〈Original Article〉

Changes in lactate dehydrogenase isozyme expression and activity during development in mouse testis

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Summary Lactate dehydrogenase (L-lactate; NAD oxidoreductase, LD) is widely distributed in vertebrate tissues and catalyzes oxidation–reduction reactions between pyruvic acid and lactic acid. LD is encoded by A, B, and C genes, and among these, the *LD-C* gene is localized in the testes of higher vertebrates. It has been speculated that LDx isozymes, which are tetramer (C4) enzymes encoded by the C gene, play important roles in the testes, yet the details remain unclear. Here we clarified the biochemical characteristics of testicular LD by analyzing expression levels and activities of LDx isozymes and *LD-C* genes during mouse development and growth.

Liver and testes tissues were excised from mice between fetal day 15 and 50 weeks after birth. Next, mRNA levels of *LD-A*, *-B*, and *-C* genes were determined using quantitative RT-PCR. Additionally, LD isozyme fractions were examined by electrophoresis and immunohistochemistry analyses.

In liver tissues, LD5 was the predominant LD and LD-A mRNA was expressed at all stages. In testes tissues, LD-A mRNA expression levels gradually decreased to 2 weeks of age, whereas LD-B mRNA expression tended to increase gradually with aging. Conversely, LD-C mRNA and LDx isozyme expression were not observed until 1 week after birth. These data indicate that the *LD-C* gene was translated into LDx during spermatogenesis.

Key words: Lactate dehydrogenase X, lactate dehydrogenase C gene, lactate dehydrogenase isozyme, testes

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1. Introduction

Lactate dehydrogenase (EC.1.1.27; LD) is a glycolytic enzyme that catalyzes oxidation–reduction reactions between pyruvic acid and lactic acid using nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide as a coenzyme. Many studies of mammalian LD have been published since the optical specificity of L-lactic acid as a substrate was described by Meyerhof *et al.*[1] in 1975.

In starch gel electrophoresis experiments, Vessel *et al*. revealed that human serum LD comprises three components[2], and identified five tissue specific LD isozymes[3,4]. These LD isozymes are present in most tissues, although their compositions differ between tissues in aerobic environments, such as those of myocardial and skeletal muscle; liver microenvironments are physiologically anaerobic. In subsequent *in vivo* studies, the human LD isozyme was shown to be encoded by the distinct LD genes A (*LD-A*) and B (*LD-B*), which are present on chromosomes 11 and 12, respectively[5].

In addition to the five isozymes, yet other LD isozymes were shown to be formed from the genes A and B[6]. Among these, the LDx isozyme was detected in human testes after adolescence, and its electrophoretic mobility was found to be between that of LD3 and LD4 isozymes. The functional LDx isozyme comprises a protein tetramer (C4) of the C subunit from the *LD-C* gene, which is present on chromosome 11. But the *LD-C* gene is not present on chromosome 11 and the LDx isozyme encoded by the C gene is not present in somatic cells. Many details of LDx remain obscure.

Studies of LDx isozymes include genetic, enzymatic, immunological, metabolic, and biochemical analyses[7-11]. LD isozyme dynamics have also been associated with the development and growth of mammals, birds, amphibians, and fish[12-16]. Yet these studies did not report systematic analyses of LD genes or proteins or their kinetics during development and growth.

In this study, we systematically investigated the dynamics of the LD-C isozyme and the *LD-C* gene during mouse development and growth and made biochemical comparisons with testes-expressed LDx isozyme.

2. Materials and methods

Mice

Male and female mice (BALB/slc) were purchased from SLC (Hamamatsu, Japan) and were isolated for 24 h after mating (gestational day 1). Fetuses were anesthetized with isoflurane *in utero*, and were then removed from their dams. From gestational days 15 to 19, mice tissues were stored at −80°C. Mice of >1 week of age were anesthetized with isoflurane for immediate laparotomy, and systemic perfusions were performed by left ventricular puncture with diethylpyrocarbonate-treated phosphate buffer (pH 7.4, PBS). Harvested organs were then washed with PBS and were rapidly cooled in liquid nitrogen and stored at −80°C until use.

Mice were individually housed in plastic cages under specific pathogen-free conditions in an environmentally controlled animal chamber at 22°C–24°C with about 50% relative humidity. The environmentally controlled animal chamber was maintained under a 12-h light/12-h dark cycle with lights on from 08:00 to 20:00. All procedures were conducted in accordance with the Guidelines for Animal Experiments at Kitasato University and were approved by the ethics committee.

RNA extraction from mice tissues

Total RNA was isolated from cells using RNeasy Mini kits (Qiagen, GmbH, Germany) according to the manufacturer's instructions. First-strand cDNA synthesis was performed by reverse transcription from RNA using a Superscript Reverse Transcription kit (Clontech, Shiga, Japan).

Semi-quantitative reverse transcription-polymerase chain reaction analyses of LD genes

LD-A, B, and C mRNA expression levels in liver and testes tissues were evaluated using reverse transcription-polymerase chain reaction (RT-PCR) with the following primers: LD-A forward,

5′-GTTGCAATCTGGATTCAGCG-3′ and reverse, 5′-CTGCAGCTCCTTCTGGATTC-3′; LD-B forward 5′-GAAGCTCATTGCGTCCGTTG-3′ and reverse, 5′-CCCAGTTGGTGTAGCCTTTG-3′; LD-C forward 5′-CCCTTGTTGACGTCGATACG-3′ and reverse 5′-CTTCCGATCACACGGCCTAC-3′; beta actin forward 5′-TCATGAAGTGTGACGTTGACATCCG T-3′ and reverse 5′-CCTAGAAGCATTTGCGGTGC ACGATG-3′. LD-A, B, and C DNAs were then amplified for 35 cycles of denaturing for 60 s at 95°C, annealing for 60 s at 53°C, and extension for 60 s at 70°C. Beta actin cDNA was amplified for 32 cycles of denaturing for 60 s at 95°C, annealing for 60 s at 65°C, and extension for 60 s at 70°C. PCR products were then loaded onto 1.5% agarose gels and were electrophoresed. Quantities of PCR products were determined semi quantitatively using an ATTO densitograph (ATTO, Tokyo, Japan) and were normalized to those of beta actin.

LD activity in mice tissues

Mouse liver and testes tissues were suspended in PBS and were homogenized using ultrasound. The resulting homogenates were then centrifuged at 22,000 G for 10 min at 4°C, and supernatants were collected. LD activity was measured in accordance with the recommended method of the Japanese Society of Clinical Chemistry. Activity values were calculated from absorption coefficient of nicotinamide adenine dinucleotide as international units. Finally, enzyme activities per 1 mg of tissue were compared.

Electrophoresis of the LD isozyme from mice tissues

LD isozyme electrophoresis was performed using a Titan gel S-LD plate (Helena, Saitama, Japan). Briefly, liver or testes tissues were homogenized and activities were adjusted to 600 U/L in PBS according to the manufacturer's instructions. After electrophoresis at 90 V for 20 min, LD activity staining was performed using Titan gel S-LD reagent (Helena).

Immunohistochemistry of LDx in mice testes

To investigate the localization of LDx in testis, immunohistochemical analyzes were performed using an anti-LDx antibody that was generated in rabbits after immunizing with the mouse LDx protein. The rabbit serum was purified using the saturated ammonium sulfate salt precipitation method. Mice testicle samples were fixed in 15% neutral buffered formalin and frozen sections were prepared. Staining was performed according to standard immunohistochemical techniques using the primary antibody from rabbit serum and a biotin-labeled anti-mouse rabbit IgG antibody (Dako, CA, USA). Sections were also stained with peroxidase conjugated streptavidin (Dako) and 3,3'-Diaminobenzidine (DAB) solution (Dako).

3. Results

Semi-quantitative RT-PCR expression analyses of mice LD mRNA

Initially, we analyzed LD-C mRNA expression levels in testes, and then compared LD-A, B, and C mRNA expression levels in liver and testes tissues. Only LD-A mRNA was expressed in the liver at all stages of embryonic development and growth, whereas liver LD-B and LD-C mRNA expression was not confirmed at any developmental stage (Fig. 1 a and c). In contrast, LD-A mRNA was expressed in testes at all stages of embryonic development and growth, and expression levels increased particularly after birth. LD-B mRNA was also detected in testes at all developmental stages, and expression levels increased particularly after birth. Testicular LD-C mRNA appeared at 1 week postnatally, and expression levels reached a plateau at 3 weeks of age (Fig. 1 b and d).

Comparison of LD activity values in mouse liver and testes tissues

In comparisons of testes and liver tissues, LD activities increased to 18 weeks of gestation, and then gradually decreased in both tissues. Testicular LD activities increased with maturation and plateau at 4 weeks of age (Fig. 2).

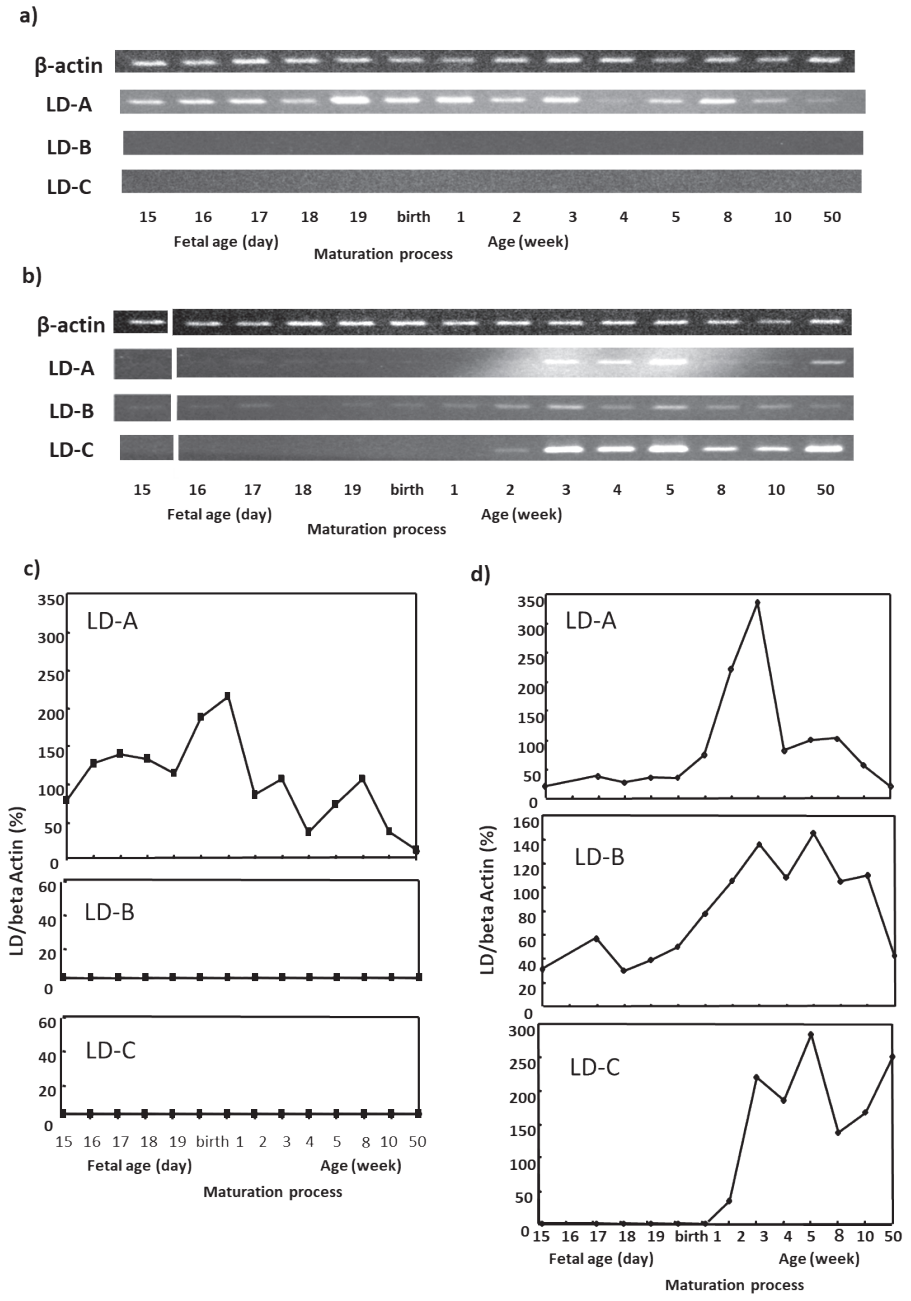

Fig. 1 LD mRNA expression levels in organs during mouse development stages
LD-A, B, and C mRNA were isolated from mouse liver and testes tissues and expression levels were determined using semi-quantitative PCR with beta actin as an internal standard. Band intensities were analyzed using an ATTO densitograph. (a) Electrophoretic images of LD and beta actin cDNA from the liver; (b) electrophoretic images of LD and beta actin in the testes; (c) LD-A, B, and C mRNA expression levels in the liver and (d) in the testes are presented for several developmental stages.

Distributions of LD isozymes in mice tissues

To confirm our observations of mRNA expression levels and LD activities, we determined LD-C protein expression levels in testes, and made comparisons with those in liver tissues. In liver tissue, only LD5 was expressed at all embryonic and growth stages, and LD1, LD2, LD3, LD4, and LDx isozymes were not detected during any develop-

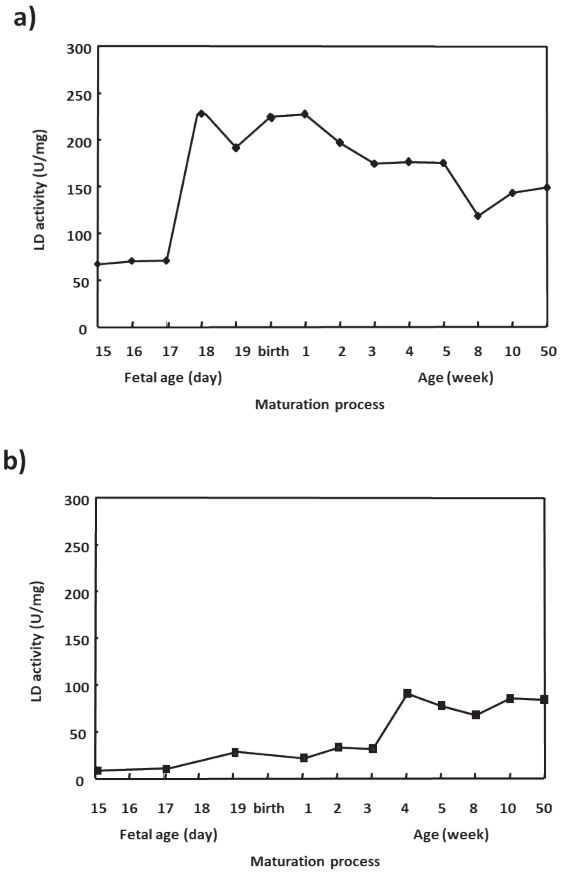

Fig. 2 LD activities in mouse organs during development

LD activities in homogenized liver and testes extracts were measured in accordance with the method recommended by the Japanese Society of Clinical Chemistry. Activity values (U/L) per 1 mg of the organ weight were compared; (a) development changes in liver LD activities; (b) development changes in testes LD activities.

mental stages (Fig. 3 a and c).

In LD isozyme fractions from testes, LD1 to LD5 proteins were present prenatally and LD5 isozyme was predominant. Subsequently, fraction ratios decreased in the order of LD4, LD3, LD2, and LD1. The fraction ratio of LD5 decreased with increasing age, whereas the LD1 isozyme increased over the same weekly analyzes. In addition, LD1 became dominant after birth, and fraction ratios of LD2, LD3, and LD4 decreased thereafter in this order. The LDx isozyme fraction appeared after 2 weeks of age, and plateaued after 3 weeks of age (Fig. 3 b and d).

Iimmunohistochemical confirmation of LDx in mouse testes

To confirm that LDx is localized in the testes of mice during maturation after birth, we performed immunohistochemical staining analyses using an anti-LDx antibody. In these experiments, LDx protein was not detectable at birth or at 2 weeks of age, but was found in some spermatocytes at 3 weeks of age, and was confirmed in sperm samples at 4 weeks of age. From 6 weeks of age, LDx protein was localized at the centers of seminiferous tubules. This localization was more pronounced at 8 weeks, and sperm were strongly stained (Fig. 4).

4. Discussion

In this study, we investigated trends of LD isozyme mRNA and protein expression in mice liver and testes tissues during gestation and maturation. We observed no changes in liver LD isozyme fractions during the study period, but progressive increases in LD activities were apparent from 2 weeks before birth, and gradual decreases were observed after birth. Only LD-A mRNA was expressed in liver, and increased expression levels were observed over the 2 weeks before birth followed by decreases after birth, corresponding with LD activities. In contrast, LD isozyme activities changed markedly over developmental stages in the testes and the LD5 fraction was dominant over the 15 weeks of gestation, whereas the LD1 fraction dominated with maturation and the LDx isozyme appeared during the 2 weeks after birth. This trend was reflected by increasing LD mRNA expression until 4 weeks of age.

In liver tissues, only LD-A mRNA expression and LD5 isozyme activity were observed at all developmental stages. The LD isozyme is encoded by LD-A and LD-B genes and is assembled from subunits of A and B as B4 (LD1), A1B3 (LD2), A2B2 (LD3), A3B1 (LD4), and B4 (LD5). Previous studies show that five LD isozymes are distributed in tissues[17-19]. Moreover, these LD isozymes are present

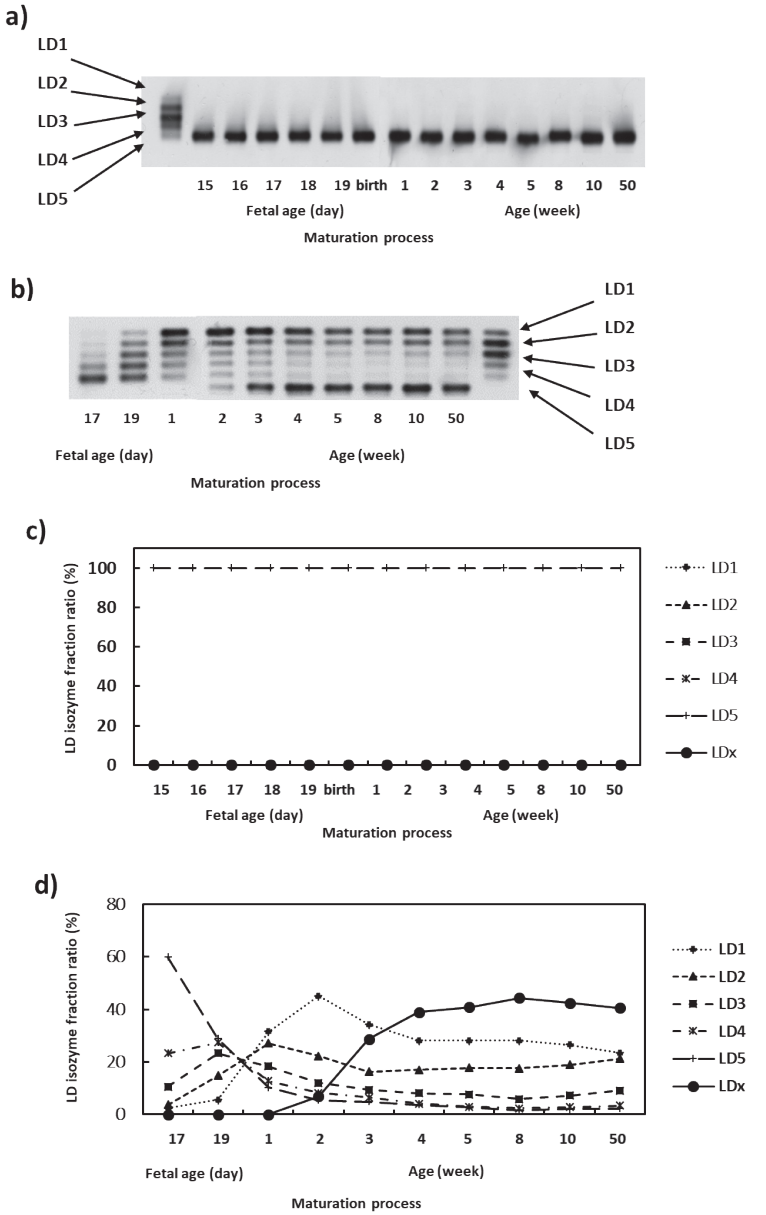

Fig. 3 Electrophoresis of LD isozymes from mouse tissues at various developmental stages

Samples were prepared by homogenizing liver and testes tissues an adjusting LD activities to 600 U/L in PBS. Electrophoresis was performed using Titan gel S-LD plates. LD activity staining was then performed using Titan gel S-LD reagent and band intensities were analyzed using an ATTO densitograph; (a) developmental changes in liver LD isozyme expression; (b) development changes in testes LD isozyme expression; quantitation of band intensities from liver (c) and testes (d) tissues over development stages.

in most tissues under physiological conditions, and in aerobic environments such as myocardial tissues, isozymes of the A subunit are active. The B subunit isozyme is active in skeletal muscle and liver tissues, which endure physiologically anaerobic conditions. In liver tissues, we confirmed that only LD5 is expressed and formed from the B subunit and is expressed at constant levels through maturation,

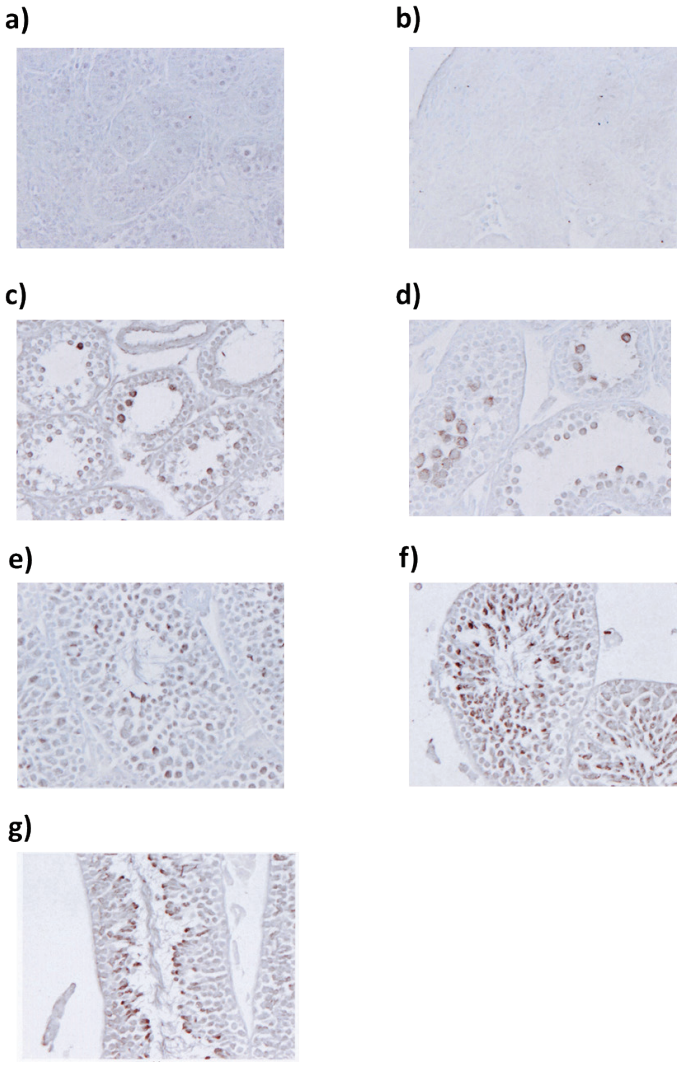

Fig. 4 Immunohistological analyses of LDx in testes during maturation

Mice testicle samples were fixed in 15% neutral buffered formalin and frozen sections were prepared at a thickness of 3 μm. Staining was performed using standard immunohistochemical techniques. Sections were stained with streptavidin biotin conjugate and DAB; LDx isozyme in testis of mice at 0 (a), 1 (b), 2 (c), 3 (d), 4 (e), 6 (f), and 8 (g) weeks after birth.

suggesting that it plays roles under anaerobic conditions.

Our data also show that LD mRNA and isozyme expression varies with developmental stages in the testes. LD5 was dominantly expressed during fetal stages, whereas LD1 expression became dominant from 2 weeks before birth. LDx appeared for the first time at 2 weeks after birth and was constantly expressed until 4 weeks after birth. Our immunohistochemical analyzes further demonstrated the presence of LDx in testes from mice of over 3 weeks of age. Blanco *et al.* detected an additional LD isozyme to the five that are encoded by genes A and B, and referred to it as LDx[6]. This isozyme was found in testicles after human puberty, and in electrophoretic mobility assays had a molecular weight between those of LD3 and LD4 isozymes. LDx was also present in the testicles of other animal species

and its electrophoretic mobility was between those of LD4 and LD5 in rabbits, and was slower than that of LD5 in mice. In guinea pigs and rats, two LDx isozymes have been identified, with electrophoretic mobility between those of LD3 and LD4 isozymes and LD4 and LD5 isozymes, respectively. Additionally, three LDx isozymes were identified in bovine testicles; one migrated between LD3 and LD4 isozymes and the other two migrated between LD4 and LD5 isozymes. However, no LDx isozymes were detected in electrophoretic studies of porcine, feline, duck, and chicken testicles[20-22]. Functional LDx comprises a tetramer (C4) of C subunits, which are encoded by the *LD-C* gene on human chromosome 11. The *LD-C* gene, however, is not present on this chromosome and the LDx isozyme encoded by the *LD-C* gene was not found in somatic cells. Although many details remain unknown, *LD-B* may have evolved through gene linkage or duplication[23,24]. Previous studies show that tissue dynamics of LD isozymes are associated with development stages. Moreover, in heart, liver, muscle, and kidney tissues, the LD5 isozyme is reportedly dominant during early stages of development, whereas LD1 appeared immediately before birth[25,26]. These data suggest that our results have changed to the LD isozyme suitable for testicular tissue as other tissues LD isozymes.

The LDx isozyme is known to be involved in mammalian spermatogenesis. Moreover, the LDx was abundant in spermatids and spermatozoa of male mice that became fertile at about 8 weeks of age[27,28]. In accordance, our immunohistochemical analyses confirmed the presence of LDx in spermatocytes and sperm, suggesting that LDx is involved in spermatogenesis.

In summary, expression levels and activities of LD isozymes change in mouse testes during maturation, with a shift from LD5 to LD1 and the emergence of LDx from 2 weeks after birth. Conversely, liver LD is only expressed from LD-A mRNA at all developmental stages, and only takes the form of LD5. Because LDx was detected only in testes, these results suggest that LD-C is related to spermatogenesis and is only expressed during this process. We also speculate that the presence of LDx reflects the energy requirements of sperm formation. This study is limited to a small sample size, which was minimized to achieve appropriate statistical power. Hence, our findings require confirmation in larger studies. Finally, to confirm changes in testes LD expression and activity with stages of maturity, further studies are required to identify and correlate regulators of LD with our observations.

Conflicts of interest

The authors declare no competing interests.

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