RESEARCH COMMUNICATION

Cathepsin-6, a novel cysteine proteinase showing homology with and co-localized expression with cathepsin J/P in the labyrinthine layer of mouse placenta

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A novel cysteine proteinase, cathepsin-6, was isolated by RNA differential display from mouse placenta. Cathepsin-6 showed the highest homology with cathepsin J (same as P) and L. The structural features including the catalytic triad of the C1 proteinase family were well conserved in cathepsin-6. The expression of cathepsin-6 and cathepsin J/P was restricted in labyrinthine trophoblasts of the placenta.

Key words: development, differential display, *in situ* hybridization, trophoblast.

INTRODUCTION

Cathepsins belong to the family of lysosomal proteinases and comprise a large number of members, e.g., 15 distinct amino acid sequences are registered in GenBank® as mouse cathepsins. They are involved in diverse biological functions including bulk protein turnover [1,2], protein processing [3], antigen presentation [4], chronic inflammation [5], bone resorption [6], and tumour formation and metastasis [7,8]. Tissue distribution of each member differs considerably [9]. The group including cathepsin B, H and L are expressed ubiquitously, whereas others such as cathepsin K, S and W are expressed only in restricted tissues and are possibly involved in more specific functions.

Placenta is one of the most active organs in tissue remodelling as well as in secretion of peptide factors and thus probably involves pleiotropic functions of different cathepsins. Some cathepsins were reported to be expressed in the placenta of different species in a cell-type- and gestational-stage-specific manner [10–12]. Afonso et al. [11] examined the expression of cathepsin B, L and D in mouse trophoblasts and found that all three enzymes were expressed in giant cells, but only cathepsin D protein was detected in spongiotrophoblast. Recently, placenta-specific cathepsins were isolated, i.e. cathepsin J [9] and P [13] from mouse, and cathepsin Q [14] and cathepsin-L-related protein from rat [15]. It turned out that cathepsin J and P are the same and are considered to be the mouse orthologue of rat cathepsin-L-related protein [9]. Cathepsin J/P (as called collectively in the present manuscript) was specifically expressed in placenta and not in any other tissues examined [9,13]. Sol-Church et al. [13] showed by reverse transcriptase (RT)–PCR that the expression of cathepsin P depends on the developmental stage of the placenta, i.e. positive bands were observed from 11.5 to 15.5 days post-coitus (dpc), but not at 8.5, 9.5 and 17.5 dpc. From the expression profile, a possible role for cathepsin J/P during implantation and early fetal development has been implied [9,13].

We have cloned a cDNA from mouse placenta that showed extensive homology with mouse cathepsins, particularly with cathepsin J/P and L. Structural features of the cDNA, named cathepsin-6, and the expression profile are described in comparison with cathepsin J/P.

EXPERIMENTAL

RNA differential display

RNA differential display was performed using a Delta Differential Display Kit (ClonTech, Palo Alto, CA, U.S.A.) under the conditions recommended by the manufacturer. Placental tissues were prepared from pregnant ICR mice (Nippon Shizuoka Laboratory Animals Centre, Hamamatsu, Japan) at 8.5, 10.5, 12.5, 14.5, and 17.5 dpc under the dissecting microscope. Total RNA samples (2 μg) were reverse-transcribed and amplified using a number of different primer combinations, among which the set 5′-ATTAACCCTCACTAAATGCTGGTGG-3′ and 5′-CATTATGCTGAGTGATATCTTTTTTTTTAG-3′ gave rise to the present cDNA fragment. The PCR products were separated by electrophoresis on a 5% (w/v) polyacrylamide gel and those showing differential intensity were eluted from the gel into boiling water, reamplified and subcloned into pCRII (Invitrogen, San Diego, CA, U.S.A.).

Cloning and sequencing of cDNA

A cDNA library was prepared from mouse placenta of 17.5 dpc using λZAP II (Stratagene, La Jolla, CA, U.S.A.) under essentially the same conditions as recommended by the manufacturer. After screening $\sim 6 \times 10^5$ independent plaques of the cDNA library with a ^{32}P-labelled cathepsin-6 probe, three positive clones were isolated and transformed into pBluescript by *in vivo* excision. DNA sequencing was performed by an automated sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden).

Abbreviation used: dpc, days post-coitus; RT, reverse transcriptase.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AF223401.

Northern blot analysis

Northern blot analysis was performed under conventional conditions. Total RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction [16] and purified by density gradient ultracentrifugation in caesium trifluoroacetic acid (Wako Pure Chemical Industries, Osaka, Japan). The RNA samples were electrophoresed, transferred on to Hybond-N membranes (Amersham Pharmacia Biotech, Uppsala, Sweden), and hybridized to ^{32}P-labelled probes. The membrane filters were finally washed three times with 0.2 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M sodium citrate), 0.1 % SDS at 43 °C for 15 min, and the signals were visualized by exposure to X-ray films. The probe used was 184 bp from the 3′ end of cathepsin-6. For comparison, 150 bp from the 3′ end of murine cathepsin J/P [9,13], amplified by RT–PCR, was also used for the analysis. A glyceraldehyde-3-phosphate dehydrogenase cDNA probe served as a control for the applied amount of RNA.

In situ hybridization

The procedure for *in situ* hybridization was reported previously [17]. Briefly, frozen sections were prepared from the placentas of pregnant mice 12.5, 14.5, 16.5 and 18.5 dpc using the Tissue-Tek O.C.T. media for freezing (Sakura Fine Technical Co., Tokyo, Japan). The probes were the same as those used for the Northern blot analysis and they were labelled with digoxigenin (Boehringer Mannheim). Both antisense and sense probes were synthesized, the latter being used as a negative control. Specific signals were visualized by alkaline phosphatase conjugated to anti-digoxigenin antibody (Roche Diagnostic, Mannheim, Germany). The slides were counterstained for 10 min with Kernechtrot stain solution (Muto Chemicals, Tokyo, Japan).

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1                ▽                30
MSPTLFLAILCLGVGSGALALDPNLNAEWH

DWKKQYEKSYTMEEEGLRRAIWEENMRMIK
                                 90
LHNWENSLGKNNFTLKMNEFGDLTPEELRK

MMNNFPIWSHKKRKIIRKRAVGDVLPKFVD
                    *           150
WRKKGYVTRVRRQKFCNSCWAFAVNGAIEG

QMFKKTGKLTPLSVQNLVDCTKTQGNDGCQ
                                210
WGDPYIAYEYVLNNGGLEAEATYPYEGKEG

PCRYNPKNSKAEITGFVSLPESEDILMEAV
                                270
ATIGPISAAVDASFNRFSFYDGGIYHQPNC

SNNTVNH*AVLVVGYGTEGNETDGNKYWLIK
*                               330
NSWGRRWGIGGYMKIIRDQNNHCGIATYAH

YPIV
  334
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Figure 1 Amino acid sequence of cathepsin-6

The amino acid sequence was deduced from the cDNA of cathepsin-6. The open and closed triangles designate the possible cleavage sites of the signal peptide and pro-sequence respectively, predicted from the alignment with other mouse cathepsins. The 'catalytic triad' of Cys^{139}, His^{277} and Asn^{301} is indicated by the stars. The nucleotide sequence was registered in GenBank® under the accession number of AF223401.

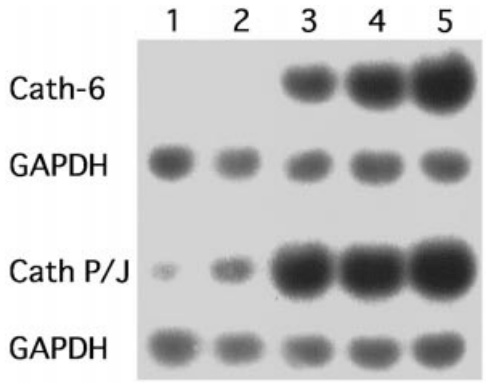

Figure 2 Expression of cathepsin-6 and cathepsin J/P in developing mouse placenta

Northern blot analysis was performed using total RNA isolated from placenta tissues (20 μg each) at different gestational days: 1, 10.5 dpc; 2, 12.5 dpc; 3, 14.5 dpc; 4, 16.5 dpc; 5, 18.5 dpc. Hybridization with GAPDH was performed on each filter as a control for the applied amounts of RNA.

RESULTS AND DISCUSSION

From RNA differential display performed on the mouse placenta or its precursor tissues from 8.5 to 17.5 dpc, a cDNA fragment with an increasing intensity during the later stages was identified and characterized. Using a 414 bp fragment as a probe, we screened a cDNA library, prepared from the mouse placenta of 17.5 dpc, and isolated and sequenced several clones. A cDNA of 1323 bp followed by a polyadenylated tail was identified; a canonical polyadenylation signal AATAAA was found 19 bp upstream from the polyadenylated tail. The size of the transcript estimated by Northern blot analysis was ~ 1.3 kb and was consistent with that of the isolated cDNA which covers nearly the full length.

Only one reasonably long reading frame was found and the deduced amino acid sequence of 334 residues with a molecular mass of 38 kDa is shown in Figure 1. The sequence showed homology with cathepsins, among which mouse cathepsin J/P and L gave the highest score. The cDNA was 78 % and 65 % identical with cathepsin J/P at the nucleotide and amino acid levels respectively. The multiple sequence alignment among the mouse cathepsins reported thus far allows the identification of the proregion and mature proteinase domain in the cDNA as indicated in Figure 1. Thus the signal peptide may be cleaved at the N-terminal side of Ala^{18}XAla and the mature proteinase may start at Leu^{115}, one residue upstream of the conserved proline. Cys^{139}, His^{277} and Asn^{301} possibly compose the 'catalytic triad' characteristic for the C1 family of cysteine proteinases [18]. Altogether, the isolated cDNA most probably encodes a novel preprocathepsin. Considering the recent situation on the nomenclature of the cathepsin family, we named the cDNA cathepsin-6.

The cathepsin-6 transcript was first detected by Northern blot analysis in the placenta at 14.5 dpc and increased towards 18.5 dpc (Figure 2). A weak but distinct positive signal for cathepsin J/P was observed at 10.5 and 12.5 dpc. The expression of cathepsin J/P was dramatically induced at 14.5 dpc. Sol-Church et al. [13] reported that cDNA fragments corresponding to cathepsin P were amplified by RT–PCR in mouse placenta samples at 11.5, 13.5 and 15.5 dpc, but not at 8.5, 9.5 and 17.5 dpc. Our Northern blot analysis and *in situ* hybridization

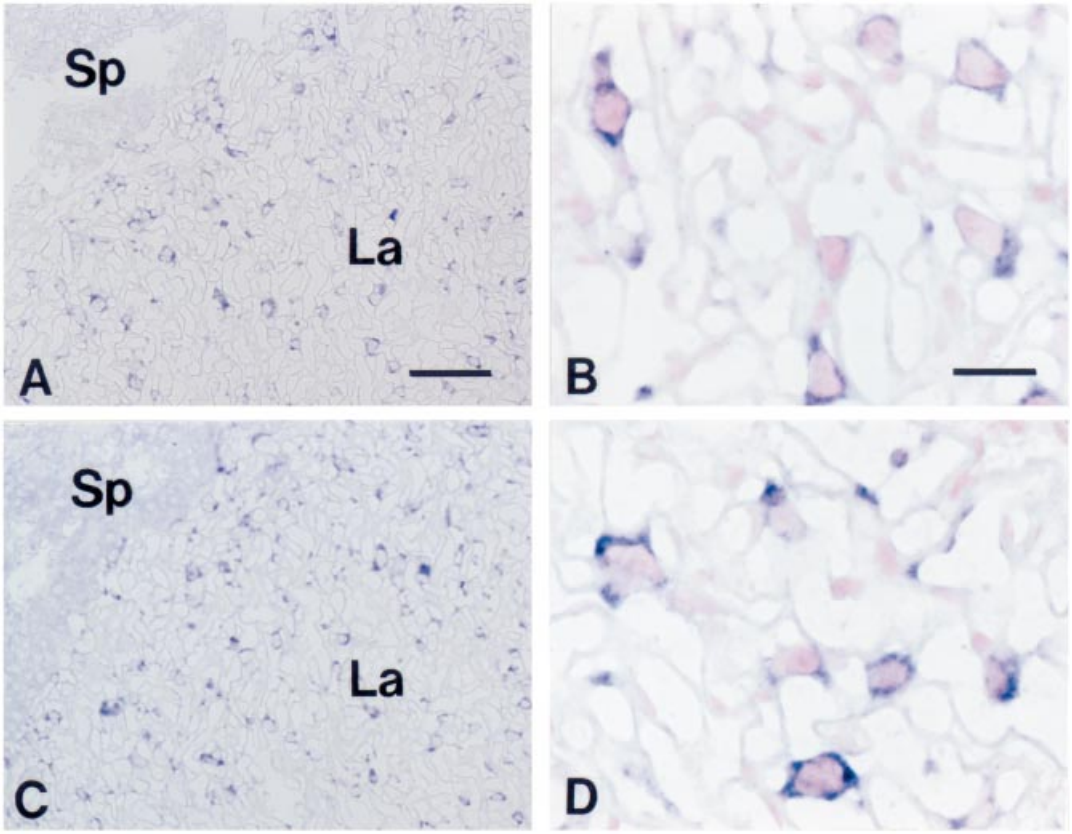

Figure 3 ***In situ*** **hybridization of cathepsin-6 and cathepsin J/P**

Tissue sections from mouse placenta at 18.5 dpc were hybridized with probes specific for cathepsin-6 (**A**,**B**) and cathepsin J/P (**C**,**D**) shown at the lower (**A**,**C**) and higher (**B**,**D**) magnification. Sp and La indicate spongiotrophoblast and the labyrinthine layer of placenta respectively. The bar in **A** (also applies for **C**) equals 100 μm. The bar in **B** (also applies for **D**) equals 25 μm.

results repeatedly confirmed the expression of cathepsin J/P at the later stage of gestation including 17.5 dpc. Although cathepsin-6 and cathepsin J/P shows extensive homology, each probe and the washing conditions we used were reasonably specific and exclude the cross hybridization of cathepsin J/P probe to cathepsin-6 transcript. Cathepsin-6 and cathepsin J/P were expressed only in placenta and no signals were detected in any other adult tissues including cerebrum, cerebellum, heart, lung, liver, kidney, stomach, small intestine, colon, uterus, and testis (results not shown).

In situ hybridization showed that the expression of cathepsin-6 was localized in the labyrinthine layer of placenta at 18.5 dpc (Figure 3A). Tissue sections from the placentas of mice at 14.5 and 16.5 dpc gave essentially the same results, but no signal was detected in the tissues from mice at 12.5 dpc. Cellular morphology at higher magnification indicated that cathepsin-6 was expressed in trophoblasts but not in endothelial cells (Figure 3B). The transcript of cathepsin J/P was detected in similar types of cells (Figures 3C and 3D). The spongiotrophoblast layer was negative for the expression of both cathepsins including the trabecular processes of spongiotrophoblast in the labyrinth. Careful microscopical examination revealed that most of the trophoblastic cells in the labyrinthine layer were positive for the expression of either cathepsin. This indicates that the same cells express both cathepsin-6 and cathepsin J/P.

Since cathepsin L, B and D were expressed in the spongiotrophoblast and the active protein was most abundant at early to middle gestation, Afonso et al. [11] considered that those cathepsins were involved in trophoblast invasion. This can be corroborated by the many reports demonstrating the role of those cathepsins in tumour invasion [7,8]. On the contrary, cathepsin-6 and cathepsin J/P, the only placenta-specific cathepsins thus far isolated, are expressed specifically in labyrinthine trophoblastic cells (Figure 3). The labyrinthine layer of mouse placenta is known to be a major place for the exchange of gas and nutrients between maternal and fetal blood. Cathepsin-6 and cathepsin J/P, therefore, may be involved in the coordination of these processes, immunological modulation, and/or processing of secretory protein factors. Studies of such differential roles among different members of the cathepsin family will lead to a better understanding of the formation and function of the placenta at the molecular level.

We are grateful to Dr Mikiro Takaishi for his technical help and critical discussion. We also thank Dr Alan Barrett, The Babraham Institute, Cambridge, for his kind suggestion on the naming of the present cDNA. This work was supported by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.

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Received 24 February 2000/30 May 2000; accepted 1 June 2000