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Technical Note

The influence of tissue pretreatment on the immunohistochemical demonstration of type I and III collagens and tenascin in fetal human tooth germs

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ABSTRACT The influence of tissue pretreatment on the PAP immunostaining for type I and III collagens and tenascin was studied in formalin-fixed and paraffin-embedded human tooth germs at the 24th and 25th weeks of fetal life. Three variables were considered: the type of buffer used (PBS or Tris), pepsin digestion and the use of normal serum as a blocking agent prior to immunostaining. All three proteins needed an enzymatic digestion to be intensely revealed. Pepsin promoted, even at low concentrations, an intracellular staining of type I collagen in the secretory odontoblasts and in the pulpal fibroblasts. Normal serum partially blocked unspecific immunoreaction when polyclonal rabbit antibodies were used. The Tris buffer increased the staining intensity of the three macromolecules and revealed an unusual tenascin-like immunoreactivity in the ameloblasts. This study demonstrated that pepsin digestion and the use of normal serum and different buffers may influence the immunoreactivity of ECM proteins.

KEY WORDS: *tooth germ, human, collagen, tenascin, immunohistochemistry*

Since the three-step peroxidase-antiperoxidase (PAP) technique was introduced by Sternberger *et al.* (1970), numerous studies have investigated factors influencing the immunostaining intensity in paraffin-processed material such as fixatives (Brandtzaeg, 1982; Burns, 1982), decalcifying solutions (Mori *et al.*, 1988), clearing agents (Matthews, 1981), detergents such as saponin or Triton X 100 (Clement *et al.*, 1985) and inhibitors of endogenous peroxidase activity (Li *et al.*, 1987). Pretreatment with proteolytic enzymes has often been reported to improve the immunohistochemical staining in formalin-fixed and paraffin-embedded tissues (Holund *et al.*, 1981; Brandtzaeg, 1982; Finley and Petrusz, 1982; Kirkpatrick and D'Ardenne, 1984). The use of normal serum has also been recommended as a blocking agent prior to the immunostaining (Sternberger, 1979; Petrusz, 1983). Up to now, no study has reported the influence of different buffers in immunohistochemistry. Therefore, the purpose of the present study was to investigate the influence of pepsin pretreatment and the use of normal serum and different buffers (PBS or Tris) on the immunostaining of extracellular matrix (ECM) proteins including type I and III collagens and tenascin in formalin-fixed and paraffin-embedded fetal human tooth germs using the PAP technique.

Our results showed that pepsin digestion was always required to demonstrate and/or to enhance the immunoreactivity of the three ECM proteins (Tables 1, 2 and 3). With 0.4% pepsin pretreatment, there was a greater immunostaining of type I collagen in the odontoblasts than in the predentin (Fig. 1). On the other hand, when pepsin was used at lower concentrations (0.05% to 0.01%), the predentin and the functional odontoblasts as well as the pulpal fibroblasts were intensely stained (Figs. 2 and 3). The dental follicle and the bone matrix showed a marked immunolabeling, whereas the preodontoblasts, ameloblasts and dental basement membrane were immunonegative. Type III collagen was intensely revealed in the dental follicle and at the periphery of the bone matrix, while a fainter immunostaining was mainly localized under the secretory odontoblasts and the preodontoblasts (Fig. 4). The functional odontoblasts were immunonegative (Fig. 5). The tenascin immunolabeling was limited to the periphery of the coronal dental mesenchyme (Fig. 6). The Tris buffer generally promoted an increased intensity in the immunostaining of the three ECM proteins (Tables 1, 2 and 3). Type I collagen was intensely detected in the epithelio-mesenchymal junction, especially in the inner dental basement membrane in pepsinized and Tris-treated sections (Fig. 7). The tenascin immunolabeling moderately spread throughout the dental papilla. However, a greater staining intensity was predominantly confined to the cuspal dental mesenchyme (Fig. 8) and particularly

Abbreviations used in this paper: PAP, peroxidase-antiperoxidase; PBS, phosphate-buffered saline; ECM, extracellular matrix; NSS, normal swine serum; NRS, normal rabbit serum.

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TABLE 1

INFLUENCE OF TISSUE PRETREATMENT ON TYPE I COLLAGEN IMMUNOSTAINING

Type I Collagen	Buffer	Without serum	NSS	Heat inactivated NSS
Staining intensity				
without enzymatic pretreatment	PBS	++	+	++
	TRIS	++	++	+++
0.4% pepsin	PBS	+++	+++	+++
	TRIS	+++	+++	+++
Background				
without enzymatic pretreatment	PBS	+	0	0
	TRIS	+	+	++
0.4% pepsin	PBS	0	0	0
	TRIS	0	+	++

The immunostaining intensity was estimated in the tissue compartment of the tooth germ showing the strongest reactivity. Normal serum was incubated for 20 min prior to the immunostaining and was substituted with Tris or PBS buffer in «serum free»-treated sections.
The background level was estimated in control sections. The primary antibody was substituted with normal serum or Tris or PBS buffer, overnight at 4°C.
The staining results were graded as follows: 0, no staining; +, slight; ++, moderate; +++, intense

in the odontoblasts where numerous fine granules appeared at the base of the cells (Fig. 9). Furthermore, a positive immunostaining was detected in the ameloblasts and in the outer enamel epithelium (Figs. 10 and 11). In all cases of pretreatment, control sections obtained by substituting the primary antibody with normal rabbit serum (NRS) or an irrelevant murine monoclonal IgG1 were negative. Normal swine serum (NSS) generally reduced the unspecific background staining in PBS-treated sections, whereas it produced a moderate background in the dental papilla and the dental follicle in Tris-treated sections (Tables 1 and 2). Heat-inactivated NSS was not effective when polyclonal rabbit antibodies were used. On the other hand, NRS never generated background staining in control sections (Table 3).

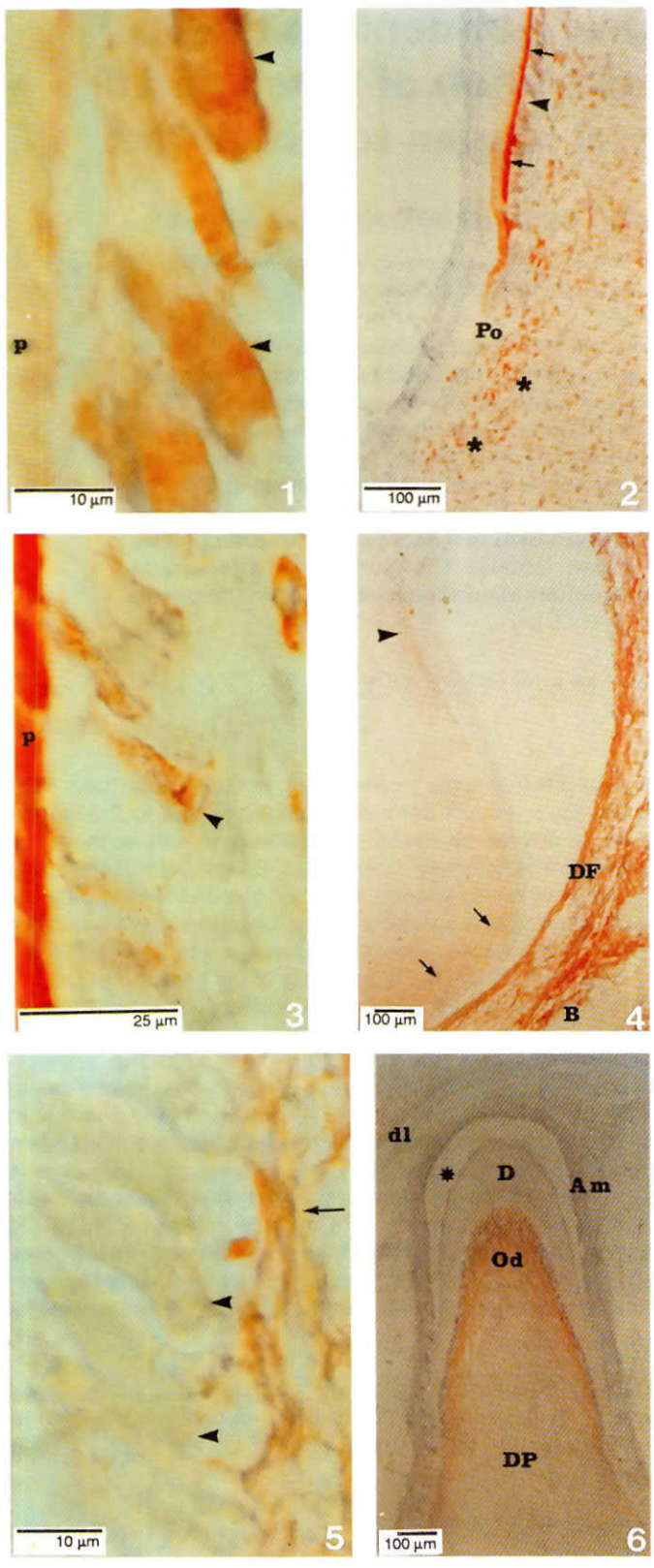


Plate 1. Influence of pepsin digestion on human fetal tooth germ sections at 24 and 25 weeks. (1-3) *PAP Immunostaining of type I collagen.* **(1)** *PBS buffer, 0.4% pepsin, heat-inactivated NSS. Type I collagen was revealed in the functional odontoblasts (arrowheads) and in the predentin (p). x1330.* **(2 and 3)** *PBS buffer, 0.01% pepsin, heat-inactivated NSS. The predentin (arrows), the odontoblasts (arrowhead) and the pulpal fibroblasts (*) were intensely stained, whereas the preodontoblasts (Po) were negative. x95 and x840.* **(4-5)** *PAP Immunostaining of type III collagen.* **(4)** *PBS buffer, 0.4% pepsin, heat-inactivated NSS. The dental follicle (DF) and the periphery of the bone (B) showed a greater staining intensity than the dental papilla in which a slight labeling was confined opposite the preodontoblasts (arrowhead) and the inner dental basement membrane up to the cervical loop (arrows). The odontoblasts and the predentin were immunonegative. x47.* **(5)** *Tris buffer, 0.4% pepsin, NSS. Functional odontoblasts (arrowheads) were immunonegative, whereas the staining was limited to the underlying mesenchyme (arrow). x1060.* **(6)** *PAP Immunostaining of tenascin. PBS buffer, 0.4% pepsin, NRS. The whole dental papilla (DP) was faintly stained, while a condensation of the labeling was detected in the secretory odontoblasts (Od) and in the coronal underlying mesenchyme. Ameloblasts (Am), secondary dental lamina (dl) and dentin (D) were negative. (*)= space resulting from decalcification of enamel. x47.*

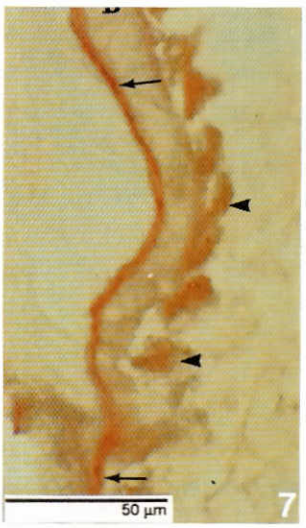
TABLE 2

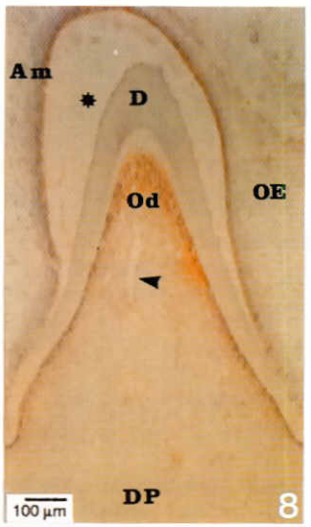
INFLUENCE OF TISSUE PRETREATMENT ON TYPE III COLLAGEN IMMUNOSTAINING

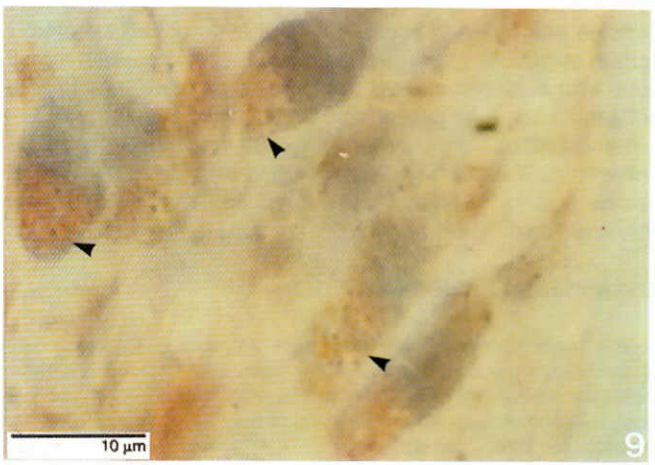
Type III Collagen	Buffer	Without serum	NSS	Heat inactivated NSS
Staining intensity				
without enzymatic	PBS	+	0	0
pretreatment	TRIS	+	+	++
0.4%	PBS	++	++	+++
pepsin	TRIS	+++	+++	+++
Background				
without enzymatic	PBS	+	0	0
pretreatment	TRIS	+	+	++
0.4%	PBS	0	0	0
pepsin	TRIS	0	+	++

For legends, see Table 1.

The effectiveness of the enzymatic treatment depends on the optimal concentration and on the time of incubation of the enzyme to be used according to the nature and species of tissue being stained: although pepsin was seen to be ineffective in liver tissue (Bedossa *et al.*, 1987), a previous investigation performed in human tooth germs showed that pepsin was the most effective enzyme to unmask antigenic sites of three basement membrane proteins while maintaining a more adequate morphology of the dental fetal tissue than pronase or trypsin (Laurent-Maquin *et al.*, 1992). Furthermore, the present study showed that pepsin could be used at lower concentrations than usually recommended. No immunostaining for type III collagen was detected in the dentin, predentin or odontoblasts, even in pepsinized and Tris-treated sections. The Tris buffer associated with pepsin digestion enhanced the staining intensity of tenascin and promoted an intracellular labeling due partially to its solubility in lipids and, thereby, its capacity to penetrate through plasma membranes. It promoted a tenascin immunoreactivity in epithelial compartments of human tooth germs with a greater intensity in the ameloblasts than in the outer enamel epithelium, the secondary dental lamina or the oral epithelium. This epithelial localization of tenascin has never been described in tooth germs. Recent studies demonstrated the presence of tenascin in the epithelium (Tervo *et al.*, 1989; Tucker *et al.*, 1991) and suggested that its origin might not be specifically mesenchymal. The presence of tenascin-like molecules in







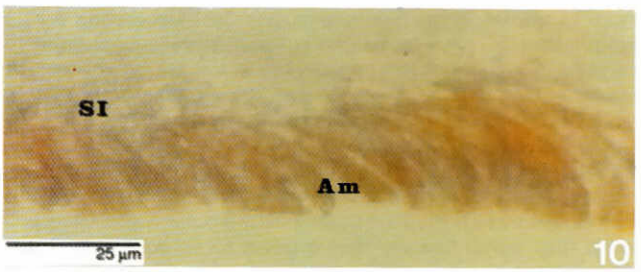


Plate 2. Influence of tris buffer on human fetal tooth germ sections at 24 and 25 weeks. (7) *PAP Immunostaining of type I collagen. Tris buffer, 0.4% pepsin NSS. Type I collagen was intensely detected in the secretory odontoblasts (arrowheads) and in the epithelio-mesenchymal junction (arrows). x416.* **(8-11)** *PAP immunostaining of tenascin. Tris buffer, 0.4% pepsin, NRS.* **(8)** *A tenascin-like immunostaining was revealed in the ameloblasts (Am) and in the outer enamel epithelium cells (OE). DP= dental papilla. Od= odontoblasts. arrowhead= blood vessel. D= dentin. (*)= space resulting from decalcification of enamel. x47.* **(9)** *The secretory odontoblasts showed an intracellular staining (arrowheads). x1660.* **(10)** *A tenascin-like immunostaining was detected in the ameloblasts (Am). SI= stratum intermedium. x665.* **(11)** *Tris buffer, 0.4% pepsin, without NRS. The tenascin-like staining remained in the ameloblasts and showed an apical localization (arrowheads). SI= stratum intermedium. x665.*

TABLE 3

INFLUENCE OF TISSUE PRETREATMENT ON TENASCIN IMMUNOSTAINING

Tenascin	Buffer	Without serum	NSS	Heat inactivated NSS
Staining intensity				
without enzymatic	PBS	0	0	0
pretreatment	TRIS	0	++	++
0.4%	PBS	++	+++	++
pepsin	TRIS	+++	+++	+++
Background				
without enzymatic	PBS	0	0	0
pretreatment	TRIS	0	0	0
0.4%	PBS	0	0	0
pepsin	TRIS	0	0	0

For legends, see Table 1.

ameloblasts could be explained by a paracrine process translocating mesenchymal tenascin molecules in the epithelial cells. On the other hand, the possibility of a common epitope shared by enamel proteins with tenascin cannot be ruled out, nor can the effects of certain conditions of pretreatment which could generate modifications in the conformational structure of proteins «creating" new antigenic sites. The use of NSS as a blocking agent prior to the immunostaining was shown to generate a moderate background unlike NRS. This interference, probably due to the adhesion of NSS components to the tissue, was not reduced when heat-inactivated NSS was used.

Experimental Procedures

The lower jaws were removed from 24- and 25-week-old normal, noninfected, human fetuses collected from spontaneous abortions or medical inductions from the Department of Developmental Biology. This study was carried out with the approval of the Regional Development and Reproduction Ethics Committee.

The fetuses were either fixed immediately by the obstetrician or brought directly to the laboratory (within less than 24 h) where the jaws were dissected and fixed in 10% neutral buffered formalin for 6 days. These samples were decalcified for approximately 3 weeks in formic acid (15 vol.) and 10% formalin (85 vol.) prior to the embedding in Paraplast at 56°C. Next, 6 µm-thick consecutive sections were cut, adhered to glass slides and dried at room temperature. Deparaffinized and rehydrated sections of tissues were incubated for 30 min at 37°C in 0.4%, 0.1%, 0.05% or 0.01% pepsin (Merck) in 0.01N HCl. These sections, together with an undigested set of sections, were treated with 3% H_2O_2 for 5 min to block endogenous peroxidase activity. Next, some sections were exposed for 20 min either to normal serum of the same species of the secondary antibody used (Dakopatts), or to normal serum, heat-inactivated at 56°C for 30 min; both were diluted to 1:5 while the other sections were immunostained without serum incubation. Throughout this process, all the incubations and washes were performed either in 0.1 M PBS, pH 7.4 or in 0.05 M Tris, pH 7.6.

The primary antibodies used were incubated in a moist chamber overnight at 4°C. a) Rabbit anti-human type I and type III collagen IgG were purchased from Sanbio and were diluted to 1:20. b) Mouse monoclonal antibody to human tenascin was obtained from Locus (clone D B7) and was diluted to 1:450. The secondary and tertiary antibodies were incubated for 20 min at room temperature. a) Swine anti-rabbit immunoglobulin and rabbit PAP IgG (Dakopatts) were diluted to 1:100 and 1:200 for type I and III collagen immunostaining respectively. b) Rabbit anti-mouse immunoglobulin and monoclonal mouse PAP (Dakopatts) were diluted to 1:50 and 1:100 for tenascin immunostaining respectively. Next, the sections were exposed for 30 min to 3-amino-9-ethylcarbazole (Dakopatts), counterstained in Masson's hemalum for 5 sec and mounted in Aquamount (Gurr). The following control incubations were performed by substituting the primary antibody overnight at 4°C with: a) PBS, pH 7.4 or Tris, pH 7.6; b) NSS or NRS; c) heat-inactivated NSS or NRS; d) an irrelevant murine monoclonal IgG1 (93G7) used as a control for tenascin immunostaining.

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References

BEDOSSA, P., BACCI, J., LEMAIGRE, G. and MARTIN, E. (1987). Effects of fixation and processing on the immunohistochemical visualization of type I, III and IV collagen in paraffin-embedded liver tissue. *Histochemistry 88:* 85-89.

BRANDTZAEG, P. (1982). Tissue preparation methods for immunohistochemistry. In *Techniques in Immunocytochemistry* (Eds. G.R. Bullock and P. Petrusz) Vol. 1. Academic Press, London, pp. 1-34.

BURNS, J. (1982). The unlabelled antibody peroxidase-antiperoxidase method (PAP). In *Techniques in Immunocytochemistry* (Eds. G.R. Bullock and P. Petrusz) Vol. 1. Academic Press, London, pp. 91-105.

CLEMENT, B., RISSEL, M., PEYROL, S., MAZURIER, Y., GRIMAUD, J.A. and GUILLOUZO, A. (1985). A procedure for light and electron microscopic intracellular immunolocalization of collagen and fibronectin in rat liver. *J. Histochem. Cytochem. 33:* 407-414.

FINLEY, J.C.W. and PETRUSZ, P. (1982). The use of proteolytic enzymes for improved localization of tissue antigens with immunocytochemistry. In *Techniques in Immunocytochemistry* (Eds. G.R. Bullock and P. Petrusz) Vol. 1. Academic Press, London, pp. 239-249.

HOLUND, B., CLAUSEN, P.P. and CLEMMENSEN, I. (1981). The influence of fixation and tissue preparation on the immunohistochemical demonstration of fibronectin in human tissue. *Histochemistry 72:* 291-299.

KIRKPATRICK, P. and D'ARDENNE A.J. (1984). Effects of fixation and enzymatic digestion on the immunohistochemical demonstration of laminin and fibronectin in paraffin embedded tissue. *J. Clin. Pathol. 37:* 639-644.

LAURENT-MAQUIN, D., DIAB EL-LADKI, R., GAILLARD, D., HARTMANN, D.J. and BOUTHORS, S. (1992). Immunohistochemical localization of laminin, type IV collagen and fibronectin in human tooth germs during odontogenesis. In *Recent Advances in Cellular and Molecular Biology* (Eds. R.J. Wegmann and R.A. Wegmann) Vol 5. Peeters Press, Leuven, pp. 109-124.

LI, C.Y., ZIESMER, S.C. and LAZCANO-VILLAREAL, O. (1987). Use of azide and hydrogen peroxide as an inhibitor for endogenous peroxidase in the immunoperoxidase method. *J. Histochem. Cytochem. 35:* 1457-1460.

MATTHEWS, J.B. (1981). Influence of clearing agent on immunohistochemical staining of paraffin embedded tissue. *J. Clin. Pathol. 34:* 103-105.

MORI, S., SAWAI, T., TESHIMA, T. and KYOGOKU, M. (1988). A new decalcifying technique for immunohistochemical studies of calcified tissue, especially applicable to cell surface marker demonstration. *J. Histochem. Cytochem. 36:* 111-114.

PETRUSZ, P. (1983). Essential requirements for the validity of immunocytochemical staining procedures. *J. Histochem. Cytochem. 31:* 177-179.

STERNBERGER, L.A. (1979). *Immunohistochemistry*, 2nd ed. J. Wiley and Sons, New York.

STERNBERGER, L.A., HARDY, P.H., CUCULIS, J.J. and MEYER, H.G. (1970). The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. *J. Histochem. Cytochem. 18:* 315-333.

TERVO, K., TERVO, T., VAN SETTEN, G.B., TARKKANEN, A. and VIRTANEN, I. (1989). Demonstration of tenascin-like immunoreactivity in rabbit corneal wounds. *Acta Ophtalmol. 67:* 347-350.

TUCKER, J.E., LEMON, R., MACKIE, E.J. and TUCKER, R.P. (1991). Immunohistochemical localization of tenascin and fibronectin in the dentine and gingiva of *canis familiaris*. *Arch. Oral Biol. 36:* 165-170.

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