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Occurrence and distribution of matrix metalloproteinase-2-immunoreactivity in human embryonic tissues

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SUMMARY

Tissue development and structure is controlled by dynamic and interactive relationships between cells and the extra-cellular matrix (ECM) which they secrete. We have investigated the occurrence and distribution of metalloproteinase-2 (MMP-2), an enzyme involved in the catabolism of ECM components, in human embryonic tissues by immunocytochemistry. Cells displaying MMP-2 immunoreactivity showed a widespread distribution in human embryonic tissues and organs. Cytoplasmic staining was detected in cells deriving from all three embryonic layers. Although further studies are needed to clarify the possible substrates of MMP-2 in developing tissues, these morphological data lend support to the hypothesis that ECM remodelling and degradation may represent a physiological counterpart of ECM deposition that occur during development.

INTRODUCTION

The composition and distribution of the extra-cellular matrix (ECM) in mature and developing tissues have been extensively studied *in vivo* and *in vitro*. Such studies have yielded convincing evidence for the role of the ECM in cell differentiation and migration, as well as cell-to-cell communication (Gospodarowicz *et al.*, 1978; Wicha *et al.*, 1979; Kleinman *et al.*, 1981; Hay, 1981, 1984; Madri and Williams, 1983; Martin *et al.*, 1984; Ingber *et al.*, 1986; Ruoslahti *et al.*, 1991). In this connection, much interest has been focused on development-related changes in the composition of the ECM that occur at specific stages of organogenesis (Spooner and Faubion, 1980; Eckblom *et al.*, 1981; Thesleff and Hurmerinta, 1981; Ruch *et al.*, 1982; Bernfield *et al.*, 1984a,b; Ruch, 1985; Sharpe and Ferguson, 1988; Sakamura, 1991). Although much information about ECM synthesis and deposition in developing and mature tissues is currently

available, little is known about the mechanisms by which ECM remodelling and degradation are controlled and regulated.

Matrix metalloproteinases (MMPs), also referred to as matrixins, are an enzyme family which specifically degrade components of the ECM (reviewed in Werb, 1989; Matrisian, 1990; Stetler-Stevenson, 1990a; Woessner, 1991). Such enzymes, besides sharing common nucleotides and amino acid sequences, contain zinc at the active center, are secreted as zymogens subsequently activated by loss of a C-terminal fragment, and can be inhibited by specific inhibitors called tissue inhibitors of metalloproteinase (TIMPs). MMP activation occurs under several experimental conditions and each enzyme of the family shows a range of activity against some ECM components, although with variable efficiency. Matrix metalloproteinase-2 (MMP-2, alternatively named type IV collagenase, 72-kDa gelatinase or gelatinase A, E.C.3.4.24.24) is characterized by a strong degradative activity of basement membrane-associated type IV collagen, but exhibits catalytic activity also toward type V and VII collagens, fibronectin and elastin (Liotta *et al.*, 1981; Collier *et al.*, 1988; Seniro *et al.*, 1991).

In consideration of the potential role of MMPs in developmental dynamics, we have investigated the possible occurrence of MMP-2 in human developing tissues by immunohistochemical methods.

MATERIALS AND METHODS

Collection and processing of human embryonic tissues

Prostaglandin-induced human fetal abortuses (n=15) of 10 to 25 weeks gestation were collected within 30 min of delivery according to the ethical standards of the "S. Matteo" Hospital, Pavia, in which they were collected. All fetuses appeared morphologically normal. Specimens were immediately fixed with 4% para-formaldehyde in phosphate buffer, pH 7.4, for 24 hrs. Samples of organs were then dehydrated through graded ethanols, cleared in xylene and embedded in paraffin. The organs studied included: brain (frontal lobe), upper and lower jaws (including forming bones, cartilages, tooth germs, mesenchyme, blood vessels, salivary glands, skin, hair follicles and nasal epithelium), lung, heart, intestine, stomach, liver, vertebral column (with spinal cord, dorsal root ganglia and spinal muscles), kidney and adrenal glands. Paraffin-embedded tissues were cut at 5-7 µm. Some sections were stained with haematoxylin and eosin for morphological analysis, others were processed for the immunocytochemical detection of MMP-2 according to the indirect immunoperoxidase method described by Hsu *et al.* (1981) with some modifications. At least five sections of each tissue were immunostained and screened.

Immunocytochemical staining

Rehydrated sections were incubated serially with the following solutions: 1) 0.3% hydrogen peroxide for 30 min to remove endogenous peroxidase activity; 2) normal goat serum, diluted 1:20, for 30 min to reduce background staining; 3) rabbit polyclonal antisera to MMP-2, at a range of dilutions (1:100 to 1:1000), overnight at 4°C; 4) biotinylated goat anti-rabbit IgG, diluted 1:100, for 1 h at room temperature; 5) streptavidin-biotinylated peroxidase complexes, diluted 1:200, at room temperature for 1 h; 6) 0.03% 3,3'-diaminobenzidine tetrahydrochloride solution to which hydrogen peroxide (0.02%) was added just before use. Each solution was prepared in 0.05 M Tris buffer, pH 7.4, containing 0.1 M NaCl (0.15 M Tris buffered saline, TBS) and between each step of the immunostaining procedure the sections were washed in the same buffer.

Immunostained sections were finally rehydrated, mounted and observed using a Leitz "Orthoplan" microscope.

Antisera and controls of the immunocytochemical reaction

Anti-MMP-2 antibodies were raised in rabbits injected with synthetic peptides corresponding to the 17 amino acid-amino terminus and the putative metal ion-binding domain of MMP-2. Preparation, affinity-purification and immunochemical characterization of these antisera with Western blot analysis and enzyme linked immunosorbent assay (ELISA) have been described previously (Monteagudo *et al.*, 1990; Levy *et al.*, 1991).

Pertinent specificity tests of the immunocytochemical reaction were performed, including adsorption of the specific antisera with related and unrelated antigens, omission of the first layer and substitution of an inappropriate antiserum or a non-immune serum for the specific primary antisera (Polak and Van Noorden, 1987).

Secondary biotinylated antibodies and streptavidin-biotinylated peroxidase complexes were purchased from Dakopatts, Glostrup, Denmark. All other reagents were obtained from Sigma Chemical Co., St. Louis, MO.

RESULTS

Cells displaying cytoplasmic MMP-2-like immunoreactivity showed a widespread distribution in human embryonic tissues and organs.

Lining epithelia (e.g. epidermis, nasal, bronchial, gastric and gut epithelia) as well as glandular epithelia (e.g. liver, salivary gland, kidney, adrenal gland epithelia) were immunostained (Fig. 1). Dental lamina (Fig. 1C) and hair follicles were positive as well.

Osteoblasts, osteoclasts and immature cartilage cells (but not mature chondrocytes and perichondrium) displayed immunostaining (Fig. 2A). Positive cells were observed within the mesenchyme and immature connective tissue (Fig. 2B). Vessels of small and medium diameter showed immunoreactivity in the endothelium.

Immunoreactivity was observed in immature muscle cells (namely myoblasts and myotubes) throughout the embryos (Fig. 3A). However, not all cells appeared immunostained within developing muscle fibres, nor did all fibres in the same embryo contain positive cells. Developing myocardium was positive as well.

Immunostaining was observed within ependymal cells, some Schwann cells and ganglion cells of the peripheral nervous system (Fig. 3B). No immunoreactivity was detected in immature neurons of the central nervous system.

In summary, cells deriving from all three germinal embryonic layers were positively stained in all embryos examined.

The pattern and tissue distribution of immunostaining were similar using antisera raised against antigenically unrelated portions of MMP-2 (amino-terminus and metal ion-binding domain); this served as a further control of the specificity of the immunohistochemical reaction.

DISCUSSION

This study demonstrates the occurrence of metalloproteinase-2-like immunoreactivity in developing human tissues *in situ*. Many cell types deriving from all three primitive embryonic layers were found to be able to synthesize and possibly secrete MMP-2 during development. According to previous immunohistochemical studies on protease tissue distribution (Monteagudo *et al.*, 1990; Levy *et al.*, 1991; Sumi *et al.*, 1992), MMP-2-immunoreactivity was detectable only within the cytoplasm of immunoreactive cells, whereas no extracellular staining could be observed. This may be due to antigenic modification of MMP-2 following secretion or activation in the extra-cellular matrix or to the association with substrate molecules that may hinder antigenic sites. An alternative explanation is that the enzyme level in the extracellular compartment is below the sensitivity threshold of the immunohistochemical methods.

Previous studies have shown consistent low expression of MMP-2 in normal adult tissues, thus suggesting that MMP-2 may be a cell component involved in normal physiologic processes such as basement membrane turnover (Monteagudo *et al.*, 1990; Stetler-Stevenson, 1990a; Levy *et al.*, 1991). The overproduction and/or unrestrained activity of MMPs have been associated with many pathologic conditions characterized by degradation of connective tissue matrix, e.g. tumour invasion and metastasis, rheumatoid arthritis, osteoarthritis, periodontitis and wound healing (Liotta *et al.*, 1980, 1983; Okada *et al.*, 1990; Overall *et al.*, 1991; Sato *et al.*, 1992). Recent biochemical studies have reported the occurrence of members of the metalloproteinase family in developing tissues and have provided evidence that the expression of such enzymes may be developmentally regulated in some tissues (Brenner *et al.*, 1989; Adler *et al.*, 1990; Weinberg *et al.*, 1990; Talhouk *et al.*, 1991). The morphological demonstration of MMP-2-immunoreactivity in human embryonic tissues lends further support to the view that metalloproteinases may be involved in developmental dynamics.

Proteases have been suspected to play a role in cell migration during development (Valinsky *et al.*, 1981; Valinsky and Le Douarin, 1985). Evidence exists that proteases are locally secreted in adhesion plaques, thus allowing the cell to move in the ECM (Chen *et al.*, 1984). According to this hypothesis, the occurrence of proteases within developing neurons has led to the suggestion that proteases may be involved

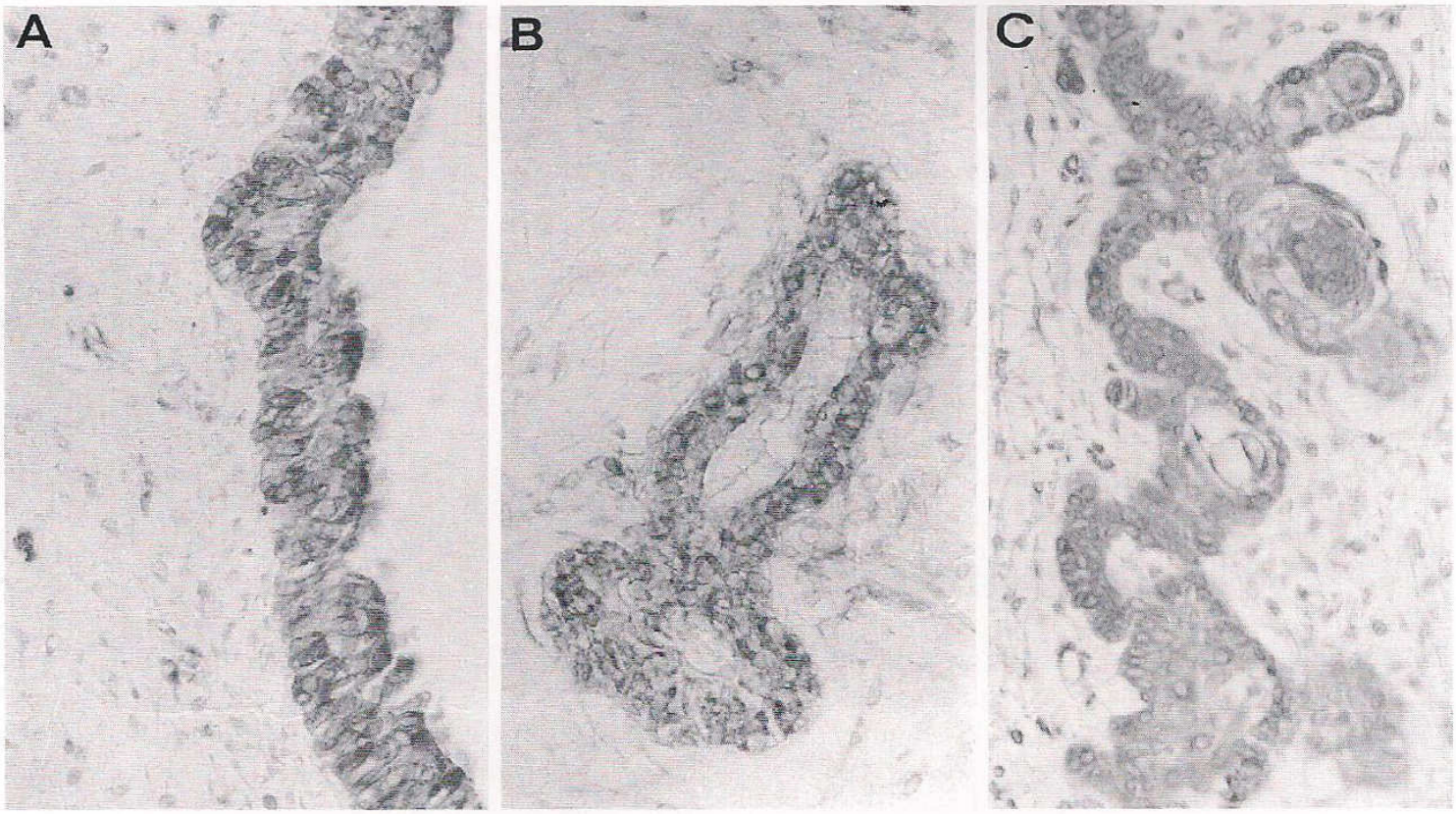

Fig. 1 - Immunocytochemical identification of matrix metalloproteinase-2-immunoreactivity in developing epithelia of human embryo. A: nasal epithelium from an embryo of about 9 weeks. B: developing salivary gland from an embryo of about 10 weeks. C: dental lamina from an embryo of about 16 weeks. Magn. 250 x (A), 400 x (B,C).

in axonal growth and tissue remodelling associated with neural development (McGuire and Seeds, 1990; Sumi *et al.*, 1992).

While the possible role of osteoclast-secreted proteases in bone resorption has been recently elucidated (Everts *et al.*, 1992), the function of proteases in bone formation is not clear. These enzymes are secreted by bone cells in culture and may play a positive role in bone formation (Galloway *et al.*, 1983; Otsuka *et al.*, 1984; Gorski *et al.*, 1990). Moreover, the involvement of MMPs in cartilage breakdown preparatory to calcification of the epiphyseal growth plate has been suggested (Howell and Dean, 1991). The finding that osteoclasts as well as obsteoblasts and immature cartilage cells display MMP-2-immunoreactivity suggests that all these cell types may participate in the remodelling of bone ECM during development.

Although further studies are needed to clarify the substrates of MMP-2 in developing tissues, biochemical analyses suggest that the enzyme may be active on a variety of components of the ECM, especially those associated with the basement membrane, viz. type IV and VII collagens and fibronectin. In this regard, it is interesting to observe MMP-2-immunoreactivity in organs, such as tooth germ and salivary glands, where changes in the composition of basement membranes and associated matrices occur according to development-regulated programs (Spooner and Faubion, 1980; Thesleff and Hurmerinta, 1981; Ruch *et al.*, 1982; Bernfield *et al.*, 1984a,b; Ruch, 1985; Thesleff *et al.*, 1987).

Although the occurrence of MMP-2 in embryonic cells suggests that this enzyme may exert its function on the adjoining ECM, the possibility of an intracellular storage of metabolically inactive enzyme cannot be ruled out. Indeed, the regulation of enzyme activity is rather complex. Multiple factors are in fact important in modulating MMP-2 activity in tissues, including transcriptional regulation of enzyme synthesis, extracellular activation of latent proenzyme and down-regulation of enzyme activity by specific tissue inhibitors of metalloproteinease (Murphy *et al.*, 1981; Stetler-Stevenson *et al.*, 1989,1990b; Howard *et al.*, 1991).

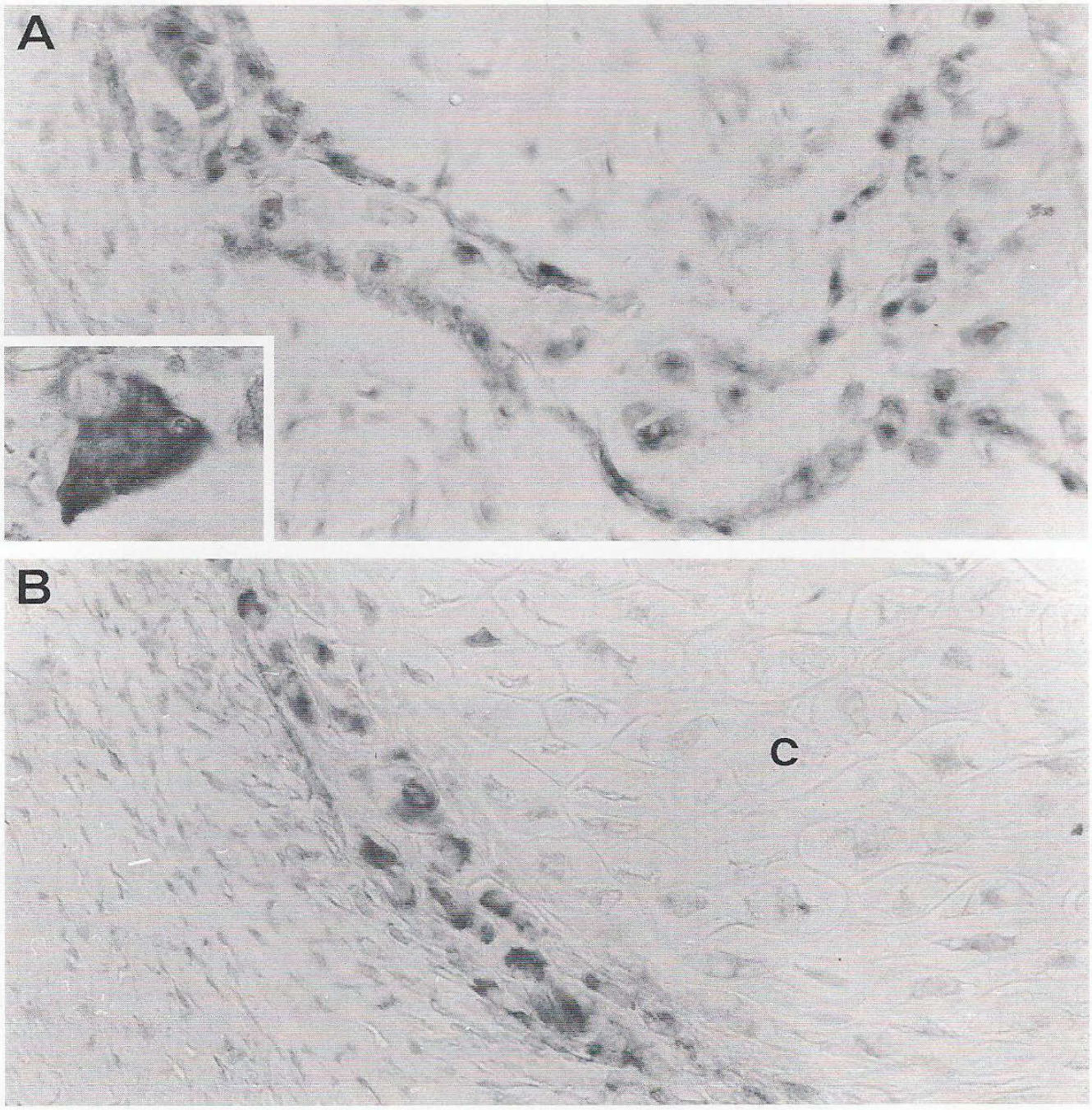

Fig. 2 - Immunoperoxidase localization of matrix metalloproteinase-2-immunoreactivity in osteoblasts (A), osteoclasts (A,inset) and immature cartilage cells (B) of a human embryo at 15 weeks. C, cartilage. Magn. 400 x.

In conclusion, the widespread expression of MMP-2 in human embryonic tissues supports the view that ECM remodelling and degradation may represent a physiological counterpart of imponent ECM deposition that occurs during development. This aspect may be important in developmental dynamics such as epithelial-mesenchymal interactions and cell migration and differentiation.

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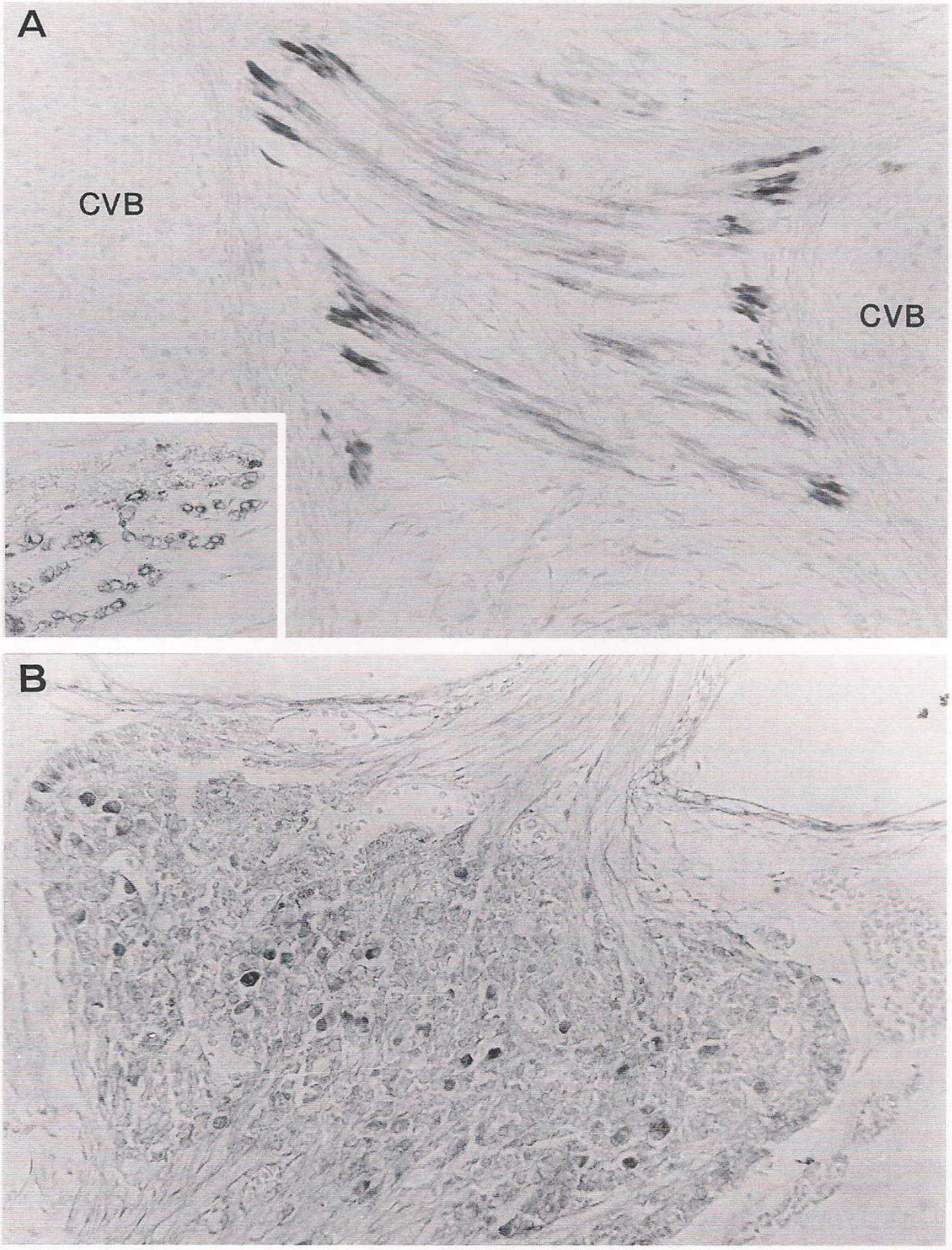

Fig. 3 - Immunocytochemical localization of matrix metalloproteinase-2-immunoreactivity in developing muscle (A) and immature ganglionic neurons (B) from a human embryo of about 13 weeks. A: immunostaining within developing muscle fibres appear more intense in the extremities of the fibres approaching cartilaginous vertebral bodies (CVB); inset: immunoreactivity in transversally-cut myotubes from the same embryo. B: immature neurons in a dorsal root ganglion displaying positive reaction within their somata. Magn. 200 x (A), 400 x (A inset) and 250 x (B).

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