INTRODUCTION

Stromelysin-3 (ST3), a new member of the matrix metalloproteinase (MMP) family (Matrisian, 1992; Murphy et al., 1993), was first described in human invasive breast carcinomas (Basset et al., 1990). Since then, abnormal ST3 expression has been observed in various carcinomas (for review, see Rouyer et al., 1994), including those of lung (Urbanski et al., 1992), skin (Wagner et al., 1992; Wolf et al., 1992) and head and neck (Muller et al., 1993, Polette et al., 1993). Moreover, it was established that ST3 overexpression was restricted to the majority of the tumors (Muller et al., 1993; Rouyer et al., 1994). However, ST3 expression is not specific to carcinomas and has also been observed during skin wound healing (Wolf et al., 1992), embryonic limb bud morphogenesis (Basset et al., 1990), postweaning involution of the mammary gland (Lefèbvre et al., 1992) and tail resorption during amphibian metamorphosis (Wang and Brown, 1993; Patterton et al., 1995), at the time where intense cell death (apoptosis) occurred.

Physiological and pathological tissue remodeling processes have been shown to result from the action of transiently expressed proteinases (for a review see Alexander and Werb, 1991). The MMPs, the plasminogen activators of urokinase-type (uPA) and of tissue-type (tPA), and their inhibitors, the tissue inhibitors of metalloproteinases (TIMPs) and the plasminogen activator inhibitors (PAIs), respectively, are thought to be involved in these processes through an interacting proteolytic cascade (for reviews see Matrisian, 1990; Alexander and Werb, 1991). It is tempting to speculate that ST3 actively participates in this proteolytic network and is implicated in the tissue remodeling that enables tumor progression and ultimately results in the formation of metastases (Stetler-Stevenson et al., 1993 and references therein).

As there are many similarities between developmental processes and malignant growth, it has been proposed that malignancy can be regarded as a disorganized reactivation of developmental mechanisms (for reviews see Sutherland et al., 1990; Cross and Dexter, 1991 and references therein). Throughout embryonic development, organogenesis is known to be regulated by epithelial-mesenchymal interactions (Kedinger et al., 1986; Birchmeier and Birchmeier, 1993) and to be associated with extensive tissue remodeling, requiring

SUMMARY

We have used northern blot analysis and in situ hybridization to study the spatial distribution of stromelysin-3 (ST3) expression during mouse embryogenesis. ST3 mRNA was observed in trophoblastic cells at the site of embryonic implantation (7.5-8.5 days) and in a variety of developing embryonic tissues. In these tissues, the highest ST3 expression levels were observed during the development of the external features of limb, tail and snout, and during bone and spinal cord morphogenesis. In limb, tail and snout, ST3 expression was specifically detected in mesenchymal cells lining the basement membrane at the junction of primitive dermis and epidermis, and adjacent to epithelial cells undergoing proliferation and/or apoptosis. In bone, ST3 was expressed in invasive mesenchymal cells and, in the spinal cord in neuroepithelial cells of the floor plate, at the time that this structure is crossed by commissural axons. Altogether, these observations suggest a role for ST3 during embryonic morphogenesis, in tissue remodeling processes associated with cell proliferation, death and/or invasion. Moreover, when compared to urokinase and tissue plasminogen activators, the spatiotemporal pattern of ST3 expression shows some similarities, but was not completely superimposable, suggesting that these genes may cooperate in some developing tissues and have specific functions in others.

Key words: matrix metalloproteinases, mouse embryo, embryonic implantation, external feature modeling, osteogenesis, nervous system morphogenesis, ECM remodeling

Abbreviations: d, day postcoitum; ECM, extracellular matrix; MMP, matrix metalloproteinase; NGF, nerve growth factor; NGFI-A, nerve growth factor induced A; NGFI-B, nerve growth factor induced B; ST3, stromelysin-3; TIMP, tissue inhibitor of metalloproteinases; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

Developmental expression of mouse stromelysin-3 mRNA

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regulated degradation of extracellular matrix (ECM) (Sappino et al., 1989). Using northern blot analysis and in situ hybridization, we have now investigated ST3 expression during mouse embryonic implantation and development. Furthermore, in the strongest ST3-positive tissues, we have analysed uPA and tPA expression patterns, since these proteinases are believed to be involved in initial steps of the proteolytic cascade leading to ECM remodeling (Mignatti et al., 1986; Alexander and Werb, 1991).

MATERIALS AND METHODS

Embryo collection

Depending on subsequent analysis, embryos were either immediately frozen in liquid nitrogen (RNA extraction), or embedded in TissueTek OCT compound (Miles Diagnostic Division, Elkhart, IN) and frozen in an ethanol/dry ice bath (in situ hybridization). Once frozen, tissues were stored at −80°C. Embryos were collected daily postconception from 9.5 days of gestation to birth. At 7.5 and 8.5 days, embryos were collected in utero.

Isolation of RNA and northern hybridization

RNA was prepared, from whole embryos, by a single-step method using acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). RNAs were fractionated by gel agarose electrophoresis (1%) in the presence of formaldehyde and transferred to nylon membranes (Hybond N, Amersham). Filters were acidified (10 minutes, 5% CH3COOH) and stained (10 minutes, 0.004% methylene blue, 0.5 M CH3COONa, pH 5.0) prior to hybridization. Double-stranded DNA was 32P-labeled by random-priming (Feinberg and Vogelstein, 1983). Northern blots were hybridized for 18 hours under stringent conditions (50% formamide, 42°C). Washings were performed in 2x SSC, 0.1%SDS at 22°C, followed by 0.1x SSC, 0.1%SDS at 55°C. The mouse ST3 probe has been previously described (Lefebvre et al., 1992). The uPA and tPA cDNA probes are a generous gift from Drs J. D. Vassalli and A. P. Sappino.

In situ hybridization

Cryosections (6 µm thick) fixed in acetone and formaldehyde were treated with proteinase K. Hybridization was for 16 hours with 35S-labeled antisense transcripts from ST3, uPA or tPA-cDNA inserts, subcloned in Bluescript II (Stratagene), and followed by RNase treatment (20 µg/ml, 30 minutes, 37°C) and two stringent washings (2x SSC, 50% formamide, 60°C, 2 hours), before autoradiography using NTB2 emulsion (Kodak). Autoradiography was for 2 to 4 weeks. Sections were counterstained using toluidine blue.

RESULTS

ST3 is expressed at the site of embryonic implantation

In order to survive and develop, mammalian embryos must penetrate the uterine wall to gain access to maternal circulation. The most external embryonic cell layer, the ectoplacental cone, gives rise to the trophoblastic giant cells which are the first differentiated cell type in mammalian embryos. The trophoblast is responsible for the invasion of the blastocyst through the uterine mucosa and is one of the most highly invasive cell types known among normal cells. The time frame of invasiveness observed for trophoblast in vivo in mouse is between 4.5 and 7.5 days (Rugh, 1968; Fisher et al., 1989). Trophoblast cells cross the basement membrane of the uterine epithelium to effect successful implantation (Strickland and Richards, 1992). Trophoblast outgrowths clear areas of matrix immediately subjacent to the cells, in a process that is TIMP-inhibitable (Librach et al., 1991; Behrendtsen et al., 1992), and consequently thought to be MMP-dependent.

ST3 in situ hybridization was performed at the end of mouse implantation at 7.5 and 8.5 days. ST3-positive areas were observed at the junction of embryonic and maternal tissues, in a loose network of cells corresponding to the giant trophoblastic cells located at the periphery of the embryo, while the egg cylinder was devoid of specific signal (Fig. 1A-C). In addition, smaller cells disseminated in the maternal tissue some distance from the embryo were also strongly ST3-positive (data not shown). Since at this stage the maternal and foetal tissues are not separated by a basement membrane, and since the adult uterus is known to be able to express ST3 (Lefebvre et al., 1992), it is impossible to ascertain whether some of the ST3-positive cells are of maternal origin.

Using antisense uPA RNA probe on serial sections, at 7.5 and 8.5 days, uPA expression was restricted to the trophoblastic cells (Fig. 1D-F), whereas at the end of embryonic development, ST3 mRNA was almost undetectable (Fig. 2, lanes 10 and 11).

Successive hybridizations of the same filters using uPA and tPA cDNA probes showed that both genes were also expressed during mouse development. The strongest expression of uPA occurred at 10.5 days of embryogenesis, slightly earlier than for ST3 expression (Fig. 2, lane 2). Moreover, uPA was clearly present at 9.5d (Fig. 2, lane 1). The tPA expression pattern was almost identical to that of ST3 (Fig. 2, lanes 1-11), except at 9.5 days (Fig. 2, lane 1) and at 19.5d (Fig. 2, lanes 10 and 11) where tPA expression was higher than that of ST3.

In order to identify which organ(s) expressed ST3, we investigated ST3 expression on sagittal sections of embryos at various stages of development (11.5-16.5 days), using in situ hybridization. At all stages, except at 11.5 days, a spatially restricted expression of the ST3 gene was obvious in various tissues located either at the periphery or inside the embryo (Fig. 3A-F). ST3 transcripts are shown at 12.5 days in snout and spinal cord (Fig. 3B), at 13.5 days in tail (Fig. 3C), at 14.5 days in limb, testis and lung (Fig. 3D) and at 16.5 days in bone (Fig. 3F,G). No signal above background was observed when using sense [35S]RNA ST3 probe (Fig. 3H). Since, using northern blot analysis, ST3 mRNA was observed in 11.5 day embryo (Fig. 2, lane 3), we assume that, at this stage, ST3 is expressed more homogeneously, but at a lower level than later in the development. Thus, ST3 expression occurred, at different times and with variable intensity, in various primitive skin locations and in a panel of organs from urogenital, pulmonary,
Developmental expression of mouse stromelysin-3 skeletal and nervous systems. The strongest ST3 expression was seen in the most distal part of developing limb, tail and snout, and during bone and spinal cord morphogenesis. These observations prompted us to perform a more detailed analysis of ST3, uPA and tPA expression in these developing organs.

**ST3, uPA and tPA expression during limb, tail and snout morphogenesis**

We have previously reported that the ST3 gene was expressed in the interdigital mesoderm of the limb bud in the human embryo (8 weeks), shortly before cell death by apoptosis is occurring in this tissue area (Basset et al., 1990). Similarly, in the present study, ST3 mRNA could be detected at 13.5 days, in the interdigital limb bud mesenchymal cells underlying the ectoderm, which was itself negative. ST3 labeling was observed until birth in developing limbs (data not shown). While several layers of mesenchymal cells expressed the ST3 gene when the interdigital region was still present, only mesenchymal cells in close contact with the basement membrane lining the primitive epiderm expressed the gene in older embryos (Fig. 4A).

In the tail, ST3 expression was seen from 13.5 days until birth. ST3 mRNA becomes detectable when the tail decreases in length so that, at 18.5 days, it is proportionately shorter than at any previous stage of development (Rugh, 1968; Kaufman, 1992). Over this period, the tail shape evolves to become pro-
portionately wider and less pointed than before. ST3 mRNA was specifically observed in the mesenchymal cells, immediately underneath the ectoderm (Fig. 4B).

ST3 expression was observed in the snout, from 12.5 days until birth. ST3 labeling was restricted to the mesenchymal cells underlying the primitive epiderm. The primordia of the vibrissae, clearly seen on the snout at 16.5 days, were ST3 negative (Fig. 4C).

Using uPA and tPA in situ hybridizations on serial sections, we were unable to detect any obvious labeling either in the limb, the tail or the snout (data not shown).

**ST3, uPA and tPA expression during embryonic osteogenesis**

Endochondral and intramembranous ossification, the two main routes of bone development, are preferentially confined to the limbs, vertebral column and long bones, and to the bones of the facial skeleton, cranial vault and clavicle respectively (for a review see Stein et al., 1990). In both cases, ossification involves cells of mesenchymal origin, the osteoblasts, which secrete and assemble matrix to generate spicules and tubes. Bone is a tissue in which matrix deposition and remodeling is particularly important and in which stringent local control of MMP activity is required (Alexander and Werb, 1991). The earliest ossification centers appear at 14-14.5 days in the base of the skull and ribs and the process continues until after birth.

Using in situ hybridization, ST3 expression was seen in osteogenic tissues, in areas of membrane bone formation as early as 15.5 days (data not shown) and, at later stages, in regions of endochondral bone formation such as the ossifica-

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Fig. 3. Tissue-specific distribution of ST3 in the mouse embryos. Dark-field photographs of midsagittal sections of: (A) 11.5, (B) 12.5, (C) 13.5, (D) 14.5, (E) 15.5 and (F) 16.5 day old embryos, hybridized with antisense [35S]RNA probe specific for mouse ST3. The arrowheads point to the presence of ST3 transcripts. From 12.5 to 16.5 days, ST3 positivity is obvious in the developing snout (B-F), spinal cord (B,C), tail (C-F), limb (D,F), testis (D), lung (D-F) and bone (F). Bright-field photographs of serial midsagittal sections of 16.5 day old embryo hybridized with antisense (G) and sense (H) mouse ST3 [35S]RNA probes. ST3 transcripts were detected with antisense probe (G), whereas no significant labeling above background was found when using sense probe (H). Bars: (A) 90 µm, (B) 100 µm, (C) 130 µm, (D) 160 µm, (E) 180 µm, (F-H) 220 µm.
tion centers of the vertebrae (Fig. 5A,B) and limbs (Fig. 6A-D). The resolution of the in situ hybridization technique did not allow us to identify unambiguously the cell type(s) expressing ST3 in developing bones. However, ST3 expression occurs at the time of the initiation of ossification, in cells either lining the periosteum or within the marrow cavity (Fig. 6B,D). This spatiotemporal pattern suggests that these ST3-expressing cells are osteoblasts (Stein et al., 1990). It is noteworthy that mesenchymal condensations leading to cartilaginous elements and cartilage were ST3 negative (Fig. 5A,B).

The pattern of uPA expression was similar to that of ST3 expression (Fig. 5C,D), whereas tPA mRNA could not be detected (Fig. 5E,F).

**ST3, uPA and tPA expression in the floor plate during spinal cord morphogenesis**

Within the neural plate, the first cells to differentiate are located at the midline and give rise to a specific set of cells which constitute the floor plate (Placzek et al., 1990, 1993). Between 12.5 and 15.5 days, the overall shape of the cord changes, so that the proportion of the white matter is gradually increased. By about 16.5-17.5 days, the floor plate disappears, leading to the ‘butterfly’ shape, characteristic of this region in the adult spinal cord. The floor plate is believed to have a role in the determination of dorsoventral polarity in the developing central nervous system (Wagner et al., 1990; Yamada et al., 1991), and in the guidance of developing axons of commissural neurons (Yaginuma et al., 1990; Bovolenta and Dodd, 1990).

In the spinal cord, ST3 expression was detected from 12.5 to 14.5 days in a small number of neuroepithelial cells corresponding to the floor plate (80-100 μm width) (Fig. 7A,B). Expression first appears in a rostral-caudal temporospatial gradient that parallels the rostral-caudal pattern of development of this structure (Rugh, 1968; Kaufman, 1992). The expression of ST3 in the nervous system suggests that the ST3 gene may be regulated by a factor(s) of neural origin. Interestingly, the mouse ST3 gene promoter shows consensus sequences for two transcriptional factors regulated by NGF (O.L., unpublished results), NGFI-A (Christy and Nathans, 1989) and NGFI-B (Wilson et al., 1991). In this respect, we note that the rat pheochromocytome PC12 cells, which are tumoral neuroepithelial cells (Greene and Tischler, 1976), also express ST3 (data not shown). These cells have been previously reported to express the rat homolog of human stromelysin-1, transin (Machida et al., 1991; Fillmore et al., 1992).

tPA was also expressed in the neural tube, but at a lower level than ST3. As previously reported in the rat (Sumi et al., 1992), tPA mRNA was restricted to the floor plate and to the adjacent ependymal cells lining the more ventral regions (Fig. 7C,D). No uPA mRNA could be detected (data not shown).

**DISCUSSION**

In the present study, we report the spatiotemporal expression of ST3 during mouse implantation and embryogenesis. Knowledge of the ST3 foetal tissue distribution may help to shed light on its role in the process of tissue remodeling, which has been addressed for both normal and malignant tissues.

At the site of mouse embryonic implantation (7.5-8.5 days), ST3 mRNA is predominantly seen in the giant trophoblastic cells. Because of their ability to cross the basement membrane in order to invade the adjacent tissue compartment, and by analogy with carcinoma cells, these cells have been previously called ‘pseudomalignant’ cells. uPA and MMPs, including the 92×10^3 M_t type IV collagenase and stromelysin-1, have already been reported to be produced by trophoblastic cells localized to regions of invasion during the embryo’s invasive phase (for
a review see Strichland and Richards, 1992). Moreover, from in vitro studies, it has been previously suggested that tPA is not involved in amnion invasion (Mignatti et al., 1986). In accordance with this view, we have observed expression of uPA but not tPA in the trophoblastic cells. Our observations support the hypothesis that ST3 may participate together with uPA and other MMPs in the molecular events leading to implantation of the embryo in maternal tissue.

ST3 is widely expressed during a wide part of mouse embryogenesis, especially during external shape determination of the embryo and in developing systems as different as urogenital, pulmonary, skeletal and nervous systems. In all ST3-positive tissues, the ST3 mRNA was not randomly distributed but restricted to one cell type, located in well-defined tissue areas. Thus, the highest levels of ST3 expression were detected in the most distal part of the limb, tail and snout, from 12.5 (snout) or 13.5 days (limb and tail) until birth, in mesenchymal cells located underneath the basement membrane at the junction of developing dermis and epidermis. Limb and tail development has been shown to involve, alongside cell proliferation, intense programmed cell death (ffrench-Constant, 1992). Interestingly, we have previously observed ST3 expression during mouse postweaning involution of the mammary gland (Lefebvre et al., 1992), a process known to involve intense apoptosis (Walker et al., 1989). Osteogenesis results from a sequential expression of cell-growth and tissue-specific genes leading to the osteoblast proliferation and differentiation, and to ECM deposition, maturation and mineralization (for a review see Stein et al., 1990 and references therein). In developing bone, ST3 is expressed independently of the type of ossification, from the time of the initiation of osteoid deposition until birth, presumably by osteoblasts. These cells are known to break out of the peristeous, traverse connective tissue and colonise the hypertrophic cartilage (Alexander and Werb, 1991). High expression by human osteoblasts of MMPs and TIMPs has been previously shown in vitro (Meikle et al., 1992). Moreover, TIMP expression has been directly correlated with ossification in vivo (Nomura et al., 1989). In the spinal cord, the ST3 gene was transiently expressed from 12.5 to 14.5 days, and expression was restricted to the neuroepithelial cells constituting the floor plate, from the rostral-most part to the caudal-most part of the neural axis. To our knowledge, this is the first report of MMP expression in the floor plate itself. However, MMPs have already been described to be released by the growth cones of the commissural axons, at the time they cross the floor plate, in order to generate extracellular spaces, in the absence of preformed channels (Pittman and Buettner, 1989; Kuwada et al., 1990). ST3 may cooperate to this ‘drilling’ process, either directly or indirectly, perhaps by activating the pro-MMPs released by growth cones.

What accounts for the expression of the ST3 gene during mouse embryonic development? At a molecular level, we can not answer to this question since the number and the nature of both the inductive factor(s) of the ST3 gene and the possible substrate(s) of the ST3 protein remain unknown. Nevertheless, tissues expressing ST3 during mouse morphogenesis present some characteristics in common and can be subdivided into two categories. The former group, including limb, tail and snout, is characterized by ST3 expression in fibroblasts adjacent to epithelial cells undergoing intense proliferation and/or degeneration (apoptosis) leading to a modification of the size of the epithelial compartment. During these processes, the

Fig. 5. In situ hybridization of ST3, uPA and tPA mRNA in 19.5 day vertebrae. Sagittal sections were hybridized with antisense [35S]RNA probes specific for mouse ST3 (A,B), uPA (C,D) and tPA (E and F). ST3 and uPA are strongly expressed in the ossification center (os) (A-D), whereas tPA was not (E and F). Cartilage (ca) did not express ST3, uPA and tPA. No significant labeling above background was found when using sense mouse ST3, uPA and tPA RNA probes (data not shown). Dark field (B,D,F) and bright field (A,C,E). Bars: (A-F) 25 µm).
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The basement membrane, which supports the epithelial cell compartment and separates it from the mesenchymal cells, has to modify its size in order to adapt and maintain a correct homeostasis of the tissues being remodeled (Streuli et al., 1991 and references therein). Since the basement membrane is not an elastic structure, it must be continuously remodeled through constant ECM destruction and redeposition (Leblond and Inoue, 1989). Accordingly, previously described ST3-expressing tissues including tadpole tail during amphibian metamorphosis (Wang and Brown, 1993; Patterton et al., 1994), mouse mammary gland during postweaning involution (Lefebvre et al., 1992), mouse uterus during postnatal involution (O.L., unpublished results), and human non-invasive carcinomas (Rouver et al., 1994), showed identical criteria. The second category of tissues expressing ST3 during embryonic development are characterized by invasion of cells from one compartment to another cell type compartment. This is the case during embryonic implantation and during bone and floor plate morphogenesis. As in tissues of the first group, the basement membrane must be remodeled during these processes, and often at a more dramatic level. Most of the human invasive tumors expressing ST3 (see Rouver et al., 1994) exhibit the characteristics of this second category. However, it is noteworthy that, in carcinomas, ST3 expression is not observed in the invasive cancer cells, but in fibroblastic cells surrounding them while, in developing tissues, ST3 can be expressed by invasive cells themselves (i.e. trophoblast cells and possibly osteoblasts).

Taken altogether, our observations indicate that ST3

Fig. 6. In situ hybridization of ST3 mRNA in 16 day left hindlimb. Sagittal section was hybridized with antisense [35S]RNA probe specific for mouse ST3 (A-D). ST3 was expressed in sites of bone formation (os) within the marrow cavity and lining the periosteum (pe) of tibia (ti) (A,C) and fibula (fi) (A-D). Cartilage (ca) did not express ST3. No significant labeling above background was found when using sense mouse ST3 RNA probe (data not shown). Bright field (A,B,D) and dark field (C). Bars: (A,C) 100 µm, (B) 50 µm and (D) 25 µm.

Fig. 7. In situ hybridization of ST3 (12.5 day) and tPA (13.5 day) mRNA in spinal cord. Mid-thoracic transverse sections were hybridized with antisense [35S]RNA probes specific for mouse ST3 (A,B) and tPA (C,D). Both probes gave a positive signal, restricted to the floor plate (fp) for ST3 (A,B) and to the floor plate and the ependymal layer (e) for tPA (C,D). The cartilage primordium (ca) of the vertebral body (centrum) and the degenerating notochord (n) were ST3 and tPA negative. No significant labeling above background was found when using sense mouse ST3 and tPA RNA probes (data not shown). Dark field (B,D) and bright field (A,C) of the same sections. Bars: (A-D) 50 µm.
expression is specifically observed in tissues associated with tissue remodeling, and particularly when modifications of the basement membrane are occurring. Thus, it is tempting to speculate that ST3 may, directly or indirectly, participate in ECM remodeling together with other extracellular proteases. Consistent with this hypothesis, expression of ST3 is concomitant with that of uPA during embryonic uterine invasion and bone ossification, and with that of tPA in the floor plate during spinal cord morphogenesis. Nevertheless, we have observed in the present study that the ST3 expression pattern was not completely superimposable onto that of uPA and tPA. ST3 was found to be expressed independently from uPA and tPA throughout limb, tail and snout development. Moreover, ST3 was never co-expressed with both plasminogen activators. These observations support the concept that tissue remodeling processes involving cell proliferation, cell death and/or cell invasion are associated with independent proteolytic pathways.

Finally, association between tissue remodeling processes and ST3 expression during embryonic development was not only observed in mouse, but also in human (Basset et al., 1990) and in amphibian (Wang and Brown, 1993; Patterton et al., 1995), suggesting a basic and evolutionarily conserved function for ST3. The observation that ST3 expression during embryonic development is related to cell proliferation, invasion and death, is consistent with the proposed role of ST3 in tissue remodeling processes leading to local tumoral progression and ultimately to metastases, in carcinomas (Basset et al., 1990).

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