40 h), in which moderate immunoreactivity, weaker than that of unfixed frozen sections, was observed throughout the sections (Fig. 5). After pepsin treatment, staining intensi-

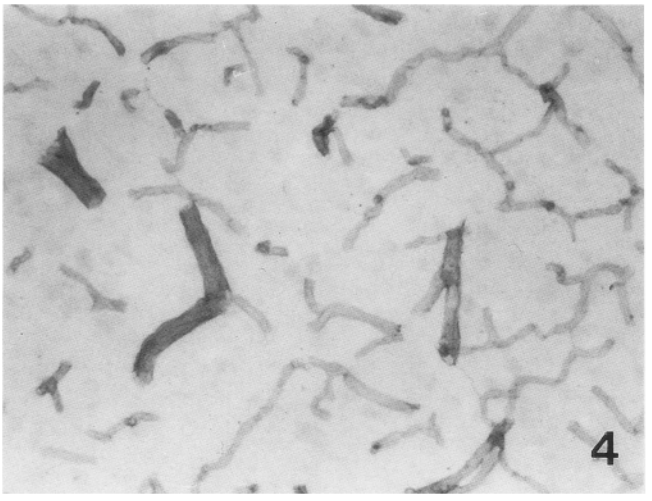

Fig. 4. Frontal cortex, human, formalin-fixed, vibratome section after pepsin treatment. Antilaminin, 1:800. Immunoreactivity has been restored in the entire section, but the reactivity in capillaries is weaker than that in larger vessels. PAP. ×250

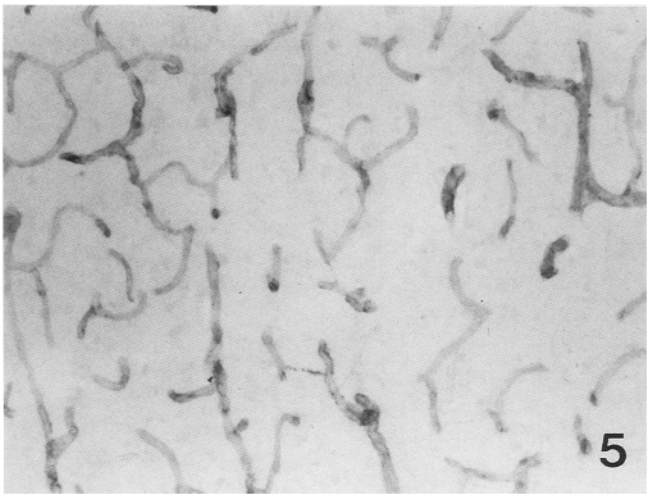

Fig. 5. Vibratome section of human frontal cortex fixed by buffered formalin 40 h postmortem. Antilaminin, 1:800. Moderate immunoreactivity is observed throughout the section. PAP. ×125

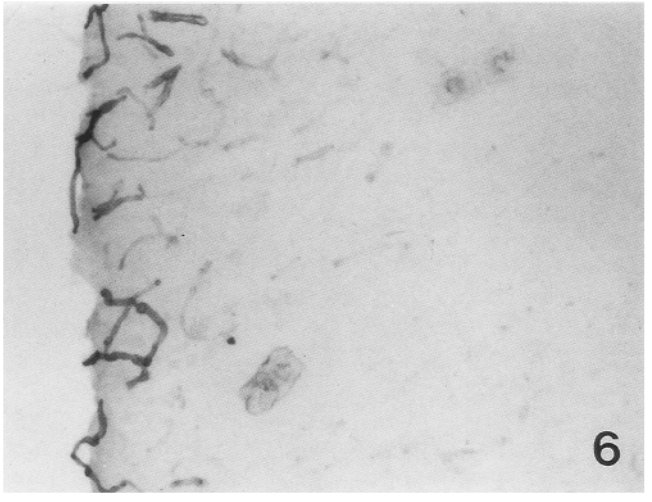

Fig. 6. Vibratome section of rat brain after 48 h fixation in 10% buffered formalin. Antilaminin, 1:1000. Immunoreactivity is restricted to the sectioned face of the block (*left margin of the section*). PAP. ×125

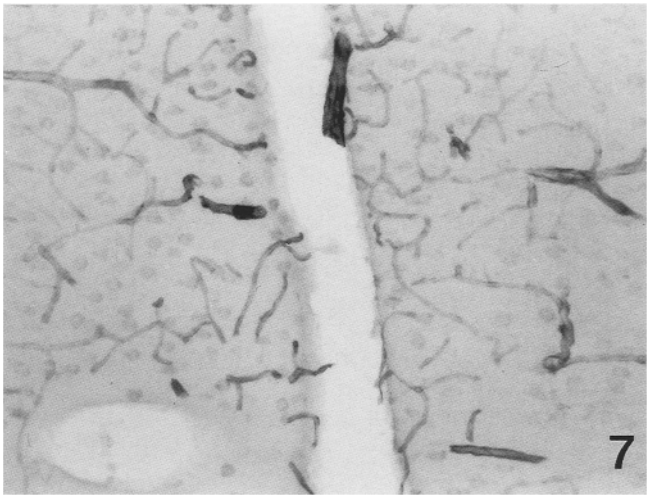

Fig. 7. Vibratome section of rat brain containing a trauma-induced cerebral cortical lesion after 48 h fixation in 10% buffered formalin. The lesion was made just after the brain was removed from the skull. Antilaminin, 1:1000. Immunoreactive blood vessels are observed around the lesion (*central field*), but not elsewhere. PAP. ×125

ty increased without changes in staining pattern (Figs. 3–5).

Rat, frozen brains, frozen sections

In spite of the length of the postfixation time, laminin immunoreactivity was invariably detected in the entire section, similar to the observations with human, frozen sections.

Rat, formalin-fixed brains, vibratome sections

Twenty-four hours after fixation, no immunoreactivity was observed in most areas including superficial cortical layers, but both capillaries and larger vessels were visualized in the cutting margins of the brain blocks (Fig. 6) and around the trauma-induced lesion (Fig. 7). Staining was not diminished, even after 72 h of fixation. Pepsin treatment restored immunoreactivity to the entire section.

Discussion

Human autopsy brain is subjected to endogenous postmortem proteolysis, and consequently reveals variable immunoreactivity from case to case

It is well known that laminin immunoreactivity (Katz et al. 1974; Foidart et al. 1980; Ekblom et al. 1982) as

well as that of several other antigens, such as factor VIII/von Willebrand factor (Burns et al. 1980; McComb et al. 1982; Sternberger 1986), while readily detectable in unfixed, frozen sections, is masked in formalin-fixed sections. Immunoreactivity can be unmasked by protease treatment of sections prior to immunostaining. It is fortuitous that these antigens, which are extremely sensitive to proteolytic enzymes, regain their antigenicity in formalin-fixed sections following enzyme treatment (Burns et al. 1980). The wide variety of proteases suitable for unmasking suggests effects on the intrachain structure of proteins, rather than directly on the uncoupling of specific aldehyde-effected secondary amino bonds that form the majority of the inter- and intramolecular bridges (Fox et al. 1985).

In human vibratome sections, weak immunoreactivity was restricted to the superficial cortical layers in most cases. The areas showing reactivity increased as the postmortem delay was prolonged. In cases in which fixation was initiated only after prolonged postmortem periods, moderate immunoreactivity was present throughout the sections. This preferential staining pattern at different postmortem periods is quite similar to that of the postmortem leakage of serum protein in human autopsy brain; in our previous study, superficial cortical layers exhibited serum protein leakage during early postmortem periods. Leakage spread with prolongation of postmortem periods (Mori et al. 1991). Conceivably, these two different phenomena are closely related to postmortem proteolysis. The masking and unmasking of laminin antigen in human vibratome sections can be explained by limiting the access of laminin antibody: in undigested areas, formalin fixation causes protein cross-linking. Consequently, access of antibody was impaired, while in the digested areas, postmortem proteolysis may have increased the distance between tissue proteins, thus preventing excessive intramolecular cross-linkage during fixation. However, even in the two cases in which moderate immunoreactivity was observed in the entire section, pepsin treatment led to an increase in immunoreactivity. This finding suggests that enzymatic digestion of tissue antigen by postmortem proteolysis is insufficient to prevent antigen masking entirely.

Postdigestion on the tissue section by enzyme treatment had effects qualitatively similar to postmortem proteolysis, but was more extensive quantitatively. Apparently, pepsin digestion could further unmask the antigen. Our observations suggest that variable data could be obtained from case to case because of the degree of postmortem predigestion of the antigen. It is conceivable that laminin immunoreactivity can be used as an indicator for masking of epitopes as a result of tissue processing.

Unfixed frozen sections are weakly digested as a result of sectioning, and masking of laminin antigen during subsequent fixation is prevented

There was no masking of laminin antigen in unfixed frozen sections of human autopsy or rat brain, even after prolonged postfixation in formalin. While visualization of laminin antigen in frozen sections may have been due to absence of fixation, the lack of masking during subsequent fixation of such sections suggests that digestion occurring either during or after thawing of the sections may have been sufficient to obviate the subsequent masking. Indeed, immunoglobulin staining reveals serum protein leakage around blood vessels in unfixed frozen sections (unpublished data). Therefore, it can be expected that laminin antigen in the vascular wall is subject to predigestion by a variety of proteases in leaked serum protein and thus postfixation of such sections does not mask the antigen.

On the other hand, in the immersion-fixed vibratome sections obtained from the rat, the antigen was masked nearly in its entirety except for the cutting margins of the brain blocks or around the surgically-induced cortical lesions, regardless of how long the tissues were kept in fixative. As far as laminin staining is concerned, the cutting margin is equivalent to the cortical lesions inflicted by the scalpel blade. Indeed, such procedures cause leakage of serum protein (Mori et al. 1991). Absence of masking of the antigen in these focal areas by subsequent fixation may have been due to predigestion of the tissue antigen by proteases in the leaked serum protein. Unfixed frozen sections also can be considered a continuity of the cutting margin of the brain blocks. Any damage which could cause serum protein leakage to unfixed brain, whether frozen or not, may avoid masking of the antigen.

Immunoreactivity of unfixed, frozen sections is more consistent than that of formalin-fixed sections pretreated with protease

The present study has elucidated laminin visualization in both unfixed, frozen sections postfixed by formalin and in the pepsin-digested formalin-fixed sections. However, more constant and intense staining was obtained in the former than in the latter.

Unmasking of the antigen in the latter depended on conditions of tissue fixation and enzyme digestion (McComb et al. 1982); tissues that were fixed for long periods of time in formalin required longer exposure to a proteolytic enzyme and were more resistent to digestion than were tissues that were fixed briefly. Therefore, it is difficult to determine the optimal conditions for pepsin digestion in each case; generally, however, in cases of weak digestion, laminin antigen could not be unmasked, and in cases of intense digestion, the antigen was found to lose its antigenicity. Pepsin digestion of laminin has been reported to release a large cystine-rich fragment which retains most of the antigenicity of the original protein (Timple et al. 1979).

Undigested, frozen sections were difficult to handle for immunocytochemical staining. When fixed in formalin for as little as 30 min, they had become hardened and adherent to the slides and were much easier to process. It is encouraging that this postfixation did not mask laminin immunoreactivity.

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