Development-Dependent Expression of Cathepsins D and E in Various Rat Tissues, with Special Reference to the High Expression of Cathepsin E in Fetal Liver

Takashi Kageyama[1]*, Masae Tatematsu[2], Masao Ichinose[3], Naohisa Yahagi[3],
Kazumasa Miki[3†], Akihiko Moriyama[4] and Satoshi Yonezawa[5]

[1]*Department of Cellular and Molecular Biology, Primate Research Institute,
Kyoto University, Inuyama 484-8506, Japan*
[2]*First Department of Pathology, Aichi Cancer Center Research Institute,
Nagoya 464-8681, Japan*
[3]*First Department of Internal Medicine, Faculty of Medicine,
The University of Tokyo, Tokyo 113-8655, Japan*
[4]*Division of Biomolecular Science, Institute of Natural Sciences,
Nagoya City University, Nagoya 467-8501, Japan*
[5]*Department of Embryology, Institute for Developmental Research,
Aichi Human Service Center, Kasugai 480-0392, Japan*

ABSTRACT—The levels of cathepsins D and E in various rat tissues during development were determined with the sensitive assay method we have developed. The level of cathepsin D increased gradually in each tissue during fetal development suggesting the gradual maturation of the lysosomal system in a cell. The level of cathepsin E differed significantly between tissues at various developmental stages. The level in liver increased rapidly from 13-day-gestation fetal stage and decreased gradually at later fetal stages. The level in other tissues such as stomach and spleen began to increase at later fetal stages or the infant stage. Cathepsin E was found in fetal hepatocytes and its gene was hypomethylated when the expression of the gene was elevated. The enzyme was found to be present mainly as a proform suggesting that, after working, an active form is rapidly inactivated.

INTRODUCTION

Proteinases have been implicated in a variety of metabolic processes that create and destroy biologically active proteins and peptides. Proteinases at early developmental stages are thought to be especially important, since various proteinous and peptide growth factors are involved in the growth and differentiation of embryonic or fetal cells and their biological activities are regulated by proteinases through processing and degradation. Some proteinases such as proteasome (Ahn *et al.*, 1991) and collagenase (Reponen *et al.*, 1992) have been shown to be expressed development-dependently. To date, however, information about proteinases which are specific for early developmental stages is much less extensive than that which is known about proteinases in the adult tissues of mammals or other vertebrates.

To clarify the roles of proteinases at early developmental stages, we undertook to determine the levels of cathepsins D and E in various fetal tissues of rat and monkey. Cathepsins D and E are known to be the major intracellular aspartic proteinases and are involved in the processing and degradation of intracellular proteins and peptides (Lapresle, 1971; Barrett, 1977; Kageyama, 1995; Kay and Tatnell, 1998). A recent report has shown that cathepsin D might be involved in activation/inactivation of signaling proteins during development (Saftig *et al.*, 1995). Little is known about the developmental change or role of cathepsin E, although we have reported some preliminary findings that cathepsin E was detected as a major proteolytic enzyme of the gastric mucosa of rat fetus (Yonezawa *et al.*, 1993) and was appreciable in some tissues of rabbit at late fetal stages (Kageyama, 1993).

Since cathepsins D and E have similar general proteolytic activities at an acidic pH, determination of their levels in cells and tissues has been performed by complicated procedures using specific antibodies (Muto *et al.*, 1988; Sakai *et al.*, 1989). We found that they have quite different hydrolytic activities against two peptide substrates, namely, β-endorphin and sub-

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* Corresponding author: Tel. +81-568-63-0578;
FAX. +81-568-63-0085.
† Present address: First Department of Internal Medicine, Toho University School of Medicine, Tokyo 143-8541, Japan.

stance P (Kageyama, 1995), and thus developed a new method for the assay of these cathepsins to determine their levels in various mammalian tissues (Kageyama *et al.*, 1996b).

In this report, we describe the developmental change of cathepsins D and E in various rat tissues determined by the method we have developed. The high level of cathepsin E in the fetal liver was noteworthy and was analyzed in detail using Southern and Northern blotting and immunohistochemistry.

MATERIALS AND METHODS

Materials

Substance P and β-endorphin were purchased from the Peptide Institute, Inc., Minoh, Japan. cDNA for rat cathepsin E (pRT732) was isolated from a cDNA library of rat gastric mucosal mRNA. All other chemicals were of reagent or analytical grade.

Preparation of tissue homogenate

SD strain rats were mated overnight and pregnancy was confirmed by examination of vaginal smears. The day that sperm were detected was defined as day 0 of gestation. Rat tissues at various developmental stages were homogenized in 0.05 M sodium phosphate buffer, pH 7.0, with a mechanical homogenizer. Each homogenate was centrifuged at 10,000 × g for 20 min and the supernatant was used for PAGE and the assay for proteolytic activity. Each supernatant was mixed with an equal volume of glycerol and kept at –20°C for long-term storage. The amount of protein in the crude homogenate supernatant was determined by the method of Lowry *et al.* (1951).

Assay of cathepsins D and E

The procedure was based on that described in our previous reports (Kageyama, 1995; Kageyama *et al.*, 1996b). In brief, the reaction mixture contained 0.2 M sodium formate buffer, pH 4, 1 μM E-64, 50 μM substance P/β-endorphin and an appropriate amount of crude homogenate supernatant. The reaction was incubated at 37°C for 1-2 hr and stopped by the addition of 60 μl of 3% perchloric acid. After removal of the precipitate, the mixture was subjected to HPLC on a column of ODS-120T (Tosoh Corp., Tokyo) to determine the hydrolysis of peptide. The relative specific activity of cathepsins D and E in each tissue was expressed as the amount of hydrolyzed substrate/ min/mg tissue protein. Although cathepsin D is usually present as an active form in a cell, cathepsin E in each tissue homogenate has been shown to be present mainly as procathepsin E (Kageyama *et al.*, 1996b). The ratio of cathepsin E to procathepsin E was estimated by the difference of substance P hydrolyzing activity at pH 4 and 5, since activation of procathepsin E was rapid at pH 4 and very slow at pH 5 (Kageyama *et al.*, 1992).

Separation of cathepsins D and E in the tissue homogenate and activity staining

Electrophoretic separation of cathepsins D and E in the crude homogenate was carried out using slab gels of polyacrylamide. The composition of the gel was the same as that described by Ornstein (1964) and Davis (1964). Detection of cathepsins D and E after electrophoresis was performed according to Furihata *et al.* (1972). Briefly, gels were immersed in 1% hemoglobin in 0.1 M sodium formate buffer, pH 3.5, for 20 min at 37°C, followed by another 2-hr incubation without the substrate solution. The gel was then stained with Coomassie brilliant blue R-250.

Analysis of the methylation of cathepsin E gene

High-molecular-weight DNA at various developmental stages of the rat liver and stomach was prepared by the method of Gross-Bellard

et al. (1973). A sample of 10 μg of DNA was digested to completion with *Hha*I. Separation of fragmented DNA by agarose gel electrophoresis and transfer to nitrocellulose paper was performed by the method of Southern (1975). The fragmented cathepsin E gene was detected with ^{32}P-labeled cDNA for rat procathepsin E (pRT732) under high-stringency conditions.

Northern blot analysis

Total RNA was extracted and purified by the guanidinium thiocyanate-cesium chloride centrifugation method (Chirgwin *et al.*, 1979). 15 μg total RNA from the rat liver at various developmental stages were denatured and subjected to electrophoresis in a 1% agarose gel that contained 1.1% formamide. After the RNA had been transferred to a Hybond-N membrane (Amersham Int.), the membrane was hybridized with ^{32}P-labeled cDNA for rat procathepsin E (pRT732) under high-stringency conditions.

Immunohistochemical analysis

The tissue was fixed in 10% phosphate-buffered formalin and embedded in paraffin. The avidin-biotin-peroxidase complex method was used to determine the localization of cathepsin E binding in various tissue sections using anti-rat cathepsin E serum prepared as described previously (Yamamoto *et al.*, 1996). Sections were counterstained with hematoxylin for microscopic examination.

RESULTS

Developmental change of cathepsins D and E in rat tissues

We have reported levels of cathepsins D and E in various tissues of 1-month-old juvenile rat in a previous paper (Kageyama *et al.*, 1996b). In this report, developmental change in the levels of cathepsins D and E in various tissues was investigated. The results show that the level of cathepsin D in each tissue increased gradually during fetal development and that of cathepsin E changed significantly during development (Fig. 1). The quite high level of cathepsin E in fetal liver from 14- to 17-day-gestation stages is particularly noteworthy. The level in other tissues such as stomach, spleen, and thymus increased at later fetal stages or the infant stage. Since the liver diverticulum first appears around day 12, this coincided with the time when the level of liver cathepsin E began to increase. Although we did not investigate in detail the change in levels of cathepsins D and E after 1-month-old juvenile stage, the levels of both cathepsins in some tissues such as liver of 3-month-old adult were largely similar to those in respective tissues of 1-month-old juvenile. The levels of cathepsins D and E in serum were also determined (Table 1). The level of serum cathepsin D was high at the fetal stage and decreased to one-half and then one-third at the infant and juvenile stages, respectively. The Level of serum cathepsin E (mainly procathepsin E) was high at the fetal stage but had decreased significantly at the infant and juvenile stages.

Cathepsins D and E in each tissue were visualized by activity staining after PAGE of the crude homogenate (Fig. 2). Throughout development, cathepsin D was present as an active form, whereas cathepsin E was present mainly as a precursor form, namely, procathepsin E. The ratio of cathepsin E to procathepsin E was estimated by assays at pH 4 and 5. The active cathepsin E was present at about 5-10% in each
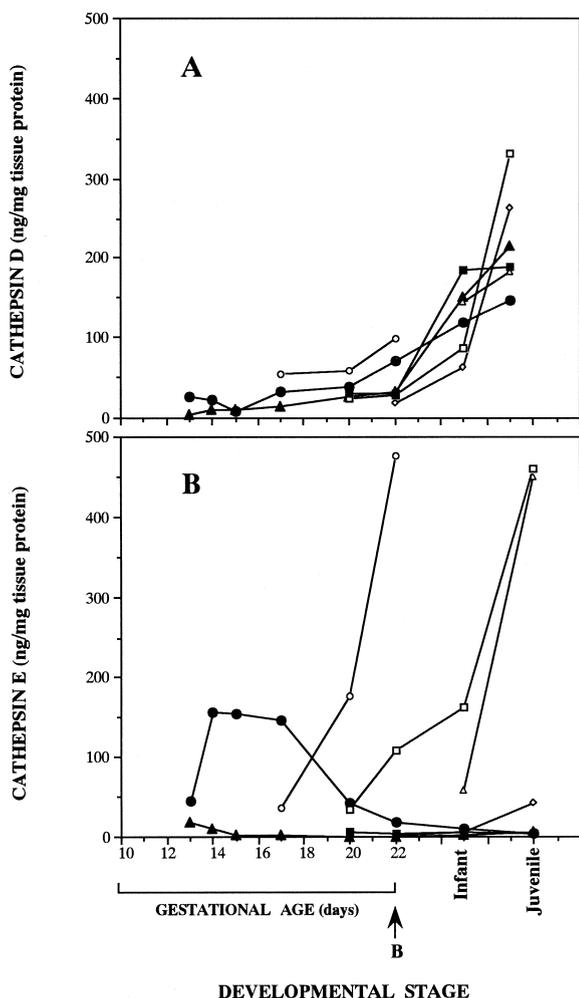
Fig. 1. Developmental change of the levels of cathepsins D (**A**) and E (**B**) in various rat tissues. ▲, Brain; △, thymus; ◇, lung; ●, liver; ○, stomach; □, spleen; ■, kidney. Arrow B shows the time of birth.

Table 1. Levels of cathepsin D and cathepsin E in the serum at three different developmental stages

Enzyme	Developmental stage		
	17-day-gestation fetus	4-day-old infant	Juvenile
	ng/ml serum		
Cathepsin D	207	102	72
Cathepsin E*	204	24	18

* The major form in the serum was procathepsin E.

tissue of the fetus and juvenile, except that a high ratio of cathepsin E (more than 40%) was found in some tissues such as juvenile kidney.

Methylation of the cathepsin E gene

For methylation analysis, a methylation-sensitive restriction enzyme *Hha*I was used. The enzyme recognizes the GCGC sequence and does not hydrolyze this sequence if
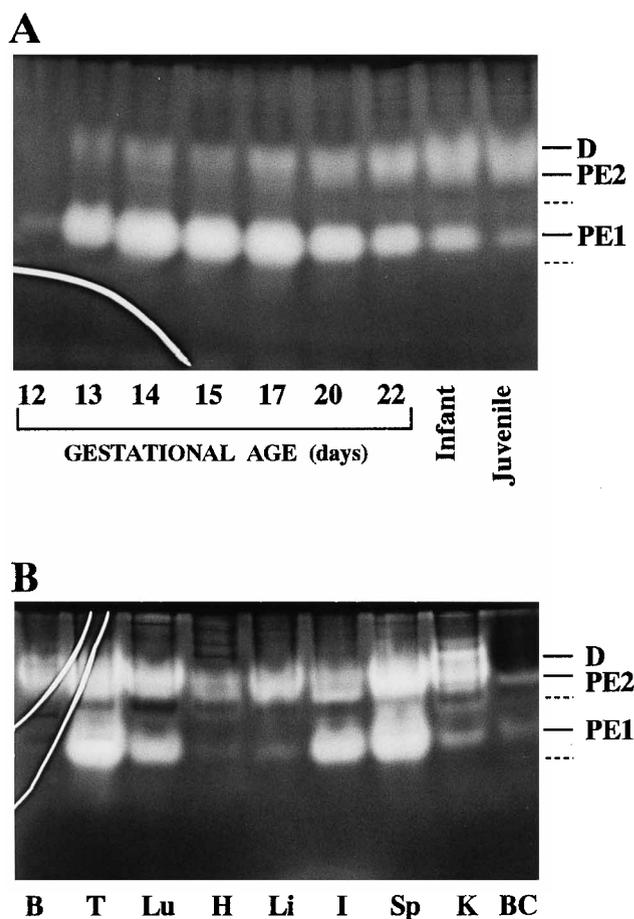
Fig. 2. Activity staining of rat cathepsins D and E. A portion of the crude homogenate supernatant was subjected to electrophoresis on a 10% polyacrylamide gel in Tris-glycine buffer, pH 8.3. After electrophoresis, activities of cathepsins D and E were stained by the method of Furihata *et al.* (1972) except that the incubation with hemoglobin was carried out at pH 3.5. (**A**) Electrophoresis of liver homogenates prepared at various developmental stages. (**B**) Electrophoresis of various tissue homogenates of 1-month-old juvenile rat. Horizontal symbols are: B, brain; T, thymus; Lu, lung; H, heart; Li, liver; I, intestine; Sp, spleen; K, kidney; BC, blood cells. Vertical symbols were: D, cathepsin D; PE1 and PE2, procathepsin E monomer and dimer, respectively. Dashed lines show the positions of cathepsin E dimer (upper) and monomer (lower), respectively. Since cathepsin E was present in lower amounts than procathepsin E, it could hardly be visualized in most tissues, except for a few such as juvenile kidney.

cytosine is methylated. It is known that genes are usually methylated in their inactive states and demethylated when expressed (Cedar, 1988). By Southern blot hybridization of *Hha*I-digested DNA of rat liver at various developmental stages, the number of lower-molecular-weight DNAs were found to be higher at 13~17-day-gestation fetal stages than at infant and juvenile stages (Fig. 3A). This result showed that hypomethylation of the cathepsin E gene occurred in liver cells at 13~17-day-gestation fetal stages and was consistent with the results of high levels of cathepsin E at these developmental stages. When stomach DNA was analyzed, the gene was gradually demethylated during fetal development being con-
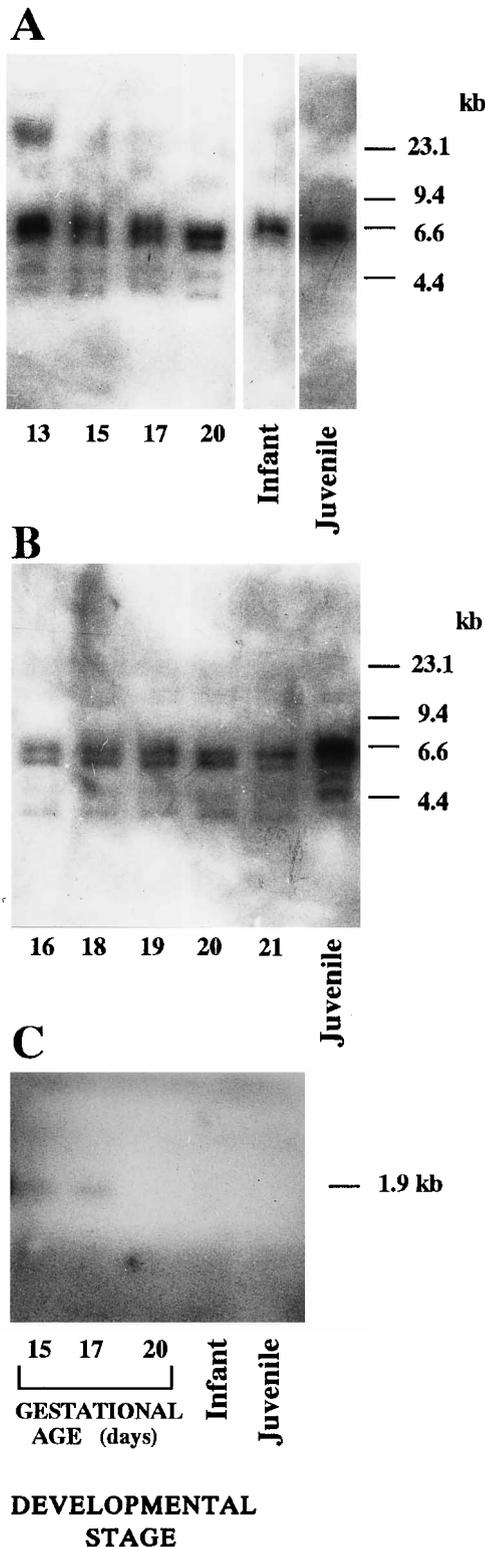
Fig. 3. Southern blot hybridization of high-molecular-weight DNAs from liver (**A**) and stomach (**B**) at various developmental stages of rat. The amount of DNA applied was 10 μg. Restriction enzymes used were *Hha*I. (**C**) Northern blot hybridization of total RNA from rat liver. The amount of total RNA applied was 15 μg. After hybridization with ^{32}P-labeled cDNA for rat cathepsin E, each membrane was washed under high stringency conditions.

sistent with the change of level of gastric cathepsin E (Fig. 3B).

Expression of the cathepsin E gene

Expression of the mRNA for cathepsin E was examined in fetal liver. A band of mRNA of about 1.9 kb was detected at 15-day and 17-day-gestation fetal stages being consistent with the high level of activity of cathepsin E at these stages (Fig. 3C). The size of mRNA for rat cathepsin E was the same with those for guinea pig (Kageyama *et al.*, 1992) and rabbit (Kageyama, 1993) cathepsins E. No appreciable band was observed at later stages, indicating that the expression of the cathepsin E gene was quite weak at these stages.

Immunohistochemical localization of cathepsin E

Sections of livers from 14- and 17-day-gestation fetus, 4-day-old infant, and 1-month-old juvenile were stained immunohistochemically using anti-cathepsin E serum (Fig. 4). Stained cells were detected in 14-day and 17-day-gestation fetuses. Although liver contained various types of cells, hepatocytes were stained exclusively showing that liver cathepsin E was synthesized in these cells.

DISCUSSION

The developmental change in levels of cathepsins D and E was clarified in various rat tissues. First, we discuss the results of cathepsin D. In our previous report (Kageyama *et al.*, 1996b), the level of cathepsin D at 1-month-old juvenile stage was determined. In the present report, the level of cathepsin D at various fetal and infant stages was investigated and it increased gradually during fetal development. Since cathepsin D is known to be localized in lysosomes (Barrett, 1977), this result suggests that the lysosomal system is incomplete at fetal stages. Cathepsin D at the fetal stage might be less essential for processing or degradation of intracellular proteins than at infant and juvenile stages. Indeed, mice deficient in cathepsin D survive during fetal development but die around 1 month after birth (Saftig *et al.*, 1995).

In the case of cathepsin E, the level in each tissue changed significantly during development. To date, tissue distribution of cathepsin E in adult or juvenile rats has been clarified and high levels were found in stomach, thymus, spleen, and bone marrow (Muto *et al.*, 1988; Sakai *et al.*, 1989; Yonezawa and Nakamura, 1991; Bennett *et al.*, 1992; Yonezawa *et al.*, 1993). The enzyme is thought to be involved to varying degrees in the physiological activities of these tissues (Lapresle, 1971; Yonezawa *et al.*, 1987; Finzi *et al.*, 1993; Nakanishi *et al.*, 1994). At early fetal stages, a high level in liver was particular noteworthy. This high level was thought to be due to the activation of the cathepsin E gene as suggested by the hypomethylation of the liver cathepsin E gene. The transcriptional activity of some tissue-specific genes has been shown to be controlled by the extent of methylation (Cedar, 1988). Cathepsin E might have an important physiological role in fetal liver, such as the regulation of liver development through
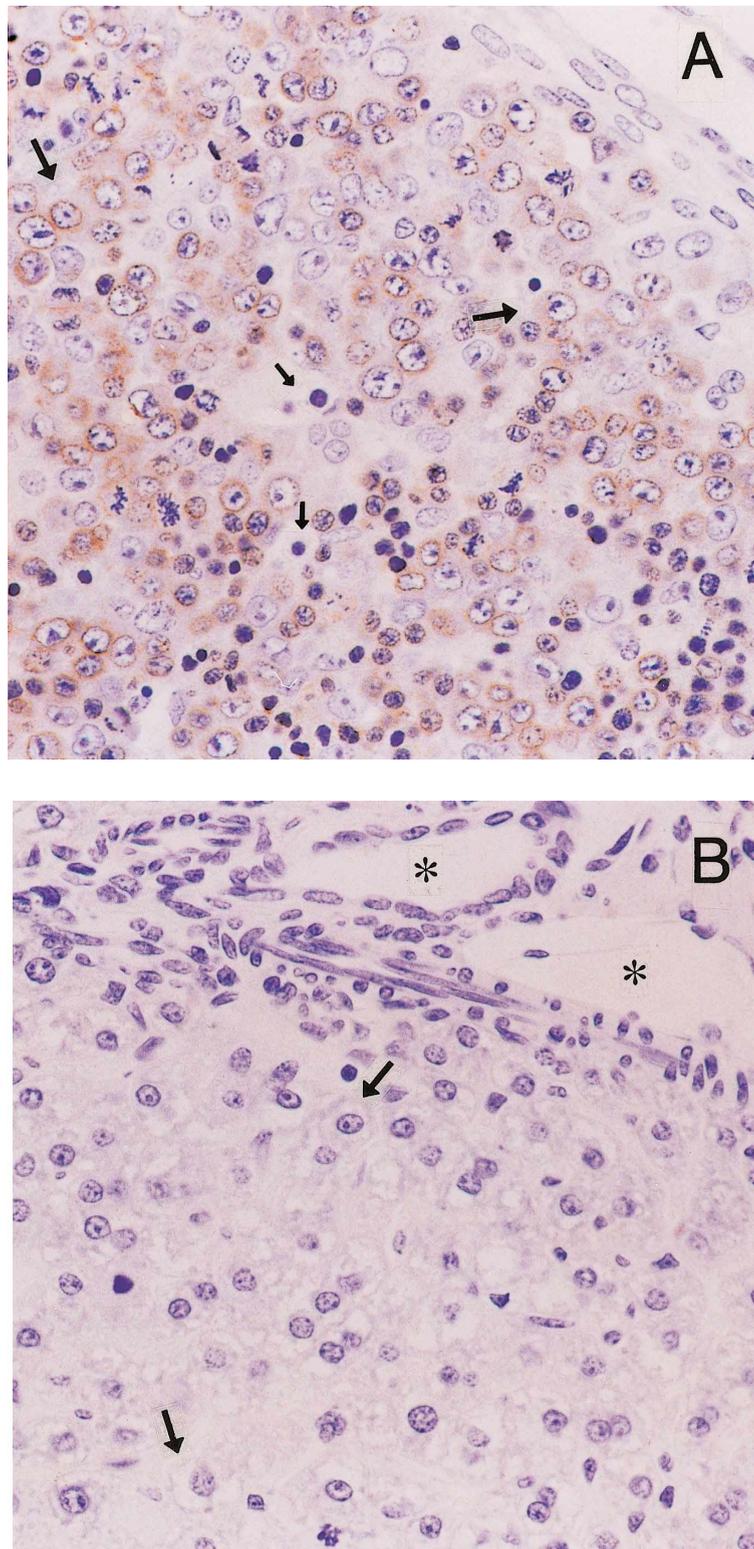
Fig. 4. Localization of cells that produce cathepsin E in rat liver of a 14-day-gestation fetus (**A**) and 1-month-old juvenile (**B**). The results of 17-day-gestation fetus and 4-day-old infant were similar overall with those of the 14-day-gestation fetus and 1-month-old juvenile, respectively, and are not shown. Large and small arrow marks show hepatocytes and hemopoietic cells, respectively. Asterisks indicate bile ducts. Magnification, ×400.

activation/inactivation of signaling proteins, since the enzyme has been shown to have high specificity for some bioactive peptides and precursor proteins that are involved in signal transduction and growth (Lees *et al.*, 1990; Kageyama, 1993, 1995; Kageyama *et al.*, 1995, 1996a). The localization of the enzyme in hepatocytes would favor this hypothesis. The enzyme might also be involved in regulating hematopoiesis at fetal stages since liver is known to be the major hematopoietic tissue in the fetus.

Cathepsin E was found to be present in each tissue mainly as a proform. Cathepsin E is synthesized as preproform and, after removal of the pre-piece, the proform is transported to the endoplasmic reticulum and endosomes (Yonezawa *et al.*, 1988; Saku *et al.*, 1991; Finley and Kornfeld, 1994). Procathepsin E might be stored in these organelles, be activated to cathepsin E in an acidic microenvironment, and act on various proteins and peptides. Since cathepsin E is unstable under neutral and weakly alkaline conditions (Kageyama *et al.*, 1992), the enzyme might be degraded rapidly after working. This process of activation and degradation is thought to be important to regulate the activity of cathepsin E in a cell.

The hypothesis that procathepsin E itself is essential at the fetal stage could not be excluded. Recently, rat pepsinogen has been shown to be a candidate for growth factors that stimulate the proliferation of gastric epithelial cells (Kishi *et al.*, 1997). Since cathepsin E is evolutionarily closest to pepsinogens between non-pepsinogen aspartic proteinases (Kageyama *et al.*, 1992), procathepsin E might serve as a growth factor in fetal liver and gastrointestinal tissues and stimulate the development of these tissues. High levels of procathepsin E in fetal serum might corroborate the possibility of this hypothesis. If this is the case, since liver originates from the gastrointestinal tract, procathepsin E might act specifically on tissues of gastrointestinal origin.

ACKNOWLEDGMENT

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