Use of Pronase and Pepsin in the Restoration of Laminin Antigenicity

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ABSTRACT. To improve the restoration of laminin antigenicity, we applied pronase E and pepsin before immunohistochemical staining. In changing the concentration and reaction times of these enzymes, we found that the best results were obtained in sections treated with a 120 min pepsin reaction followed by 5 to 10 min pronase E treatment. Constant and consistent results were obtained with this procedure, and it is expected to improve the immunohistochemical study of laminin.

Key words : immunohistochemistry — laminin — pronase — pepsin

Certain antigens (proteins) may be preserved in formalin-fixed and paraffin-embedded tissues, and their presence can be detected in tissue sections with immunohistochemical techniques. The antigenicity of some antigens, however, is blocked by formaldehyde, paraffin or other factors inherent to the fixation and embedding process. As a result their immunohistochemical detection is hindered. Many procedures have been devised to overcome this problem, including treatment with such proteinases such as trypsin and pepsin, and the application of microwaves.[1-5]

Recently, while studying laminin in liver tissue, we found that this antigen was not constantly preserved by the routine immunohistochemical methods without pretreatment of proteinases. Therefore, we used pronase E and pepsin to see how effectively they might restore laminin antigenicity. In our experiment, pretreatment of sections with pronase E for 5 to 10 min followed by pepsin for 2 hr provided the best result.

MATERIALS AND METHODS

Tissue blocks from 20 surgically removed liver tissue specimens and 20 specimens from the colon were utilized for this study. The outline of the immunohistochemical staining method we used is shown in Fig. 1. Both pepsin (SIGMA P-6887) and pronase E (P-6911) were purchased from SIGMA Chemical Company (St. Louis, USA). Pepsin was diluted by 0.1 mol/l hydrochloride to a 1:500 dilution,[1] and pronase E by PBS to 1:500 through 1:1000 (Fig. 2).[2] The primary antiserum against laminin (rabbit anti-human laminin polyclonal antibody) and the avidin-biotinylated peroxidase complex (ABC) Kit were obtained from Bio-science products AG (Emmenbrücke,

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Switzerland) and Vector Laboratories Inc., (Burlingame, USA), respectively. The primary antiserum was diluted to 1:200 in PBS. After deparaffinization with xylene, 4 μm thick sections were treated in either pepsin solution or pronase E solution. Pepsin treatment was done at 37°C. The reaction times for pepsin were 2, 5, 10, 30, 60 and 120 min, and 4, 8, 16 and 24 hrs. Those for

1. deparaffinization of specimen
2. pretreatment by proteinases
3. blocking of intrinsic peroxydase
4. reaction with primary antibody
5. reaction with secondary antibody
6. reaction with avidin-biotin complex
7. color development by 3, 3'-diaminobentidine tetrahydrochloride

Fig. 1. Outline of pretreatment and immunohistochemical staining

the pronase E solution processed at room temperature were 2, 5, 10, 30, 60 and 120 min. The treatment was always done in a moist chamber as were the other processes described below. Then the intrinsic peroxidase activity was blocked by 0.3% hydrogen peroxide, and subsequently followed by reaction with the primary antiserum, the secondary antiserum and the avidin-biotinylated peroxidase complex. 3, 3'-diaminobenzidine tetrahydrochloride (DAB) was

① Pepsin : 0.1% w/v (0.01 N-HCl)
② Pronase E : 0.1~0.2% w/v (PBS)

Fig. 2. The concentration of proteinases

used for color development, and linear dark brown colorization was considered as a positive reaction. Sections pretreated with pepsin alone for 120 min were regarded as control and were compared with results in the other preparations. The staining intensity was scored as follows; no staining (−); weaker than the control (±); staining equivalent to the control (+); and staining stronger than the control (++).

RESULTS

Results are tabulated in Table 1. When enzyme treatment was applied for a longer period of time; specifically longer than 4 hr for pepsin and longer

TABLE 1. Duration of enzyme reaction and intensity of color development.

Pepsin \ Pronase E	0	2	5	10	30	60 (min)
0	−	−	−	−	±	−
30	−	−	±	±	−	−
60	+	+	+	+	−	−
90	+	+	+	+	−	−
120 (min)	+	+	++	++	−	−

than 30 min for pronase E, tissue sections were digested or detached from glass slides. Without any pretreatment, laminin immunopositivity was not achieved no matter how long the primary antiserum was applied or how much was used (Fig. 3). The best staining result was obtained in sections treated with pepsin for 120 min followed by 5 to 10 min pronase E treatment (Fig. 4). Application of pepsin for 120 min prior to 5 to 10 min pronase E treatment did not change the result. The effect of pepsin was steady in the presence of hydrochloride, whereas the effect of pronase E was better in PBS. When a mixture of pepsin and pronase E solutions was applied, laminin was not stained.

DISCUSSION

Enzyme treatment before immunohistochemical staining is sometimes effective in restoring antigenicity which has been blocked or in intensifying the staining reaction.[1-5] In fact, we have successfully shown that enzymes, either pepsin or pronase E, worked well for the restoration of laminin, a component of basement membrane, in the liver and colonic tissue. Among preparations we have tried, the best color development was obtained in sections treated with a 120 min pepsin reaction followed by a 5 to 10 min pronase E reaction. The order of the reaction with these enzymes was not critical, and prior treatment with pronase E gave the same result.

Interestingly enough, however, when both pepsin and pronase E were applied at the same time, we were unsuccessful. This was probably because those enzymes competed with each other. The pH of the pepsin-pronase mixture seems critical. The strongest effect of pepsin appears at pH 2 to 3, while that of pronase E appears at pH 7 to 9.[6] Therefore the pH of the mixed solution was not suitable for either enzyme. Furthermore, their reaction sites differ. Although pronase E has no specific action sites in the proteins, pepsin does. This difference may explain why the order of their application did not change the result.

Enzyme treatment of a high concentration destroys tissue connection and

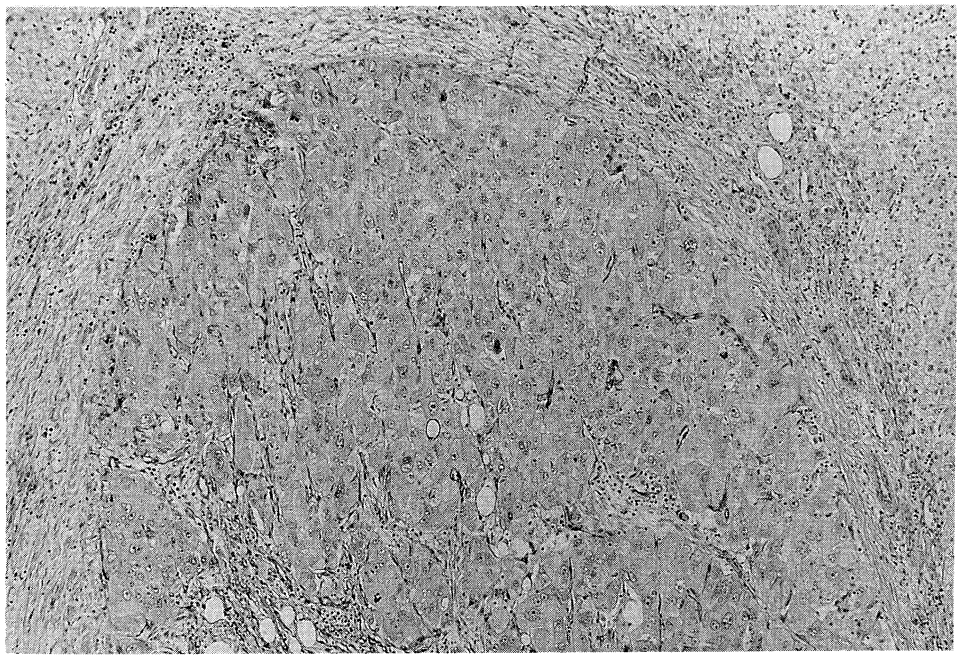

Fig. 3. Immunohistochemistry of the laminin stained without enzyme pretreatment. The nodular area represents a hepatocellular carcinoma. Note that laminin immunopositivity is not present in either carcinomatous or non-neoplastic areas. (×150)

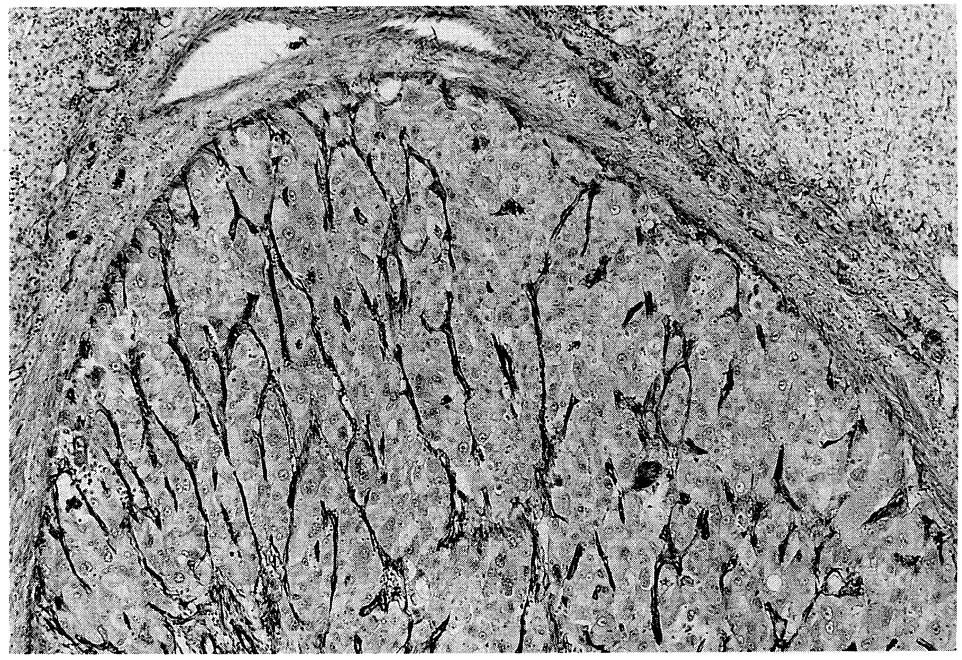

Fig. 4. Immunohistochemistry of laminin after treatment with a 120 min pepsin reaction followed by a 10 min pronase E reaction. This photograph depicts the same nodule as shown in Fig. 3. Note that laminin is strongly stained in the carcinomatous area while it is negative in normal liver tissue. (×150)

there is a loss of tissue before staining. Therefore, to obtain better results it was necessary to dilute the enzymes as low as possible and to let them react for a longer duration.

At any rate, our present experiment indicates that the application of both enzymes in the aforementioned order provides a better restoration of laminin antigenicity in comparison with other procedures reported to date[5] and with ones we have tried. However, it would be fair to say that further studies on the enzyme concentration as well as the reaction time may improve results. In addition, further investigations with other techniques, such as microwaves or with other enzymes, should be carried out.

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REFERENCES

1) Reading M: A digestion technique for the reduction of background staining in the immunoperoxidase method. J Clin Pathol **30**: 88-90, 1982
2) Finley JCW, Grossman GH, Dimeo P, Petrusz P: Somatostatin-containing neurons in the rat brain: Widespread distribution revealed by immunocytochemistry after pretreatment with pronase. Am J Anat **153**: 483-488, 1978
3) Curren RC, Gregory J: Demonstration of immunoglobulin in cryostat and paraffin sections of human tonsil by immunofluorescence and immunoperoxidase techniques. Effects of processing on immunohistochemical performance of tissue and on the use of proteolytic enzymes to unmask antigens in sections. J Clin Pathol **31**: 974-983, 1978
4) Suzuki Y, Maesawa A, Matsui K, Shimizu F, Oite T, Koda Y, Suzuki S, Ueki K, Fukagawa M, Arakawa M: Restoration of antigenicity of tissue antigens, cell-bound immunoglobulins and immune deposits in paraffin-embedded tissue. The influence of fixation and proteolytic enzymatic digestion. Acta Pathol Jpn **34**: 563-574, 1984
5) Watanabe K, Nakane K: Enzyme-labeled antibody method. 3rd ed, Tokyo, Gakusai-kikaku Inc. 1992, pp 115-123, pp 188-196
6) Lehninger AL: Biochemistry. 2nd ed, New York, Worth Publishers Inc. 1975, pp 106-107, p 196