The Fatty Liver Dystrophy (fld) Mutation

A NEW MUTANT MOUSE WITH A DEVELOPMENTAL ABNORMALITY IN TRIGLYCERIDE METABOLISM AND ASSOCIATED TISSUE-SPECIFIC DEFECTS IN LIPOPROTEIN LIPASE AND HEPATIC LIPASE ACTIVITIES*

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An autosomal recessive mutation, termed fatty liver dystrophy (fld), can be identified in neonatal mice by their enlarged and fatty liver (Sweet, H. O., Birkenmeier, E. H., and Davisson, M. T. (1988) Mouse News Letter 81, 69). We have examined the underlying metabolic abnormalities in fld/fld mice from postnatal days 3–40. Serum and hepatic triglyceride levels were elevated 5-fold in suckling fld/fld mice compared to their +/− littermates but abruptly resolved at the suckling/weaning transition. In contrast, hepatic lipase (HL) mRNA levels and activity were significantly reduced in fld/fld livers and sera, respectively, during the suckling period. Mapping studies show the fld locus to be distinct from loci encoding LPL, HL, and apoA-IV, and those responsible for the combined lipase deficiencies in cld/cld and W/W′ mice. These data suggest that the fld mutation is associated with developmental programmed tissue-specific defects in the neonatal expression of LPL and HL activities and provide evidence for a new regulatory locus which affects these lipase activities. This mutation could serve as a useful model for (i) analyzing the homeostatic mechanisms controlling lipid metabolism in newborn mice and (ii) understanding and treating certain inborn errors in human triglyceride metabolism.

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Immediately after birth, suckling rats and mice must be able to effectively utilize dietary triglycerides (97% of rat milk lipids) to meet their energy requirements (Fernando-Warnakulasuriya et al., 1981). At the suckling/weaning transition (postnatal days 13–15) they must begin to accommodate a low fat high carbohydrate chow diet. These developmental changes are mediated by a complex genetic program which is activated prior to parturition and involves genes encoding extracellular and intracellular lipid transport proteins as well as lipid-modifying enzymes that are expressed in several tissues. Factors which induce and modulate these developmental adaptations have been difficult to define, largely because of the absence of any well defined model system. To date, analyses have been limited mainly to cataloging ontologic changes in specific mRNA levels and the concentrations of their primary translation products. These studies have occasionally provided clues about the functions of the gene products. For example, induction of apolipoprotein (apo)1 A-IV mRNA accumulation in both rat intestine and liver coincides with their ability to export triglyceride, suggesting a potential role for this apolipoprotein in assembly and/or secretion of triglyceride-rich lipoproteins (Frost et al., 1983; Elshourbagy et al., 1985).

There are two naturally occurring mutations in mice that are associated with profound disturbances in triglyceride metabolism. These affect (i) lipoprotein lipase (LPL), the enzyme responsible for hydrolysis of core triglycerides present in chylomicrons and very low density lipoproteins (VLDL) and (ii) hepatic lipase (HL), which is postulated to participate in the hydrolysis of phospholipids and triglycerides in high and intermediate density lipoproteins (Landin et al., 1984; Kuusi et al., 1979; Jansen et al., 1980; Van Tol et al., 1980; Murase and Itakura, 1981; Grosse et al., 1981; Goldberg et al., 1982). The cld/cld mouse has a combined deficiency of HL and LPL. The cld mutation resides within the T/t complex of mouse chromosome 17 (Paterniti et al., 1983), while the structural genes for these lipases have been located on chromosomes 8 (LPL) and chromosomes 9 or 11 (HL) (Kirschberger et al., 1989). If allowed to suckle, homozygous cld/cld mice do not survive beyond the third postnatal day (Paterniti et al., 1983). This mutation is therefore not useful for analyzing the influence of these lipases on lipid metabolism through the suckling and weaning periods. Hyperlipidemic W/W′ mice have reduced activities of lipoprotein lipase and hepatic lipase

1 The abbreviations used are: apo, apolipoprotein; LPL, lipoprotein lipase; HL, hepatic lipase; VLDL, very low density lipoproteins; HPLC, high pressure liquid chromatography.
in postheparin plasma but normal tissue activity (heart, adipose, and liver). These mice lack mast cells suggesting that the associated heparin deficiency may contribute to the hypertriglyceridemia by affecting targeting of these lipases to endothelial surfaces (Hatanaka et al., 1986). The consequences of this mutation on lipid processing during the neonatal period have not been documented.

We present here the initial biochemical characterization of a recently identified mouse mutant (Sweet et al., 1988). Homozygotes for this autosomal recessive mutation, known as fatty liver dystrophy (fld), have severe hypertriglyceridemia which resolves at the suckling/weaning transition. An associated tissue-specific deficiency in the activity of hepatic and lipoprotein lipase coincides with this developmentally programmed hypertriglyceridemia.

**EXPERIMENTAL PROCEDURES**

**Animals**

All mice were produced and reared in the Mouse Mutant Resource colony at The Jackson Laboratory. Mutant fld/fld mice were distinguished from their +/+ littermates during the first postnatal day by their swollen abdomens and large pale livers. Confirmation of phenotype was based on subsequent growth retardation, characteristic hair changes (reduced coat growth at 7 days, unkempt and ruffled appearance by 6 weeks) as well as neurologic symptoms (see "Results"). Pregnant and lactating females as well as their weaned offspring were fed The Jackson Laboratory's standard 9G6 chow diet. All mice were maintained under strict light cycling conditions (lights on from 0600 to 1800 h).

**Measurement of Serum and Tissue Lipids**

Triglyceride and cholesterol levels were determined in sera obtained from fld/fld mice and their age-matched +/+ siblings using Wako triglyceride G and cholesterol C colorimetric assay kits (Wako Pure Chemical Industries, Ltd.). Sera were obtained at the time of death (1000-1400 h) from littermates (n = 1-7/time point).

Total lipids were extracted from the mouse livers (Bligh and Dyer, 1959) and then fractionated into neutral and polar lipids by Unisil gel thin layer chromatography (Christie, 1982). Neutral lipids were further analyzed by thin layer chromatography employing a benzenecidiethyl ether:ethyl acetate:acetic acid (80:10:2) solvent system (Storry and Tuckley, 1967). Two-dimensional thin layer chromatography of phospholipids was performed using a chloroform:methanol:acetic acid (65:35:5) solvent system in the first dimension and a chloroform:methanol:formic acid (70:25:5) system in the second dimension (Esko and Raetz, 1980). Lipids were visualized after spraying silica gel thin layer chromatography plates with 50% sulfuric acid and charring (Marsh and Weinstein, 1966).

**Plasma Lipoprotein Analysis**

Plasma was isolated from the blood of 10- to 15-day-old fld/fld and +/+ littermates at the time of death (1000-1400 h). Lipoprotein species in 10-μl aliquots of plasma were characterized by agarose gel (0.5% w/v) electrophoresis and by HPLC (Carroll and Rudel, 1983), as described in Daugherty et al. (1988).

**Analysis of Serum Apolipoprotein A IV by Two-dimensional Gel Electrophoresis and Western Blotting**

Sera (0.5 μl) from fld/fld mice and their +/+ littermates were subjected to two-dimensional gel electrophoresis (Speicher et al., 1984) followed by electrophoretic transfer to nitrocellulose filters (Burnette, 1981). Blots were pretreated with Blotto buffer (5% (w/v) nonfat dry milk, 150 mM NaCl, 10 mM Tris (pH 7.5), 0.03% antifoam A (Sigma), 0.1% sodium azide, 0.04% Tween 20 (polyoxyethylene sorbitan monolaurate)) and then incubated with a polyclonal monospecific rabbit anti-rat apoA-IV serum (Apfelbaum et al., 1987) diluted 1:4000 in Blotto (Johnson et al., 1984). Antigen-antibody complexes that had formed after hybridization at room temperature were visualized using 125I-protein A and subsequent autoradiography (Burnette, 1981; Green et al., 1988).

**RNA Blot Hybridization Analysis**

Heart, small intestine, and liver were harvested from individual fld/fld mice and their +/+ littermates at various ages of postnatal life. Total cellular RNA was extracted with guanidine thiocyanate (Chirgwin et al., 1979). RNA integrity was assessed by electrophoresis through denaturing methylmercury/agarose gels (Bailey and Davidson, 1976). RNA dot blots were constructed as previously described (White and Bancroft, 1982; Levin et al., 1987). Filters contained four amounts of each tissue RNA sample (0.5, 1, 2, and 5 μg). Yeast tRNA was added to each tissue RNA sample prior to denaturation to ensure that each dot blot also contained 0.1 3.0 ng of liver RNA prepared from 7-day-old fld/fld and +/+ mice as external standards. Northern blots of total cellular RNA were prepared following formaldehyde-agarose gel electrophoresis (Thomas, 1980).

cDNAs encoding rat apoA-I (Boguski et al., 1985), rat apoA-IV (Boguski et al., 1984), rat apoE (Luiss et al., 1985), mouse apoE, all dots on a filter contained a total of 3 ng of RNA. Each dot blot also contained 0.1 3.0 ng of liver RNA prepared from 7-day-old fld/fld and +/+ mice as external standards. Northern blots of total cellular RNA were prepared following formaldehyde-agarose gel electrophoresis (Thomas, 1980).

**Measurement of Lipase Activities**

All tissue samples were frozen immediately after dissection and stored at −80 °C until assayed. Tissues from homozygous mutant and their age-matched +/+ littermates were dissected and suspended in 0.25 M sucrose, 10 mM Tris (pH 7.5), 0.1 mM phenylmethylsulfonyl fluoride) as follows: 10% w/v for heart and liver, 5% for brown adipose tissue, and 2% for white adipose tissue. Assays for LPL were performed as described by Nilsson-Ehle and Schotz (1976). The assay for serum HL (Two et al., 1984) was carried out at 1 mg NaCl to ensure inhibition of any LPL activity. All homogenates were diluted at least 20-fold in the assay system to reduce Triglyceride X-100 interference. Protein concentration was estimated by the method of Bradford (1976). Each sample was assayed in duplicate and results expressed as milliunits/mg protein (for tissue samples) or milliunits/ml (for serum samples). One milliunit of LPL or HL = release of 1 mmol of oleic acid/min at 37 °C.
The Fatty Liver Dystrophy (fld/fld) Mouse

Measurement of L-Glycerol-3-phosphate Dehydrogenase Activity in White and Brown Adipose Tissue

Adipose tissue was dissected from the epidydimal fat pads and interscapular brown fat of fld/fld and +/+ littermates at various stages of development. Samples were immediately frozen and stored at -80°C until assayed. Frozen tissue was pulverized and then homogenized in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 1 mM β-mercaptoethanol (5 μl of buffer/mg of tissue (white adipose tissue) or 10 μl of buffer/mg of tissue (brown adipose tissue)). The assay of L-glycerol-3-phosphate dehydrogenase activity has been previously described (Kozak and Jensen, 1974). Protein concentrations were determined by the method of Lowry et al. (1951). Samples were assayed in duplicate and results expressed as μg of protein/mg.

Dietary Studies

Prolonged Suckling Experiment—Beginning at 1 week of age, a litter of fld/fld and +/+ mice and their mother were transferred to a clean cage lacking chow. To allow the mother access to chow, she and a normal unrelated postpartum female were exchanged every 12 h between this first cage containing the “mutant” litter and a second cage containing an age-matched litter of normal unrelated mice plus chow. Mutant and normal littermates were killed at postnatal days 11, 15, and 20.

Effects of High Fat Diet—Young adult (4-6-week-old) fld/fld mice and their +/+ littermates were given, for 2 weeks, a high fat diet made by modifying an American Institute of Nutrition semi-synthetic diet so that the sucrose contribution was decreased to 41% and fat was elevated to 30% (of calories) by the addition of Crisco. A control group of fld/fld and +/+ mice from the same litter were kept on the standard 96W chow diet. Sera were collected at the time of death for quantitation of cholesterol and triglyceride concentrations. Total cellular RNA was prepared from intestine and liver to determine the levels of apoA-IV and apoC-II expression.

Statistical Analyses

Data from age-matched groups of fld/fld and +/+ animals were analyzed by the Student’s t test. A t value greater than to.06 was considered significant (p < 0.06).

Chromosome Mapping Studies

Linkage analysis is being done by crossing fld/fld females to linkage testing stocks carrying specific marker loci or inbred strains differing at several polymorphic loci. F1 mice heterozygous for fld and multiple marker and polymorphic loci are crossed to fld/fld females (backcross) or fld/+ males (partial backcross). All progeny are scored for segregating visible markers, and fld/fld mutants are typed for polymorphic markers.

RESULTS

Identification and General Description of the Mutant—Fatty liver dystrophy (fld) is a recessive spontaneous mutation that arose in the inbred BALB/cByJ strain in the Animal Resources colony of The Jackson Laboratory in 1981. The mutant phenotype can be identified soon after birth because mice homozygous for this autosomal recessive mutation have swollen abdomens and pronounced hepatomegaly. The liver has a characteristic pallor. These features of the mutant phenotype can be identified soon after birth because mice homozygous for this autosomal recessive mutation have swollen abdomens and pronounced hepatomegaly. The liver has a characteristic pallor. These features of the mutant phenotype disappear by the time the mutant is approximately 28 days of age. By 3 days of age the mutant (fld/fld) is noticeably smaller in size than its normal littermates, and growth of the mutant remains retarded throughout life. Hair growth is retarded beginning at 7 days of age. The coat of the homozygous fld mouse at 6 weeks of age appears unkempt and ruffled, while the coat of the normal littermate appears smooth and sleek. By the 14th postnatal day, a generalized tremor and unsteady gait develop and progress slowly throughout life. When held up by the tail, the mutant clenches the toes of its rear feet and attempts to clasp its hind legs together. Many homozygotes die between the 19th and 35th postnatal days. Survival of the homozygote is not enhanced by culling the litter. Survival of the homozygote is not enhanced by culling the litter. Male infertility may be a result of behavioral impairment.

Genetic Characterization of the fld Mutation—Fatty liver dystrophy (fld) is inherited as a recessive mutation. Homozygous fld/fld females mated to homozygous normal (+/+ male) produce all normal progeny. Matings between the resulting heterozygotes (+/fld) produce normal and affected progeny in a 3:1 ratio as expected for a recessive gene. Results are summarized in Table I. The gene is not sex-linked. In all crosses the male sex ratio is 47% and is in concurrence with the male sex ratio of 46.4% given for the reproductive performance of the BALB/cByJ inbred strain (Les, 1982). Linkage test crosses using known gene markers on each autosomal chromosome have thus far failed to identify the chromosomal location of the fld locus. Further linkage tests are in progress since the entire length of each chromosome has not been examined to date.

fld/fld Mice Develop Hypertriglyceridemia during the Suckling Period—Sera from fld/fld mice and their phenotypically normal +/+ littermates were analyzed at various stages of postnatal development for triglyceride and cholesterol levels. Paired normal and mutant animals were bled at the same time to reduce variations arising from different feeding schedules. Sera from preweaning stage fld/fld mice were strikingly more turbid and milky in appearance than those obtained from their +/+ siblings. The serum triglyceride concentrations of fld/fld animals were markedly elevated throughout the suckling period, reaching levels as high as 1000 mg/dl. These concentrations were 2-6 times higher than those of their unaffected littermates (panel A of Fig. 1). In contrast, choles-

![Fig. 1. Concentration of serum triglyceride and cholesterol in fld/fld and +/+ mice. Sera from age-matched fld/fld and +/+ littermates were obtained at the time of death. When more than one animal was analyzed at a particular time point, data are presented as the average (+1 S.D.) for sera (n = two to seven animals). Adult mice were 4-6 weeks old. Concentrations of triglyceride (panel A) and cholesterol (panel B) are expressed as mg/dl serum.](image-url)
terol levels were relatively less elevated (<2-fold) in fdl/fdl mice during this period (panel 8). The hypertriglyceridemia in fdl/fdl mice resolved abruptly at the suckling/weaning transition (days 13–15). Differences between +/− and fdl/fdl littermates entirely disappear before weaning is completed. Adult fdl/fdl and +/− littermates consuming a standard chow diet have indistinguishable serum triglyceride levels (Fig. 1).

Samples of +/− and fdl/fdl plasma were analyzed to determine which lipoprotein species contained the vast excess of triglyceride noted in affected animals. The plasma lipoprotein profile of 10-day-old fdl/fdl mice as defined by agarose gel electrophoresis indicated that these animals had an under-representation of high density lipoproteins but an apparent overabundance of VLDL or chylomicron remnants compared to their +/− littermates (data not shown). To further differentiate between these two possibilities, the samples were fractionated by HPLC. Plasma from fdl/fdl mice contained a dominant large lipoprotein species whose elution profile was consistent with that of chylomicron remnants. The concentration of this lipoprotein species was 15–30-fold higher than in +/− plasma (data not shown).

To determine which cell type in the liver contained excess triglyceride, tissue sections were prepared from normal and affected animals and stained with Oil Red O. Huge lipid droplets were observed within the hepatocytes of 12-day-old fdl/fdl mice. No other cell types appeared to be affected. These changes were not present in the hepatocytes of phenotypically normal +/− littermates (data not shown). Hematoxylin- and eosin-stained sections did not reveal any inflammatory infiltrate or fibrosis associated with the triglyceride overload. Light microscopic analysis of 5–10-μm sections of adult (65–day-old) fdl/fdl and +/− mice failed to disclose any pathologic changes, suggesting that the massive triglyceride accumulation observed in the early postnatal period did not have any lasting effects on hepatic architecture.

Expression of Apolipoprotein and Cellular Fatty Acid Binding Protein Genes in fdl/fdl Mice and Their +/− Littermates—To understand the basis for this neonatal hyperlipidemia, the patterns of accumulation of a number of mRNAs, encoding proteins which participate in lipid transport and modification, were examined. Cloned cDNAs were used to probe dot blots containing samples of total cellular RNA prepared from the livers and small intestines of age-matched fdl/fdl and +/− mice. Concurrent with the fall in serum triglyceride concentrations at the suckling/weaning transition, hepatic apoA-IV mRNA levels in fdl/fdl mice began to decrease, reaching concentrations comparable to those in their normolipidemic littermates during the weaning period (panel A). These differences in apoA-IV mRNA levels were tissue-specific. No significant differences in small intestinal apoA-IV mRNA concentrations were noted between fdl/fdl and +/− littermates at any of the 11 different postnatal time points surveyed (panel B of Fig. 3). This was apparent at postnatal day 3, the earliest time point assayed. Levels rose to a peak of 25 pg/pg of total cellular RNA during the second postnatal week. This value is ~100 times greater than that documented for age- and/or gender-matched +/− littermates.

There was no evidence that the fdl/fdl apoA-IV mRNA or its primary translation product was abnormal. Northern blot hybridization analysis of RNAs prepared from suckling and weaning fdl/fdl and +/− mouse liver and intestine revealed a single ~1500-nucleotide-long mRNA that was similar in size

FIG. 2. Developmental patterns of accumulation of specific mRNA in the liver and intestine of fdl/fdl and +/− mice. Dot blots containing four different amounts of each total cellular RNA sample prepared from the (pooled) livers or intestines of age-matched fdl/fdl or +/− littermates (n = one to five normal or affected mice/time point) were probed with several cDNAs encoding proteins involved in lipid metabolism. Relative mRNA concentrations in a given tissue are expressed as arbitrary densitometric units. These units are not comparable between probes and thus cannot be used to determine the relative abundance of the different mRNAs in a given sample of liver or intestinal RNA. L-FABP, intestinal fatty acid binding protein; L-FABP, liver fatty acid binding protein.

(Reviewed in Sweetser et al., 1987).

By contrast, profound elevations of apoA-IV mRNA levels were observed in the livers of fdl/fdl mice (panel A of Fig. 3). This was apparent at postnatal day 3, the earliest time point assayed. Levels rose to a peak of 25 pg/pg of total cellular RNA during the second postnatal week. This value is ~100 times greater than that documented for age- and/or gender-matched +/− littermates. Concurrent with the fall in serum triglyceride concentrations at the suckling/weaning transition, hepatic apoA-IV mRNA levels in fdl/fdl mice began to decrease, reaching concentrations comparable to those in their normolipidemic littermates during the weaning period (panel A). These differences in apoA-IV mRNA levels were tissue-specific. No significant differences in small intestinal apoA-IV mRNA concentrations were noted between fdl/fdl and +/− littermates at any of the 11 different postnatal time points surveyed (panel B of Fig. 3).

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in all samples. In addition, sera from 7- and 24-day-old unaffected and affected animals were fractionated by two-dimensional gel electrophoresis and the proteins transferred to nitrocellulose filters. When these Western blots were probed with a polyclonal monospecific rat apoA-IV antibody, no differences in the size or pl of this apolipoprotein could be demonstrated between fld/fld mice and their +/− littermates (data not shown). Furthermore, single dimension Western blots of sera prepared from 7-, 10-, 15-, and 24-day-old animals and liver homogenates from 9-day-old mice indicated that there were less than 2-fold differences in the steady state levels of apoA-IV between fld/fld and +/− mice (data not shown).

In addition to the abnormal developmental pattern of apoA-IV mRNA accumulation found in mice homozygous for the fld mutation, expression of the apoC-II gene also appeared to be affected. A progressive rise in hepatic apoC-II mRNA concentrations was documented in fld/fld mice that peaked just prior to the suckling/weaning transition (postnatal day 10 in panel C of Fig. 3). ApoC-II mRNA concentrations subsequently fell in these mice during the weaning period. This "profile" contrasts with the pattern observed in their +/− littermates. In these normolipidemic mice, liver apoC-II mRNA levels decreased slightly (~2-fold) from the early suckling period to the end of the weaning. The net result of these contrasting ontologic changes is that by the 10th postnatal day, there is a ~7-fold "elevation" in apoC-II mRNA in the livers of fld/fld mice. As with apoA-IV mRNA, this difference is tissue-specific with only modest differences noted in the intestines of normal and affected littermates (compare panels C and D of Fig. 3).

The Hypertriglyceridemia and Abnormalities in ApoA-IV and ApoC-II mRNA Accumulation during Development Appear to Be Genetically Programmed—To determine what effect dietary lipids had in establishing and perpetuating the hypertriglyceridemia observed in fld/fld mice, two experiments were performed. The first experiment was designed to examine whether the triglyceride elevation could be elicited in adult fld/fld mice by switching them from a low fat high carbohydrate chow diet to one highly enriched in triglycerides. Four- to six-week-old fld/fld mice and their normal +/− littermates were placed on a high fat diet (see "Experimental Procedures") or maintained on a standard rodent chow for 2 weeks. At the conclusion of this dietary manipulation, no significant differences in serum triglyceride or cholesterol concentrations were noted among any animals on either diet (data not shown). Liver apoA-IV mRNA levels were doubled by the high fat diet, but there was no discernible difference between fld/fld and their +/− littermates (data not shown). These results suggested that neither the hypertriglyceridemia nor the profound changes in liver apoA-IV mRNA concentrations could be reinduced in adult fld/fld mice by increasing the lipid content of the diet.

A complementary experiment was designed to determine if prolongation of the suckling phase could perpetuate the elevation in serum triglyceride levels in fld/fld mice beyond the 13th–15th day when it "normally" begins to resolve. fld/fld mice and their +/− littermates were either maintained solely...
on mother's milk for up to 20 days after birth using the protocol described under “Experimental Procedures” or they were weaned in the normal fashion. Prolongation of suckling was achieved by a regular (every 12 h) exchange of the dam and a lactating foster mother that also had a litter of similar size and age. Since the rotating mothers were housed in separate cages and only the cage with the “normal” litter contained chow, we could be assured that the litter with mutant fld/fld mice had no access to chow. The feeding behavior of the pups did not seem to be affected by the exchange of lactating females. Animals were killed at the 11th, 15th, and 20th postnatal day. Prolonged suckling did not perpetuate hypertriglyceridemia nor the relative increases in hepatic apoA-IV or apoC-II mRNA concentrations (Fig. 4). In fact, prolongation of suckling appeared to cause an earlier resolution of the increase in apoA-IV mRNA levels so that by day 20, its concentration in fld/fld mice and their +/+ littermates was similar (in contrast to the 17-fold differences between these groups when they were weaned normally). Together these data support the notion that resolution of these markers of the fld phenotype (increased serum triglycerides, increased liver apoA-IV, and apoC-II mRNA concentrations) is genetically programmed and not dependent upon (i) exposure to the high concentrations of triglycerides in mother's milk, (ii) the act of suckling itself, or (iii) any other component transferred via mother's milk.

**Analysis of Lipid-modifying Enzymes in fld/fld and +/+ Mice**—ApoC-II is a cofactor of lipoprotein lipase (Havel et al., 1970; LaRosa et al., 1970; Nilsson-Ehle et al., 1980). Human patients with true LPL enzyme deficiency have elevated plasma apoC-II levels (Nikkilä, 1983). Hence, the elevations in serum triglycerides and apoC-II mRNA levels raised the possibility that the fld mutation reflected, at least in part, abnormalities in the transcription, synthesis, targeting, or structure of lipoprotein lipase or hepatic lipase. Cloned cDNAs encoding LPL and HL were therefore used for blot hybridization analysis of liver and heart RNAs prepared from normal and affected mice at various stages of postnatal development (Fig. 5). The levels of both mRNAs in liver appeared to be approximately 2-fold lower in fld/fld mice compared to their littermates throughout the suckling period (panels A and C). The differences in liver HL but not LPL mRNA concentrations appear to resolve prior to weaning. Interestingly, homozygous fld mice manifest persistently lower levels of hepatic LPL mRNA until the midweaning period at which time they abruptly increase so that their concentrations exceed that of +/+ littermates (by 6-fold on day 20, 2-fold on postnatal day 23, and ~50% higher by day 24, see Fig. 5A). Only modest (<2-fold) differences in heart LPL mRNA accumulation were noted between mutant and normal animals at postnatal days 9, 10, 17, 22, and 25 (Fig. 5B).

LPL activity was then measured in liver plus several tissues known to have high levels of this lipase (i.e. heart, brown and white adipose tissue). Developmental studies were done on several different litters at various postnatal stages, allowing an analysis of two to six mutant or +/+ littermates/time point. The results are summarized in Fig. 6. No statistically significant differences were noted in the activity of this lipase in the livers of 7–40-day-old fld/fld and their +/+ littermates. LPL activity in heart was slightly lower in 7-, 10-, and 11-day-old fld/fld mice compared to their phenotypically normal littermates with the difference at days 10 (p < 0.05) and 11 (p < 0.01) being statistically significant. These differences were not noted in the hearts of young adult (34–40-day-old) mice. No differences in LPL activities were noted in brown adipose tissue harvested from 10-, 11-, and 27-day-old or young adult fld/fld mice and their +/+ littermates.

A dramatic, albeit transient, difference in LPL activity was noted between the epididymal fat pads of suckling fld/fld and +/+ mice. The enzyme’s activity is 16-fold lower in 7-day-old homozygous mutant compared to normal mice. At 10–11 days of age, LPL activity is 7 times lower (p < 0.001). Interestingly, these differences have resolved by postnatal days 17–18.

The epididymal fat pads of suckling fld/fld mice appeared to be deficient in fat, e.g. their weight in 10–11-day-old animals was ~17-fold less in homozygotes compared to +/+ littermates while their total protein content was ~3-fold greater. This reflects a diminished fat/protein ratio in their adipocytes. Due to the very small size of this white adipose tissue in sucking fld/fld mice, we have not been able to examine LPL mRNA levels. The pronounced deficiency in LPL activity could reflect a decrease in the activity of the enzyme/adipocyte. (This in turn could be due to alterations in LPL gene transcription, mRNA stability, or a post-translational effect.) Alternatively, the decrease in enzyme activity could reflect a “relative” decrease in the total number of adipocytes in the epididymal fat pads of fld/fld mice. L-Glycerol-3-phosphate dehydrogenase was used as an independent marker of normal adipocyte development and function to distinguish between these two possibilities. The specific activities of L-glycerol-3-phosphate dehydrogenase in epididymal fat pads harvested from 10–15-day-old fld/fld and

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**Fig. 4. Effects of the prolonged suckling experiment on serum lipid and hepatic apolipoprotein mRNA levels.** See “Experimental Procedures” for a description of the experimental design. The number of animals examined at each time point in each treatment group is shown in parentheses above each bar unless only one animal was used. When n ≥ 2, an average value (+S.D.) is given. PS, prolonged suckling.
Although hepatic lipase is synthesized in mouse liver, it appears to be rapidly secreted into the circulation. Therefore, unlike rat and human subjects, it is abundant in preheparin serum (Peterson et al., 1986). Fig. 7 shows that sera from 7- and 9-day-old fld/fld mice have HL activity levels which are ~6-fold lower than their normal littermates (p < 0.001). This difference resolves by the 16th postnatal day; no significant differences in serum HL activity were noted between fld/fld mice and their +/− littermates between days 16 and 30.

Neither the LPL, HL, or ApoA-IV Locus Appear to Be Altered in fld/fld Mice—The chromosome mapping studies described under “Experimental Procedures” indicate that it is unlikely that fld is a mutation in one of the LPL, HL, or ApoA-IV structural loci. Recently, the LPL locus has been mapped to chromosome 8 in mice where it is closely linked to the apoA-IV structural loci. Recently, the LPL locus has been mapped to chromosome 8 in mice where it is closely linked to the apoA-I/A-IV/C-I11 gene cluster. A linkage test with apoA-I (Apoa-1) is in progress to verify this negative data. Using a limited panel of somatic cell hybrids, Kirchgessner et al. (1989) have recently localized the mouse HL gene to mouse chromosome 9 or 11. The fld mutation is not linked to oligosyndactyly (os) on proximal chromosome 8, indicating that the fld mutation does not correspond to the structural gene encoding LPL. Negative linkage data with short ear (se) on chromosome 9 suggest that fld is not near the apoA-I/A-IV/C-III gene cluster. A linkage test with apoA-I (Apoa-1) is in progress to verify this negative data. Using a limited panel of somatic cell hybrids, Kirchgessner et al. (1989) have recently localized the mouse HL gene to mouse chromosome 9 or 11. The fld mutation is not linked to se or transferrin (Trf) on chromosome 9 or to four loci on chromosome 11 (hemoglobin α, Hba; waved-2, wa-2; rex, Re; esterase-3, Es-3).

DISCUSSION

We have studied a newly described mutant strain of mice with an autosomal recessive mutation, termed fatty liver dystrophy, that is characterized by a transient neonatal hypertriglyceridemia and a fatty enlarged liver. Tissue-specific reductions in lipoprotein lipase activity as well as a decrease in hepatic lipase may play important pathogenetic roles in this congenital abnormality. fld/fld mice and their phenotypically normal +/− littermates provide a potentially powerful model system for (i) analyzing the homeostatic mechanisms...
which exist in newborn mice to deal with the massive quantities of dietary triglycerides normally delivered during early postnatal life and (ii) understanding and treating certain inborn errors in human lipid metabolism characterized by hypertriglyceridemia.

Newborn rodents process enormous amounts of exogenous triglyceride. The diet of the suckling animal contains 10–20 times more fat than the adult (Frost et al., 1983). The concentration of triglyceride-rich lipoproteins in their lymph is 100 times higher than in plasma. Nonetheless, the plasma lipoprotein compositions of suckling and adult rats are remarkably similar (Fernando-Warnakulasuriya et al., 1983) reflecting at least in part the remarkably rapid clearance of plasma triglycerides in the neonate (t<sub>1/2</sub> = 1.4 min (Frost et al., 1983)). Such efficient processing of triglycerides is an essential metabolic capability not only because of the massive amounts presented to the gut epithelium but also because of the functional immaturity of the neonatal digestive system (Henning, 1987). Pancreatic lipase activity is low prior to the completion of weaning (Snook, 1971; Deschot-Lanckman et al., 1974), and the sucking rat or mouse must apparently rely on lingual lipase, an enzyme which is released from the serous glands of the tongue and is active in the stomach, for limited hydrolysis of milk lipids (Hamosh, 1979; Staggers et al., 1981; DeNigris et al., 1988). In addition, the neonatal rodent small intestine has enhanced permeability (Henning, 1987). Intact globules of milk lipids are absorbed by pinocytosis giving rise to large lipoprotein particles in enterocytes (Berendsen and Blanchette-Mackie, 1979; Mak and Trier, 1979). Frost et al. (1983) have shown that because of the ineffective secretion of very low density lipoproteins by the liver, nearly all triglycerides found in suckling rat plasma are derived from this intestinal input. As the animal enters the sucking/weaning transition (days 13–15), an “adult” micellar mode of lipid absorption becomes active, fewer large lipoprotein particles are found in enterocytes (Mak and Trier, 1979), and the export of triglyceride-rich VLDL from the liver commences (Mahley et al., 1984).

As noted in the Introduction, while this general scheme for processing of dietary triglyceride in the neonatal rodent is known, little information is available about the mechanisms/factors which regulate the developmental metabolic program, due in part to the lack of mutations that affect one or more sites of triglyceride processing active during the neonatal period. The fld mutation described in this paper represents a prototype of such a class of naturally occurring developmental mutations.

Several general hypotheses can be derived to explain the observed developmental abnormality in suckling fld/fld mice. The mutation could affect a function that is only expressed from birth through the sucking/weaning transition. In this case, the congenital defect would not be encountered in adult animals because the function is not expressed after this developmental period. Alternatively, this functional abnormality could persist throughout life but is masked because of the induction of another gene(s) (or a modification of the original defective gene product) at the sucking/weaning transition. These other adult genes could either be normally expressed at later developmental stages or their expression could be unique to the fld/fld mouse. Last, the deficiency could be masked in adult animals by the profound differences in the lipid content of mother’s milk and chow.

Our studies indicate that the fld mutation appears to affect a genetic program that is important in the regulation of triglyceride metabolism during development. Experiments that prolonged the suckling period to the 20th postnatal day or increased dietary lipids presented to adult mice for a 2-week period failed to either prolong or elicit hypertriglyceridemia, respectively. These two experiments appear to rule out the third hypothesis proposed above and suggest that the mutation is under the developmental control of some factor(s) other than diet or the act of suckling. These experiments do not permit us to distinguish between the first and second hypotheses.

It is interesting to speculate which features of the fld mutation could represent the primary genetic lesion and what phenotypic traits are merely adaptive (i.e. secondary) responses. Several markers of the fld/fld phenotype have been identified: increased serum and hepatic triglyceride levels, increased concentrations of apoA-IV and apoC-II mRNA in liver, as well as decreases in lipoprotein and hepatic lipase activities.

The enormous (up to 100-fold) increase in hepatic apoA-IV mRNA concentrations observed from the third postnatal day to the sucking/weaning transition in fld/fld mice probably represents such an adaptive response. Analysis of apoA-IV mRNA by Northern blot analysis and its translation product by two-dimensional gel electrophoresis of plasma failed to reveal any differences between fld/fld mice and their phenotypically normal +/+ littermates. Furthermore, mapping studies with se on mouse chromosome 9 suggest that fld is not closely linked to the apoA-IV and apoA-I genes (Lusis et al., 1983). Thus, these data indicate that there is no structural abnormality in apoA-IV or a mutation in a cis-acting DNA sequence which affects the regulation of its gene. Moreover, the prolonged sucking experiment suggests that resolution of the elevated levels of apoA-IV mRNA in the livers of fld/fld mice is not due to a change in diet or cessation of sucking but rather some other alteration. If apoA-IV facilitates the assembly and/or export of triglyceride-rich lipoproteins from a liver massively overloaded with this class of lipid, as suggested by Elshourbagy et al. (1985), the “precocious” tissuespecific increase in hepatic apoA-IV mRNA concentration in mice homozygous for the fld mutation may represent an attempt by that organ to accommodate the vast excess of triglycerides present. The lack of apparent correlation between the increase in steady-state levels of apoA-IV mRNA and protein in sera and liver may, for example, reflect the effects of translational control mechanisms or differences in protein stability (turnover).

The degree of augmentation of apoA-IV mRNA levels in suckling fld/fld liver is particularly noteworthy. First, while studies in adult rats indicate that acute or chronic fat feeding only produces a modest (≤2-fold) increase in intestinal protein and mRNA levels (Apfelbaum et al., 1987; Gordon et al., 1982), comparable studies in either rat or mouse liver have not been reported. The massive increase in apoA-IV mRNA levels observed in fld/fld mice indicates that expression of the apoA-IV gene in the liver is remarkably responsive to physiologic stimuli during the neonatal period. The lack of a response in fld/fld intestine suggests that different factors may modulate apoA-IV gene expression in liver and intestine. Despite these dramatic changes early in life, treatment of adult normal or mutant mice with a high fat diet failed to produce a large increase in mRNA concentration. These results are consistent with a previous analysis of strain-specific differences in the response of the apoA-IV gene to high fat diets which indicated that adult BALB/c mice (the background on which the fld mutation arose) are relatively nonresponsive compared to other strains such as C57BL/6 and that this responsiveness is controlled by a locus distinct from that of the apoA-IV structural gene (Williams et al., 1986).
Apoc-II serves as a necessary cofactor for lipoprotein lipase (Havel et al., 1970; LeRosa et al., 1978; Nilsson-Ehle et al., 1980). We are not aware of any analysis of developmental changes in apoc-II mRNA accumulation in liver or intestine prior to this study. Our results indicate that in both suckling and weaning fld/fld mice and their normal littermates apoc-II mRNA concentrations are 2-18 times higher in intestine than in liver. Levels in the intestine reach a peak just prior to the suckling/weaning transition and subsequently decline during the weaning period. An up to 7-fold increase in apoc-II mRNA levels occurs in the livers of fld/fld mice. As in the case of apoa-IV mRNA, the effect was tissue-specific, and the greatest difference between fld/fld and mice and their +/- littermates occurred during the late suckling period. This difference in liver apoc-II gene expression could also be an adaptive change. Elevations in plasma apoc-II concentrations are commonly seen in patients with true lipoprotein lipase deficiency (Nikkila, 1983) where this change is regarded as compensatory.

The fld/fld mouse exhibits a tissue-specific deficiency in LPL activity. LPL is normally synthesized in rodent adipocytes and myocytes as well as several other cell types. The highest concentrations of LPL mRNA and enzyme activity are encountered in heart, skeletal muscle, and adipose tissues (Garfinkel and Schotz, 1987; Kirchgesner et al., 1988). LPL is exported from these sites of synthesis and transported to its physiologic site of action, the endothelial surfaces of capillaries (Nilsson-Ehle et al., 1980), where it binds via interactions with heparan sulfate proteoglycans (Cheng et al., 1981; Shimada et al., 1981). Activity levels of LPL are significantly lower in the heart and white adipose tissue of suckling fld/fld mice compared to their normal +/- littermates, while no significant differences in the LPL activity levels were demonstrable in liver or brown adipose tissue. The extent of the difference in LPL activity in the white adipose tissue of suckling fld/fld and +/- mice is much greater than in heart (16-fold versus <2-fold, see Fig. 7). This is interesting in light of the fact that LPL activity in these two tissues is known to be under separate genetic control (Ben-Zeev et al., 1983).

Analysis of fld/fld mice and their +/- littermates also revealed a decrease in liver HL mRNA levels in hypertriglyceridemic suckling fld/fld mice as well as concomitant reduction in their serum HL activity, all of which resolve by the end of the weaning period.

Since the fld mutation does not map to either the LPL or HL loci, it appears that this mutation is associated with an abnormality in regulation of these two lipases rather than in their structures. As noted in the Introduction, two mouse mutants which have combined deficiencies of HL and LPL have been previously reported: cl/cld and W/W*. Both appear to involve loci that are separate from the LPL and HL genes. The dominant white spotting (W) locus which encodes the c-kit proto-oncogene (Chabot et al., 1988; Geissler et al., 1988) has been mapped to mouse chromosome 5 while the cld locus is located on mouse chromosome 17. Our mapping studies of the fld mutation show that it is not linked to hammertoe (Hm) on proximal chromosome 5 or head tilt (het) on proximal chromosome 17, indicating that it is distinct from these two other mutations which affect HL and LPL activities. The fld/fld mouse provides additional evidence that HL and LPL have common controlling mechanisms within a tissue that is distinct from their structural loci. The fld locus appears to express the affected genes during early postnatal development.

Although the primary lesion in the fld mouse has not yet been established, the developmental defects in LPL and HL activities could be sufficient in themselves to produce the lipid abnormalities noted in suckling fld/fld mice. The hypertriglyceridemia could be caused by a lack of hydrolysis by HL and LPL. The specific defect in white adipose tissue LPL could block the ability of adipocytes to store the fatty acids normally hydrolyzed from circulating triglycerides. The lack of such a reservoir for dietary triglycerides is supported by our observation that the epididymal fat pads are apparently devoid of lipid and that fld/fld mice are not obese even in the face of massive hypertriglyceridemia. The fatty liver could be due to a number of underlying derangements: (i) inadequate extrahepatic metabolic processing of the chylomicrons coupled with an altered chylomicron remnant receptor activity; (ii) abnormalities in the metabolic processing of chylomicron remnants within hepatocytes; (iii) aberrant excessive synthesis of triglycerol in the fld/fld liver; and/or (iv) an associated inability to release VLDL. The plasma lipoprotein studies suggest an excess of chylomicron remnants in the fld/fld mouse, supporting the second possibility, although the others cannot yet be excluded.

The fld mutation appears to have some similarities to certain inherited human disorders of triglyceride metabolism. The classically autosomal recessive familial LPL deficiency (type 1 hyperlipoproteinaemia) presents in childhood with hypertriglyceridemia and associated increases in plasma chylomicrons, low to absent LPL activity in all tissues where it has been studied (type 1 hyperlipoproteinemia) presents in childhood with hypertriglyceridemia and associated increases in plasma chylomicrons, low to absent LPL activity in all tissues where it has been studied. Although the primary lesion in the fld mouse appears to affect the expression of these genes during early postnatal development.

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