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Original Article

Localization of Type IV collagen α 1 to α 6 chains in basement membrane during mouse molar germ development

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ABSTRACT The dental basement membrane (BM) putatively mediates epithelial-mesenchymal interactions during tooth morphogenesis and cytodifferentiation. Type IV collagen α chains, a major network-forming protein of the dental BM, was studied and results disclosed distinct expression patterns at different stages of mouse molar germ development. At the dental placode and bud stage, the BM of the oral epithelium expressed α 1, α 2, α 5 and α 6 chains while the gubernaculum dentis, in addition to the above four chains, also expressed α 4 chain. An asymmetrical expression for α 4, α 5 and α 6 chains was observed at the bud stage. At the early bell stage, the BM associated with the inner enamel epithelium (IEE) of molar germ expressed α 1, α 2 and α 4 chains while the BM of the outer enamel epithelium (OEE) expressed only α 1 and α 2 chains. With the onset of dentinogenesis, the collagen α chain profile of the IEE BM gradually disappeared. However from the early to late bell stage, the gubernaculum dentis consistently expressed α 1, α 2, α 5 and α 6 chains resembling fetal oral mucosa. These findings suggest that stage- and position- specific distribution of type IV collagen α subunits occur during molar germ development and that these changes are essential for molar morphogenesis and cytodifferentiation.

KEY WORDS: *Type IV collagen, α chains, molar germ development, basement membrane, immunofluorescence localization*

Introduction

The dental basement membrane (BM) interposed between the dental epithelium and dental mesenchyme mediates the sequential and reciprocal epithelial-mesenchymal interactions essential for tooth morphogenesis and cytodifferentiation. It contains isoforms of type IV collagen, laminin, nidogen/entactin, heparan sulfate proteoglycan, fibronectin, and type III collagen (Lesot *et al.,* 1981; Thesleff *et al.,* 1981; Kubler *et al.,* 1988). Type IV collagen molecules, a major network-forming protein of BM, are heterotrimers composed of three α chains that exist in six genetically distinct forms (α1 to α6) and with at least three molecular forms i.e. $[\alpha1(IV)]_2\alpha2(IV)$ which is ubiquitous in all BM, α3(IV) α4(IV) α5(IV) which is abundant in glomerular BM, and α5(IV)/α6(IV) which is found in skin epithelium (Hudson *et al.,* 1993; Yurchenco and O'Rear, 1994; Ninomiya *et al.,* 1995; Sado *et al.,* 1995; Kagawa *et al.,* 1997). So far, there is no report on the expression patterns of type IV collagen molecular forms in molar germ organogenesis nor on the marked stage-specific changes in the distribution of type IV collagen during early tooth development, up to the disappearance of the BM during the initiation of terminal differentiation of ameloblasts (Lesot *et al.,* 1981; Kubler *et al.,* 1988; Heikinheimo and Salo., 1995). In the present study, indirect immunofluorescence on cryosections was used to characterize the changes in molecular forms of collagen by localizing the α1 to α6 subunits of type IV collagen during mouse molar germ development.

Results

The stages of molar germ development of the samples are shown in Fig. 2 A,B,C and D. Samples collected on embryonic days (ED) 11, 13, 15 and postnatal day (PD) 1 corresponded to the dental placode stage, bud stage, early bell stage, and late bell stage with initial dentine and enamel matrix formation, respec-

Abbreviations used in this paper: BM, basement membrane; ED, embryonic day; IDE, inner dental epithelium; IEE, inner enamel epithelium; OEE, outer enamel epithelium; PBS, phosphate buffered saline; PD, postnatal day.

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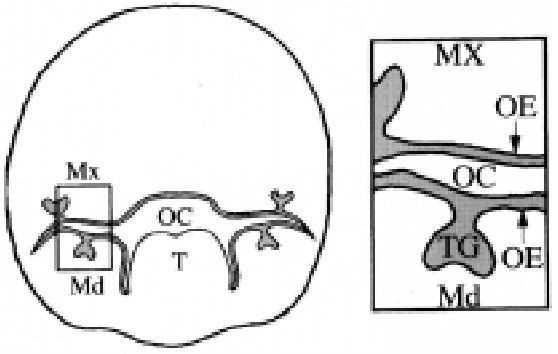

Fig. 1. Orientation and region of observation in frozen section. *Mx, maxilla; Md, mandible; T, tongue; TG, tooth germ (1st mandibular molar); OE, oral epithelium; OC, oral cavity.*

tively. During all stages examined from ED11 to PD1, the BM of oral epithelium in the fetal oral cavity showed positive immunostaining for α1, α2, α5 and α6 chains while the BM of capillaries showed positive reactions for α1 and α2 chains in all areas of mesenchymal tissues examined. Representative immunostaining patterns of various type IV collagen α chains in each developmental stage are shown in Figs. 3 to 6 and described below.

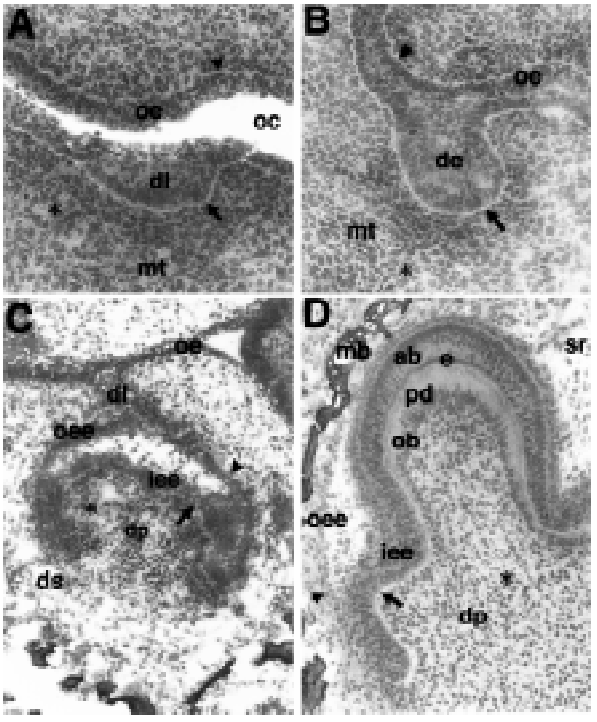

Fig 2. Histological findings of frontal cryo-section at the 1st molar level of mouse embryo. (A) *Dental placode stage (ED11) 150x.* **(B)** *Bud stage (ED13) 75x.* **(C)** *Early bell stage (ED15) 75x.* **(D)** *Late bell stage (PD1) 90x. Abbreviations: ab, ameloblast; de, dental epithelium; dl, gubernaculum dentis; dp, dental papilla; ds, dental sac; e, enamel matrix formation; mb, mandibular bone; mt, mesenchymal tissue of mandible; iee, inner enamel epithelium; oe, oral epithelium; oee, outer enamel epithelium; pd, predentine; sr, stellate reticulum; *, capillary. Arrow head, basement membrane of maxillary oe (A,B), iee (C) and oee (D); Arrow, basement membrane of dl (A,B), iee (C) and oee (D). In (B) and (C), fusion of oral epithelium of maxillary and mandible is due to a technical artifact. (H.E.)*

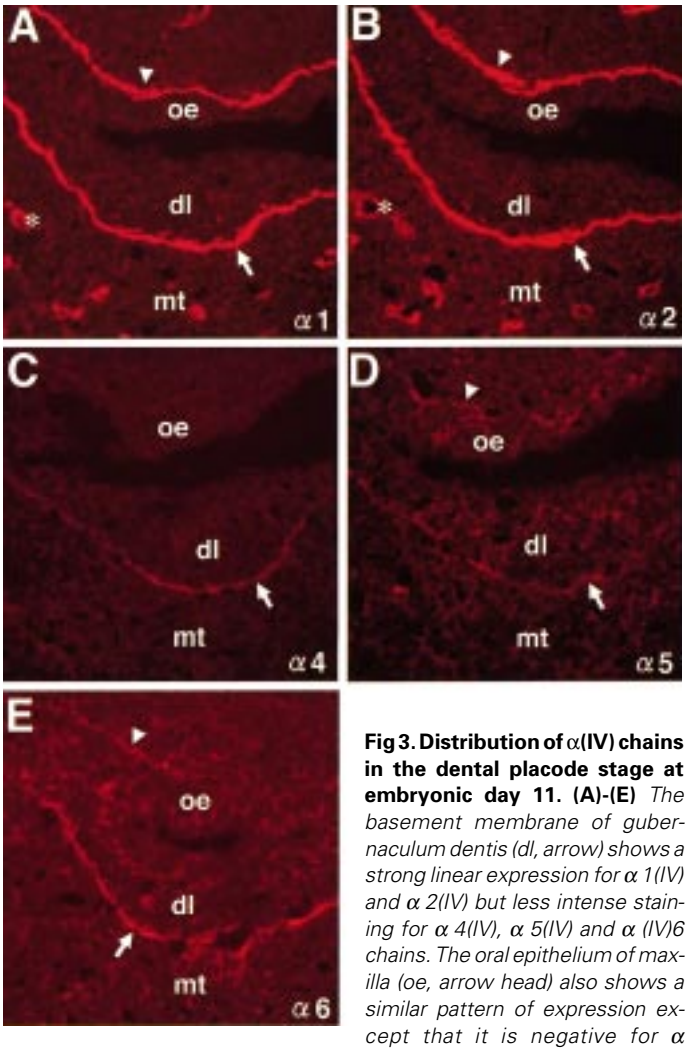

Fig 3. Distribution of α(IV) chains in the dental placode stage at embryonic day 11. (A)-(E) *The basement membrane of gubernaculum dentis (dl, arrow) shows a strong linear expression for α 1(IV) and α 2(IV) but less intense staining for α 4(IV), α 5(IV) and α (IV)6 chains. The oral epithelium of maxilla (oe, arrow head) also shows a similar pattern of expression except that it is negative for α 4(IV)chains. The vascular basement membrane (*) stains for only α 1(IV) and α 2(IV). mt, mesenchymal tissue of mandible, 250x.*

Dental placode stage (ED11) and bud stage (ED13)

Antibodies against α1, α2, α4, α5 and α6 chains stained the BM along the gubernaculum dentis (ED11) (Fig. 3 A-E and Fig. 4 A-E). At the bud stage, expression of α4, α5 and α6 chains was stronger along the BM of the dental epithelium on the buccal than on the lingual aspect of mouse molar germ (Fig. 4 C,D and E). Antibody against α3 chain showed no staining of the BM at all stages of the odontogenic epithelial development.

Early bell stage (ED 15)

The BM associated with the inner enamel epithelium (IEE) was positive for α1, α2 and α4 chains, while the BM adjacent to the outer enamel epithelium (OEE) was positive for α1, α2 but not α4 chain (Fig. 5 A,B and C). The gubernaculum dentis of molar germ was positive for α1, α2, α5 and α6 (Fig. 5 A,B,D and E).

Late bell stage with initial matrix formation (PD1)

In the late bell stage, antibodies against α1, α2, and α4 chains showed a continuous staining reaction along the BM of the IEE (Fig. 6 A,B and C), while only α1 and α2 chains were demonstrated in the BM of OEE. When functional ameloblasts started to

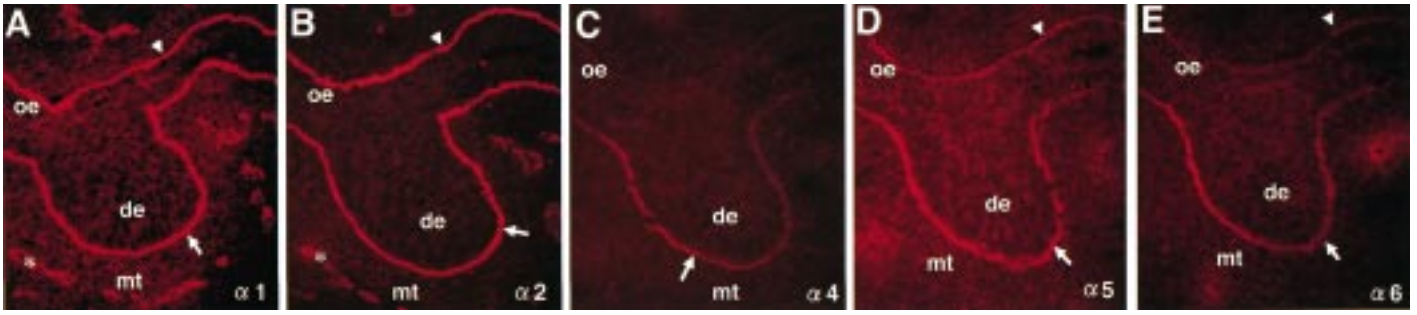

Fig 4. Distribution of α(IV) chains in the bud stage at embryonic day 13. (A,B) *The basement membrane of the dental epithelium (de, arrow) shows a strong linear expression for α1(IV) and α2(IV).* **(C-E)** *Staining for α4(IV), α5(IV) and α6(IV) chains was more intense along the basement membrane of the de on the buccal aspect of molar germ. (A-E)The oral epithelium of maxilla (oe, arrow head) also shows a similar expression pattern except that it is negative for α4(IV)chains. The vascular basement membrane (*) stains for only α1(IV) and α2(IV). mt, mesenchymal tissue of mandible, 125x.*

secrete the initial enamel matrix in the cuspal area, the distribution of α1, α2, and α4 chains in IEE became discontinuous and gradually disappeared towards the tip of the cusp (Fig. 6 A,B and C). αThe changes in the distribution of α1 to α6 chains at each stage and area of mouse molar germ morphogenesis are summarized in Fig. 7.

Discussion

The expression patterns of collagen IV α chains during mouse molar germ development were visualized using indirect immunofluorescence. Our findings showed that the BM in the early stages (the dental placode and bud stage) expressed five out of the six genetically distinct molecular forms of collagen IV α chains i.e. α1(IV), α2(IV), α4(IV), α5(IV) and α6(IV) except α3(IV)chain. In the absence of α3(IV) chain, the α4(IV)chain probably exists as a homotrimer in the molecular form $[\alpha4(IV)]_3$. So far evidence has only been obtained for heterotrimers having chain composition of $[\alpha1(IV)]_2\alpha2(IV)$, α3(IV) α4(IV) α5(IV), and α5(IV)/ α6(IV) (Yurchenco and O'Rear, 1994; Ninomiya *et al.,* 1995; Sado *et al.,* 1995;) and homotrimers of $[\alpha1(IV)]_3$ and $[\alpha3(IV)]_3$ (Saus *et al.,* 1988). In this study the staining for α1(IV) and α2(IV) was generally much stronger than those for the other three α chains for all stages of tooth germ development. We attribute this to the predominant presence of α1(IV) and α2(IV) chains in the dental BM.

At the bud stage, expression for type IV collagen α4, α5, and α6 chains was stronger along the BM of the dental epithelium on the buccal than on the lingual aspect of the mouse molar germ. This asymmetrical expression might be related to molar germ morphogenesis and to the differences in histogenesis and cytodifferentiation that exist between the buccal and the lingual aspects of the enamel organ (Yoshiba *et al.,* 2000). The cytokines which are not homogeneously distributed in the adjacent dental mesenchyme may also modulate this asymmetrical expression (Ruch *et al.,* 1981; Ruch, 1987; Thesleff *et al.,* 1981, 1995; Nagai *et al.,* 1995; Paralkar *et al.,* 1991; Aberg *et al.,* 1997). BMP-4 which modulates dental epithelium differentiation during tooth morphogenesis shows stronger expression on the buccal than on the lingual side of the enamel organ (Aberg *et al.,* 1997). Recent reports have suggested that cellular interactions with collagen IV are also mediated by integrin ligation of specific regions within the central triple helical domain (Miner and Sanes, 1996). In vascular BM, non-collagenous domains of the α2, α3 and α6 chains of collagen IV can directly interact with endothelial cells by engaging distinct α_v and α_1 integrin receptors (Petitclerc *et al.,* 2000). However the role of integrins in modulating the distribution of collagen IV molecules in the dental BM remains to be elucidated.

The collagen IV α chain profile of the gubernaculum dentis BM from the bud stage to the early bell stage fundamentally resembled those of the BM of oral epithelium in expressing α1, α2, α5 and α6 (Fleischmajer *et al.,* 1997). In the early bell stage, the BM of IEE expressed α1, α2 and α4 chains, with disappearance of α5 and α6 chains whereas BM of OEE expressed only α1 and α2 chains resembling the BM of capillary which is composed basically of α1

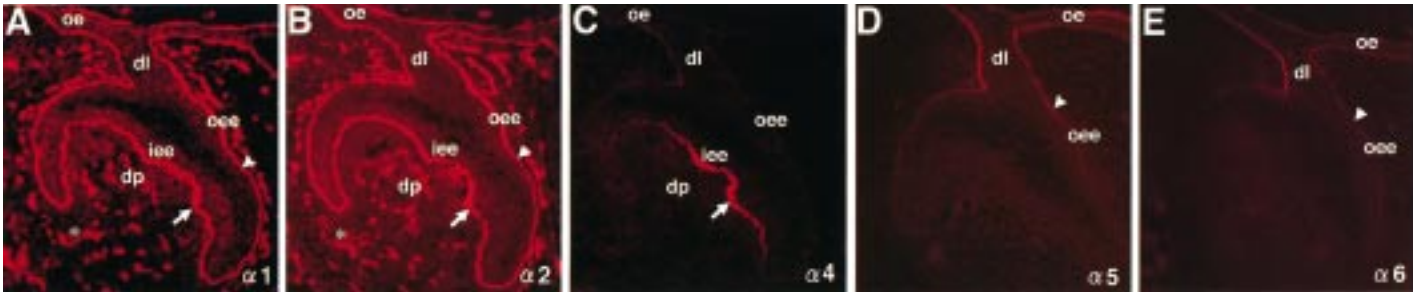

Fig 5. Distribution of α(IV) chains in early bell stage at embryonic day 15. (A-C) *The basement membrane of inner enamel epithelium (iee, arrow) stains for α1(IV), α2(IV) and α4(IV) chains. Vascular basement membranes (*) stain for α1(IV) and α2(IV) chains. (A-E) The basement membrane of outer enamel epithelium (oee, arrow head) stains for α1(IV) and α2(IV)chains, and partially for α5(IV) and α6(IV) chains. This same pattern of expression is also seen in the gubernaculum dentis (dl) and oral epithelium (oe). 125x.*

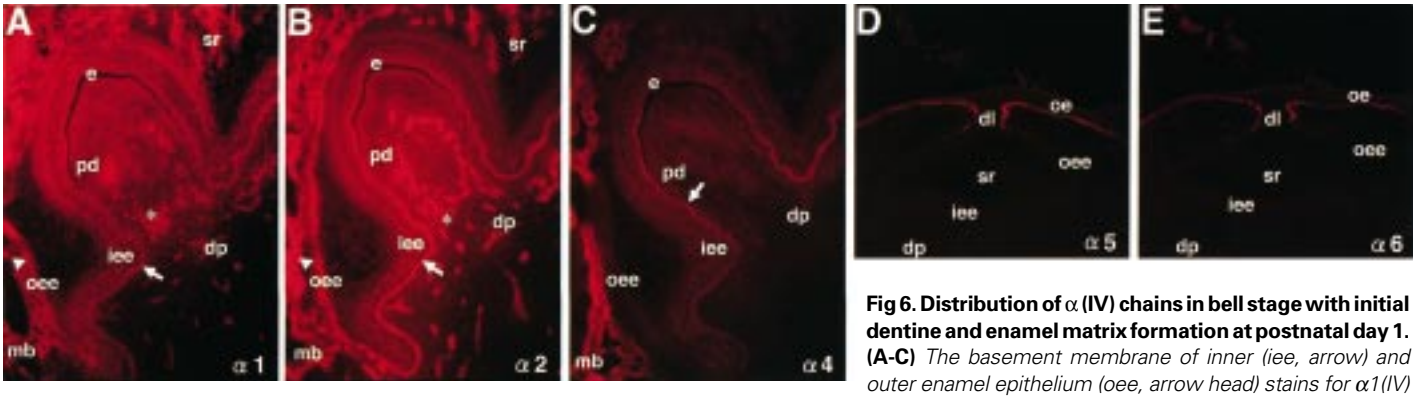

Fig 6. Distribution of α (IV) chains in bell stage with initial dentine and enamel matrix formation at postnatal day 1. (A-C) *The basement membrane of inner (iee, arrow) and outer enamel epithelium (oee, arrow head) stains for α1(IV) and α2(IV) while α4(IV) chains specifically stain the iee basement membrane. These reactions gradually disappear in the enamel matrix (e) and predentine matrix formative area (pd) of the cusp. Vascular basement membranes (*) in dental papilla (dp) and stellate reticulum (sr) are positive for α1(IV) and α2(IV) chains. (Mb, mandibular bone shows non-specific natural fluorescence).* **(D-E)** *The basement membrane of gubernaculum dentis (dl) and oral epithelium (oe) stain for α5(IV) and α6(IV) chains. 125x.*

and α2 chains. These findings suggest that stage- and position-specific α chain isoforms exist in the BM in molar germ development. Laminins are the other major network-forming proteins of the dental BM. They are hetrotrimers consisting of α, α and α subunit chains with eleven isoforms (Yoshiba *et al.,* 1998). Laminin-5 is composed of three subunit chains α3, α3 and α2 encoded by three different genes. Collagen IV and laminin form homotypic polymers that are stabilized by nidogen bridging (Yurchenko *et al.,* 1992). The distribution ofα1, α2 and α4 chains of BM collagen IV in the IEE may be linked to laminin-5 which demonstrated similar transcription patterns for alpha3, beta3 and gamma2 subunits in the incisor and molar germs inner dental epithelium (IDE)(Yoshiba *et al.,* 1998; 2000). This differential expression suggests that laminin-5 subunits in the IDE might be involved in the histogenesis of the IDE and ameloblast differentiation (Yoshiba *et al.,* 1998; 2000). Our study suggests that α1, α 2 and α4 chains of type IV collagen in the BM of IEE at the early bell stage may function as a trapping and delivery system by sequestering factors involved in epithelial-mesenchymal interactions during tooth germ histomorphodifferentiation notably preodontoblast differentiation.

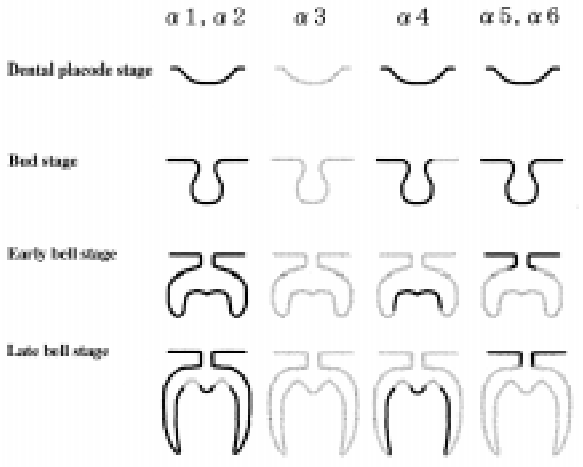

Fig. 7. Changes in the distribution of type IV collagen α chains in the basement membrane during mouse tooth germ development. *At the dental placode and bud stages, the basement membrane of the gubernaculum dentis stained positively for all but α3(IV) chains. However, at the early and late bell stages, the basement membrane of the inner enamel epithelium showed positive staining for α1(IV), α2(IV) and α4(IV) chains, while the basement membrane of the outer enamel epithelium was positive for α1(IV) and α2(IV) chains. Bold lines indicate positive staining reaction and pale lines indicate negative staining reaction.*

Materials and Methods

Monoclonal antibodies

Rat monoclonal antibodies, H11, H22, H31, H43, M54 and M69, recognizing type IV collagen αl, α2, α3, α4, α5 and α6 chains, respectively, were raised against synthetic peptides of non-consensus amino acid sequences of the human (IV) chains. Their specificity against the individual human (IV) chains was confirmed by ELISA and immunoblotting, and epitopes were determined earlier by multipin-peptide scanning (Sado *et al.,* 1995). These antibodies gave the same pattern reaction with mouse tissues (Seki *et al.,* 1998).

Preparation of tissues

ICR mouse embryos (detection of vaginal plug: day 0) and newborn mice were used. The heads of mice at embryonic day 11 to 17 and postnatal day 1 were embedded in OCT compound (Tissue-Tek, Sakura Finetechnical Co., Tokyo, Japan) and frozen in liquid nitrogen. The frozen samples were then cut into 4 μm sagittal sections (Fig. 1) and mounted on gelatin-coated glass slides. One set of the cryosections were stained with hematoxylin and eosin for histological determination of the developmental stage of molar germ and orientation for observation (Fig. 2).

Immunohistochemistry

Sections were fixed with acetone for 10 min, followed by 20 min treatment with 6 M urea in 0.1 M glycine-HCI buffer (pH 3.5) as a denaturation step to expose epitopes, as described previously (Ninomiya *et al.,* 1995), and washed in PBS. After blocking with normal mouse serum for 20 min, the sections were incubated with the primary antibody for 1 h at room temperature, and then rinsed in phosphate-buffered saline solution (PBS). The sections were incubated with Cy3-conjugated mouse anti-rat lgG (Jackson Immunoresearch, West Grove, PA) as a second antibody (diluted l:300) for 50 min at room temperature. The specimens were mounted with PermaFluorTM Aqueous Mounting Medium (IMMUNON, Pittsburgh, PA) and then examined using a fluorescence microscope. For negative control, sections were reacted with normal rat serum or with the second antibody alone. All the control sections were negative.

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