LDHC: The Ultimate Testis-Specific Gene

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ABSTRACT: Lactate dehydrogenase C (LDHC) was, to the best of our knowledge, the first testis-specific isozyme discovered in male germ cells. In fact, this was accomplished shortly before “isozymes or isoenzymes” became a field of study. LDHC was detected initially in human spermatozoa and spermatogenic cells of the testes by gel electrophoresis. Immunohistochemistry was used to localize LDHC first in early-pachytene primary spermatocytes, with an apparent increase in quantity after meiosis, to its final localization in and on the principal piece of the sperm tail. After several decades of biologic, biochemical, and genetic investigations, we now know that the lactate dehydrogenase isozymes are ubiquitous in vertebrates, developmentally regulated, tissue and cell specific, and multifunctional. Here, we will review the history of LDHC and the work that demonstrates clearly that it is required for sperm to accomplish their ultimate goal, fertilization.

Key words: Lactate dehydrogenase C, glycolysis, male fertility, sperm.

J Androl 2010;31:86–94

The isozyme concept owes its development to Clement Markert (Markert and Appella, 1961), who was one of the first to recognize the significance of multiple molecular forms of enzymes, and since this time, isozymes have been extensively studied or used as markers for normal and abnormal cell function. One of the earliest studied and best examples is the lactate dehydrogenase (LDH) enzyme family, which catalyzes the interconversion of pyruvate to lactate with the concomitant oxidation/reduction of NADH to NAD⁺ (Everse and Kaplan, 1973). The first evidence of the presence of multiple forms of LDH was found using the technique of protein electrophoresis (at this time in a starch matrix) elaborated by Smithies (1959), coupled with cytochemical visualization of LDH activity (Dewey and Conklin, 1960; Allen, 1961). We learned that LDH consists of A and B subunits that assemble into homotetramers or heterotetramers that are distributed in the body in combinations reflecting the metabolic requirements of different tissues and consistently with the catalytic properties of the isozymes. For example, LDHA is most abundant in skeletal muscle, where oxygen deficiency from exercise requires glycolysis to satisfy metabolic needs, whereas LDHB is expressed abundantly in cardiac muscle, which is dependent on aerobic metabolic pathways.

Our entrance into the field in its infancy was when we asked whether a single cell type, the spermatozoan, contained one or multiple forms of LDH (Goldberg, 1963). That question began this journey with the discovery of LDH-X (now known as LDHC). This unique band corresponding to the homotetramer LDH-C₄ was only revealed in testis and spermatozoa and not in other tissues or cells. It was, to our knowledge, the first testis-specific isozyme described (Blanco and Zinkham, 1963; Goldberg, 1963, 1964). Since that time, the question of why testes and sperm need this unique form of LDH remains to be discovered.

We learned that different forms of LDH are the product of 3 different genes: Ldha, Ldhb, and Ldhc, which encode A, B, and C subunits, respectively (Li, 1989). In the human and mouse genomes, Ldha and Ldhc genes are located in tandem on chromosomes 11 and 7, respectively (Edwards et al, 1989), and Ldhb gene is on chromosomes 12 (human) and 6 (mouse; Takeno and Li, 1989). In terms of gene evolution, the current model is that in vertebrate evolution, the original Ldh gene soon duplicated, giving rise to Ldha and Ldhb genes (Markert et al, 1975). The Ldhc gene then arose from a second independent gene duplication event, by duplication of the Ldhb gene in fish and columbid birds...
(Zinkham et al, 1969; Mannen et al, 1997) and by duplication of the \textit{Ldha} gene during mammalian evolution (Li et al, 1983b, 2002; Millan et al, 1987). Divergences in the structure, function, and localization of the protein and in gene regulation must have conferred a genetic advantage, because LDHC was conserved from its appearance throughout evolution. Finally, direct evidence of the importance of LDHC was demonstrated by the generation of an \textit{Ldhc} knockout mouse model, but even though this model gave us some answers, it also raised new questions. LDHC still did not reveal all of its mysteries.

**LDHC: Characteristics of the Protein**

**Structure and Enzymatic Characteristics of LDHC**—The LDH isozymes differ in net charge, which gave us the ability to electrophoretically distinguish them. In terms of activity, earlier studies showed that the kinetics of catalysis were different between the LDH isozymes (Goldberg, 1972). Mouse LDH-C₄ structure was characterized by crystallography (Goldberg, 1972; Musick and Rossmann, 1979), and complete sequences for LDH-C₄ became available (Pan et al, 1983). These structural studies confirmed that LDHC is homologous to the LDHA and LDHB subunits (72.5\% and 75.3\% identity with LDHA, and 64.5\% and 69.8\% identity with LDHB in mouse and human, respectively). However, LDH-C₄ displays substitutions in amino acid sequence and some modifications in 3-dimensional structure that give LDH-C₄ unique structural (Hogrefe et al, 1987) and functional properties (Blanco et al, 1976; Li et al, 1983a). LDH-C₄ has high thermostability (Goldberg, 1972) and has a broader range of substrates; for example, LDH-C₄ is able to metabolize \(\alpha\)-hydroxyvalerate (Allen, 1961; Goldberg, 1965; Blanco et al, 1976). However, the differences between LDH isozymes are not in the orders of magnitude sufficient to confer advantage to 1 of the 3 relative to germ cell metabolism (Goldberg, 1972).

**Tissue Localization**—We showed the presence of LDHC protein in the testis using gel electrophoretic (Goldberg and Hawtrey, 1967) and immunohistochemical (Hintz and Goldberg, 1977) techniques (Figure 1). By using isolated fractions of mouse germ cells, the protein was detected first in preleptotene spermatocytes and was abundant in spermatids and spermatozoa (Li et al, 1989). LDHA also is present in pachytene spermatocytes, but most LDH activity in male germ cells is from LDH-C₄ (Erickson et al, 1975a; Li et al, 1989; Odet et al, 2008), and heterotetramers containing both A and C subunits are not detected in murine or human testes (Goldberg and Hawtrey, 1967; Li et al, 1989). Our initial experiments suggested that LDHC was the only LDH isozyme present in spermatozoa (Goldberg,
1965). However, recent studies have shown that LDHA is also present in spermatozoa (Sleight et al., 2005; Krisfalusi et al., 2006; Odet et al., 2008). However, LDH-C4 is responsible for more than 80% of the total LDH activity in mouse spermatozoa (Odet et al., 2008).

Originally, LDH-C4 was considered specific to male germ cells (Blanco and Zinkham, 1963; Goldberg, 1963), whereas LDHB was described as the predominant LDH isozyme in oocytes (Roller et al., 1989). However, Coonrod et al. (2006) showed recently that LDHC protein can be detected by immunohistochemistry in germinal vesicle-stage oocytes and fertilized eggs, and it persists to the preimplantation blastocyst. However, the enzymatic activity of LDH-C4 was not detectable in egg extracts, presumably because of its low level, and Ldhc transcript levels are substantially lower in oocytes (102 per 1 million transcripts) than in whole testis (2844 per 1 million transcripts; UniGene build no. 168, UniGene accession no. for Ldhc: Mm.16563). The function(s) of LDH-C4 during oogenesis, oocyte maturation, or early development is (are) still unclear. We do know that Ldhc-null females are fertile (Odet et al., 2008).

Intracellular Distribution of LDHC—Subcellular localization of a protein results in exposure to a specific microenvironment that might affect its function. Immunohistochemical studies have shown that LDHC is present in the cytosol of spermatocytes and spermatids, and in the principal piece of spermatozoa (Figure 2; Blanco et al., 1976; Alvarez and Storey, 1984; Wheat and Goldberg, 1984; Burgos et al., 1995). In the sperm principal piece, LDHA but not LDHC was bound tightly to the fibrous sheath (Krisfalusi et al., 2006). This difference of intracellular localization between LDHA and LDHC might explain the absence of in vivo heterotetramers of A-C, whereas different tetramer combinations were seen in vitro (Goldberg, 1965).

The relative hydrophobicity of LDHC (Millan et al., 1987) suggested that LDHC might be located close to the plasma membrane. Localization of LDHC in the cortical region in oocytes and preimplantation embryos (pronuclear zygotes to blastocysts) was observed (Coonrod et al., 2006). This is difficult to determine in sperm because of the small volume of the cytosol. However, by isolating different subcellular fractions of rabbit spermatozoa, Alvarez and Storey (1984) found 10% of LDHC in the plasma membrane fraction. Consistent with this result, LDHC was also found at the surface of the spermatozoa (Erickson et al., 1975a; Beyler et al., 1985), possibly because of the high diffusibility of LDHC (Zinkham et al., 1964). However, the presence of extracellular LDHC might also have resulted from the release of LDHC during the isolation procedure from damaged cells.

Several studies have provided evidence that LDHC might be localized in the matrix of sperm-type mitochondria (STMs; Alvarez and Storey, 1984; Montamat et al., 1988; Burgos et al., 1995). However, most of these data were based on indirect methods of detection, and because of the high quantity of LDHC proteins present in spermatozoa, cross-contamination between subcellular fractions is quite possible. Immunohistofluorescence studies with antibodies produced with peptides designated MC5-15 and MC211-220 (Beyler et al., 1985) detected strong signals in the principal piece of the spermatozoon (Goldberg, 1975; Duan and Goldberg, 2003; Odet et al., 2008) but only a weak signal in the midpiece region where the STMs are localized (Figure 2). This weak signal was also found in Ldhc-null sperm, suggesting that it was only background (Odet, unpublished observation). However, LDHC localized in STM might have been lost because of the permeabilization and other treatments during preparation of the sperm smear. High-resolution localization experiments using gold immunolabeling, a specific antibody, and spermatozoa from Ldhc-null mice as negative controls are needed to resolve this controversy. However, it is worth mentioning that mitochondrial activity as measured by JC-1 staining and oxygen consumption (in medium, with lactate as the only substrate) is normal in Ldhc-null spermatozoa (Odet, unpublished observations); therefore, even if a small amount of LDHC is present in the matrix of STM, we were unable to detect any function for LDHC in the respiration process.

Gene Expression Pattern and Regulation of Ldhc Gene
Gene Expression Pattern in Male Germ Cells—The level of Ldhc transcripts is higher in whole testis (2844 per 1 million transcripts; Unigene Mm.16563) than Ldha transcripts (584 per 1 million; Mm.29324) and Ldhb transcripts (160 per 1 million; Mm.29324). This is consistent with LDHC being the major LDH in germ cells and with germ cells being the major component of adult testis (>80%, mainly in the meiotic and postmeiotic phases). Like the protein, Ldhc transcripts were found in mouse testis only after 11 days postpartum with the appearance of spermatocytes, and then further increased with the appearance of spermatids (Goldberg, 1990; Thomas et al., 1990; Alcivar et al., 1991). This specific time course expression pattern of Ldhc was confirmed by microarray studies in the Griswold laboratory (Figure 3; Shima et al., 2004). By using isolated testicular cell types, they were also able to confirm that the Ldhc gene is expressed first in leptotene-zygote spermatocytes, with the highest level of Ldhc mRNA being present in spermatids (Figure 4). Although Ldbh and Ldha transcripts were found in germ
cells, they were at considerably lower levels than *Ldhc*. *Ldhb* was found mostly in spermatogonia.

**Ldhc Gene: Transcriptional Regulation**—The human *LDHC* and murine *Ldhc* genes were cloned and sequenced (Millan et al, 1987; Cooker et al, 1993), and their promoter sequences were defined (Zhou et al, 1994). Transcriptional analysis of these and other genes expressed in germ cells is compromised by the lack of a reliable cell culture system. Nevertheless, we have made progress in elucidating the regulation of *Ldhc* gene expression. Our approaches, construction of transgenic mice (Li et al, 1998; Markert et al, 1998), transfection of heterologous cell systems, and injection of expression plasmids directly into seminiferous tubules via the rete testes have defined both *cis*- and *trans*-acting factors responsible for *Ldhc* transcription (Kroft et al, 2003; Tang et al, 2008; Tang and Goldberg, 2009). We confirmed and extended prior reports that transcription of the *Ldhc* gene begins with the onset of meiosis in male germ cells. The potential *cis*-regulatory elements identified in studies using transgenic animals include a palindrome (−21 to +10; PAL), GC box (−70 to −65), and CRE sites (−53 to −49, −39 to −35). We described a functional role for these sequences by expression of mutated transgenes in vivo. Our results (Tang et al, 2008) revealed for the first time that mutation of the GC box did not abolish promoter activity, which remained testis specific. Mutation of GC box or CRE sites resulted in a 73% or 74% reduction in promoter activity, respectively, in transient transfection of germ cells and in vivo by electroporation; the combination of GC box and CRE site mutations eliminated promoter activity, whereas mutation of PAL had no effect. Therefore, we concluded that simultaneous occupancy of the GC box and CRE sites in the core promoter was necessary for full expression of *Ldhc* in the testis. Our working hypothesis proposes a partnership between the Sp and CRE-binding protein (CREB) factors mediated by an activator protein. We
have been able to extend our studies to the human Ldhc promoter because it is activated in a malignant melanoma cell line. We determined that the murine and human promoters are only partially conserved, because the latter sequence lacks a consensus TATA element upstream of the transcriptional start site. However, regulatory factors are similar for the murine and human Ldhc promoters (Tang and Goldberg, 2009). These findings are significant for developing an understanding of gene regulation in the human testis. Human material would be difficult, if not impossible, to obtain for such research. Overall, it appears that the role of the CRE site is essential for spermatocyte-specific gene expression.

Ldhc Gene: Nontranscriptional Regulation—It has been demonstrated that the high level of Ldhc mRNA in germ cells is also the consequence of mRNA stability due to longer polyadenylation tails appearing with the meiotic divisions (Fujimoto et al, 1988). However, it seems that Ldhc mRNA stability is also species specific and dependent on the presence of an AU-rich motif in the 3′ untranslated region (Salehi-Ashtiani and Goldberg, 1995). This information leads one to wonder whether LDHC might be posttranscriptionally regulated by an inhibition of translation or an increase of mRNA stability by the new and exciting way of regulating transcript levels with noncoding RNAs as microRNAs.

In the last decade, the importance of epigenetic regulation has emerged, especially during the highly regulated process of spermatogenesis (Zamudio et al, 2008). Genes frequently are hypomethylated in the testis (Oakes et al, 2007). In earlier studies, no differences were found in the methylation patterns of the Ldhc gene between somatic and germ cells (Alcivar et al, 1991), but the technique of restriction analysis of DNA methylation overlooked many potential methylation sites (Alcivar et al, 1991). Other studies demonstrated that the human promoter contains a mini-CpG island (Bonny and Goldberg, 1995), and that its methylation in nonexpressing cells serves as the likely mechanism to suppress Ldhc gene activation (Tang and Goldberg, 2009).

Function(s) of Ldhc Revealed (or Not) by Its Absence

Male Germ Cell Metabolism: Why a Need for Special Glycolytic Isozymes?—LDHC was the first germ cell–specific glycolytic isozyme discovered, but subsequent studies revealed that it was not the only one. Two other genes encoding isozymes for glyceraldehyde-3-phosphate dehydrogenase type S (GAPDHS; Welch et al, 1992, 2000) and phosphoglycerate kinase type 2 (PGK2; Boer et al, 1987; McCarrey and Thomas, 1987) in mouse and human were found to be expressed only in spermatogenic cells. In addition, several glycolytic enzymes with unique properties were detected in male germ cells (Figure 5; reviewed in Miki, 2007).

Several studies have shown that meiotic and postmeiotic male germ cells preferentially use lactate and pyruvate over glucose as an energy substrate (reviewed in Jutte et al, 1982; Mita and Hall, 1982; Grootegoed et al, 1984; Boussouar and Benahmed, 2004), suggesting that lactate oxidation by LDH isozymes has a significant role in energy metabolism during the middle and later stages of spermatogenesis. Although these observations would fit with the abundance of LDHC in these stages of spermatogenesis, there is no direct evidence to support this contention. On the other hand, it has been shown that glycolysable substrates are essential for sperm motility (Williams and Ford, 2001; Mukai and Okuno, 2004), for the protein tyrosine phosphorylation indicative of capacitation (Travis et al, 2001; Urner et al, 2001; Williams and Ford, 2001; Urner and Sakkas, 2003), and for fertilization (Urner and Sakkas, 1996; Bone et al, 2000). This was proven when inactivation of the gene for the sperm-specific glycolysis pathway enzyme GAPDH (Miki et al, 2004) dramatically reduced the level of adenosine triphosphate (ATP) in sperm, caused severe defects in progressive sperm motility and resulted in male infertility.

These data were consistent with our hypothesis that LDHC is essential for lactate metabolism during spermatogenesis, and for the conversion of pyruvate to lactate accompanied by the generation of reduced NAD⁺ (coenzyme essential for GAPDHS activity) in spermatozoa.

How to Assess LDHC Function(s)?—One approach to assess LDHC function in spermatozoa was the use of an LDH inhibitor. In bovine sperm, O’Flaherty et al (2002) demonstrated that treatment with sodium ox-
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amante (NaOx; pyruvate analog and LDH inhibitor) suppressed sperm capacitation. Similar results were obtained with mouse sperm (Duan and Goldberg, 2003). However, in addition to concerns about the specificity of the inhibitor, we found that it inhibited LDH activity efficiently in protein extracts but not in intact sperm (Odet, unpublished observations). An alternative and ultimately successful approach was to assess LDHC function by targeted disruption of the Ldhc gene (Odet et al, 2008). Initial attempts were not successful, probably because of the abundance of repetitive sequence elements in the Ldhc gene (Olsson et al, 2003). However, when the Ldhc-null mouse was generated, it proved to be an exciting model of male infertility and a unique biologic resource that could lead to more complete understanding of the biochemical mechanisms underlying sperm function.

Ldhc-Null Mice and Spermatogenesis—Our first observation was that spermatogenesis appeared normal in Ldhc-null mice. The testis histology looked unaffected, and daily sperm production and sperm counts in the cauda epididymis were normal. To identify potential transcriptional modifications in Ldhc-null testis, a microarray was performed in collaboration with Dr Michael Griswold (Washington State University, Pullman, Washington). However, preliminary results indicate that only Ldhc transcript levels were significantly different between wild-type and null testes by this method and by real-time polymerase chain reaction (Odet, unpublished observations). Although these results suggest that LDH-C4 does not play an essential role in the maintenance of spermatogenesis, they do not rule out that the absence of LDH-C4 has a subtle effect on spermatogenesis that subsequently compromises sperm function.

Ldhc-Null Male Mice and Fertilization—Although spermatogenesis appeared normal, the fertility of Ldhc-null males was severely compromised. Our initial study was performed with animals from the F(3) and F(5) back-cross generations on the C57Bl/6N background (predicted 12.5% and 3.13% residual 129svEv background, respectively). From a total of 11 different Ldhc-null males mated with more than 44 wild-type females over a 2- to 4-month period, only 2 litters of 1 and 3 pups were recovered (Odet et al, 2008).

Other studies demonstrated that this extreme subfertility was a consequence of a defect(s) in sperm function. Motility of Ldhc-null sperm was impaired, and their progressive motility decreased over time, suggesting that Ldhc-null sperm are unable to swim through the female tract (Odet et al, 2008). Moreover, even if Ldhc-null sperm were able to reach the eggs, in vitro fertilization experiments indicated that they were unable to fertilize oocytes. Ldhc-null sperm did not undergo the protein tyrosine phosphorylation changes characteristic of capacitation and were unable to develop hyperactivated motility, processes essential for fertilization. When the zona pellucida was removed, Ldhc-null sperm fertilized 29% of eggs. This was a significantly lower percentage of fertilization than occurred with sperm from heterozygote mice, suggesting that in addition to a defect in the mechanical process of oocyte penetration through the zona pellucida, there is also a defect in fusion to oocytes by Ldhc-null sperm.

Ldhc-Null Mice and Sperm Metabolism—Although it was clear that sperm defects were responsible for the infertility, the causes of the defects remained to be determined. A high rate of ATP production in sperm is known to be essential for maintaining a high level of motility for a prolonged period of time, as well as to induce sperm capacitation and hyperactivity. We hypothesized that lack of LDHC would induce a defect in ATP production by inhibition of glycolysis, the principal source of ATP in most mammalian sperm. Consistent with this hypothesis, we found rapid decreases in progressive motility and ATP levels over time in Ldhc-null sperm rather than in wild-type sperm (Odet et al, 2008). By following the transformation of 13C-labeled glucose using nuclear magnetic resonance (NMR) spectroscopy, we found that unlike wild-type sperm, Ldhc-null sperm were unable to consume glucose (Odet, unpublished observations). However, by using direct (consumption of 13C-pyruvate, NMR spectroscopy) and indirect methods (amount and rate of lactate production from pyruvate), we also observed an unexpected result: Ldhc-null sperm were able to convert pyruvate into lactate with the same rapid kinetics as WT sperm (Odet, unpublished observations). Because LDHA has been found in association with the isolated fibrous sheath, these results strongly suggest that LDHA is responsible for some or most of the LDH activity in Ldhc-null sperm.

It is worth mentioning that sperm from mice heterozygous for the Ldhc mutation were fertile, whereas Ldhc transcript levels were reduced by 40% in the testes of these mice, and global LDH activity was reduced by 19.1% in testis and 24.7% in sperm. These observations indicate that sperm contain substantially more LDH-C4 than is required to maintain normal fertility.

Conclusion

Finding that the function of sperm from Ldhc-null mice is severely compromised confirms the importance of this isozyme and strongly justifies further study of the role it plays in conferring or maintaining sperm-fertilizing capacity. As has been noted previously, these studies are of translational relevance to problems of male
infertility (Gavella and Cvitkovic, 1985; Orlando et al., 1988) and to the development of a male contraceptive (Goldberg, 1973; Erickson et al., 1975b; Mahi-Brown et al., 1990; O’Hern et al., 1995; Goldberg et al., 2001).

Therefore, we still must ask why sperm contain such a large quantity of LDHC. What could be the function of LDHC if LDH-A alone can provide the terminal reaction of glycolysis? Many questions about LDHC remain to be elucidated.

Acknowledgment

We thank Dr Michael Griswold for providing Figures 3–5.

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