Hypercholesterolemia in ENU-induced mouse mutants

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Abstract Hypercholesterolemia is caused by multiple environmental factors and genetic predispositions, and plays an important role in the development and pathogenesis of various human diseases. In this study, we aimed to establish randomly mutant mouse lines showing hypercholesterolemia for their further use in the detection of novel causative alleles. In the Munich ENU Mouse Mutagenesis Project, clinical chemistry blood analysis was performed on more than 15,000 G1 mice and 230 G3 pedigrees of chemically mutagenized mice to detect dominant and recessive mutations leading to an increased plasma total cholesterol level. Using inbred C3HeB/FeJ mice we identified more than 100 animals consistently showing hypercholesterolemia. Transmission of the altered phenotype to the subsequent generations led to the production of nine hypercholesterolemic lines. A single line showed further obvious deviations in the analysis of additional clinical chemistry blood parameters. Thus, the lines produced will contribute to the search for alleles that selectively cause primary hypercholesterolemia.—Mohr, M., M. Klempt, B. Rathkolb, M. Hrabé de Angelis, E. Wolf, and B. Aigner. **Hypercholesterolemia in ENU-induced mouse mutants.** ***J. Lipid Res.*** **2004.** 45: **2132–2137.**

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Few appropriate laboratory animal models exist for research on multifactorial and polygenic human diseases, due to their complex and/or species-specific phenotypes. Genetic engineering techniques allow the defined alteration of the mouse genome; however, the resulting phenotype of the mutant mice cannot be predicted. A complementary phenotype-driven strategy for the search of the genes involved in the appearance of a defined phenotypic alteration consists of the production of a great pool of randomly mutant mice and the subsequent selection of relevant lines according to the similarities in the pathology of animal model and human patients (1).

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ENU (*N*-ethyl-*N*-nitrosourea) has been used in various mouse mutagenesis programs to produce random mutations. Specific pathologic states have been identified by appropriate routine procedures allowing the screening of large numbers of mice for a broad spectrum of parameters (2, 3). Forward genetics techniques result in the detection of the chromosomal mapping site and the subsequent identification of the causative mutation in the established lines (4). Successful detection of the causative mutations in the phenotypically altered mice has been demonstrated in already examined ENU-induced mutant mouse lines (5).

In the Munich ENU Mouse Mutagenesis Project, a screening profile of clinical chemistry blood parameters was established for the analysis of offspring of chemically mutagenized mice in order to detect phenotypic variants with defects of diverse organ systems or changes in metabolic pathways. Breeding of the affected mice and screening of the offspring confirmed the transmission of the altered phenotype to the subsequent generations, thereby revealing a mutation as cause for the aberrant phenotype (6, 7). Mutant lines from the Munich ENU project with the causative mutation already identified are successfully used in research (e.g., Refs. 8, 9).

Hypercholesterolemia including increased values of the normal proportion of low density lipoprotein cholesterol (LDL-C) and high density lipoprotein cholesterol (HDL-C) acts as a main factor in the development of human diseases such as cardiovascular disease (10) and Alzheimer's disease (11). Beneath a subset of rare monogenic forms, in most cases hypercholesterolemia occurs as a polygenic and multifactorial disorder (12, 13). Humans show predominantly LDL-C and are sensitive to diet-induced elevations of LDL-C. In contrast, mice exhibit high amounts of HDL-C and a low LDL-C level (14). Many transgenic

Abbreviations: C3H, C3HeB/FeJ [inbred mouse strain]; ENU, *N*-ethyl-*N*-nitrosourea; G*n*, generation number; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol.

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mouse lines showing hypercholesterolemia have been produced that mimic the human situation (15–17).

Here, we present the generation and the phenotypic description of nine mutant lines derived from the phenotype-driven high-throughput hypercholesterolemia screen in the Munich ENU Mouse Mutagenesis Project for their subsequent use in the identification of novel alleles leading to increased plasma total cholesterol.

MATERIALS AND METHODS

Mutagenesis and breeding of mice

The experiments were conducted on the inbred C3HeB/FeJ (C3H) genetic background. C3H mice are described as a highly atherosclerosis-resistant strain (Ref. 18 and references therein). Ten-week-old male mice (generation G0) were injected intraperitoneally with ENU (3 × 90 mg/kg at weekly intervals).

The screen for dominant mutations was performed on G1 animals derived from the mating of the mutagenized G0 males to wild-type C3H females. Inheritance of the observed abnormal phenotype in the inbred C3H genetic background was tested on G2 mice derived from the mating of the affected G1 mouse exhibiting the altered phenotype and wild-type mice.

The screen for recessive mutations was performed on G3 mice produced in a two-step breeding scheme from G1 mice. G1 males known not to harbor dominant mutations were mated to wild-type females for the production of G2 animals. Subsequently, six to eight G2 females were backcrossed to the G1 male to produce the G3 mice of the pedigree. The analysis of the inheritance of an observed abnormal phenotype in G3 mice was done on G5 mice. Therefore, the affected G3 mouse presumably harboring a homozygous recessive mutation was mated to a wild-type mouse for the production of the presumably heterozygous mutant G4 mice showing an inconspicuous phenotype. Subsequently, the G5 mice derived from the mating of G4 mice to each other were tested for the abnormal phenotype. Alternatively, G5 mice derived from the backcross of a G4 mouse to the affected G3 animal were examined.

Once the causative mutation is identified, the internal names of the established lines will be replaced according to the official nomenclature. Mouse husbandry was done under a continuously controlled specific-pathogen-free (SPF) hygiene standard according to the Federation of European Laboratory Animal Science Associations (FELASA) protocols (http://www.felasa.org/recommendations.htm). Standard rodent diet (Altromin, Lage, Germany) and water were provided ad libitum. All animal experiments were conducted under the approval of the responsible animal welfare authority.

Clinical chemistry analysis

Blood samples from 3-month-old G1 and G3 mice fasted overnight were obtained by puncture of the retroorbital sinus under ether anesthesia. Physiologic parameter values were determined in male and female C3H controls. Plasma from Li-heparin-treated blood was analyzed using an Olympus AU400 autoanalyzer (Olympus, Hamburg, Germany) and the adapted reagents (Olympus, Hamburg, Germany). Calibration and quality control were performed according to the manufacturer's protocols. Total cholesterol, HDL-C, and LDL-C were examined by enzymatic colorimetry tests using the reagents OSR6116, OSR6187, and OSR6183 (Olympus, Hamburg, Germany) with the linear measurement ranges of 25–700 mg/dl, 2–180 mg/dl, and 10–400 mg/dl, respectively, for human samples.

In addition, the clinical chemistry screen included the following 21 plasma parameters: *a*) substrates: creatinine, ferritin, glucose, total protein, transferrin, triglycerides, urea, and uric acid; *b*) electrolytes: calcium, chloride, iron, phosphorus, potassium, and sodium; *c*) enzymes: alanine aminotransferase, alkaline phosphatase, amylase, aspartate aminotransferase, creatine kinase, lactate dehydrogenase, and lipase.

Furthermore, the following 13 hematologic parameters were measured in EDTA-treated blood using an ABC blood analyzer (Scil, Viernheim, Germany): *a*) red blood cells: hematocrit, hemoglobin, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, mean corpuscular volume, red blood cell count, and red blood cell distribution width; and *b*) white blood cells: granulocytes, lymphocytes, mean platelet volume, monocytes, platelets, and white blood cell count.

RESULTS

ENU-induced phenotypic variants showing increased plasma total cholesterol

The search for mutant mice showing increased plasma total cholesterol concentrations in the Munich ENU Mouse Mutagenesis Project was conducted on the inbred C3H genetic background 3 months post partum after overnight fasting of the animals. Determination of the physiologic range in male and female controls resulted in mean total cholesterol levels of 132 and 109 mg/dl, respectively. The 95% range of the values covered the data range including two standard deviations above and below the mean, indicating the Gaussian distribution of the data. The values within this range were defined to be physiologic, thereby eliminating outlier data (15). Thus, hypercholesterolemia was defined in our test for male and female mice showing values above the cutoff level of 160 mg/dl and 140 mg/dl, respectively, in two measurements within a 3 week interval (**Table 1**).

In the 6.5 year examination period, 15,624 G1 offspring of ENU-treated mice were screened for dominant mutations. Of the mice screened for dominant mutations, 60% were male animals; 57 male and 3 female mice showed increased mean plasma total cholesterol levels of the two measurements between 162 mg/dl and 358 mg/dl and between 141 mg/dl and 180 mg/dl, respectively. For revealing recessive mutations, 6,531 G3 mice were examined from 233 different pedigrees of ENU-treated animals. Of these, 35 male and 11 female mice exhibited increased mean plasma total cholesterol levels of the two measurements between 160 mg/dl and 312 mg/dl and between 175 mg/dl and 351 mg/dl, respectively. The lower number of female phenotypic variants may be caused by sex-

TABLE 1. Physiologic range of plasma total cholesterol (mg/dl) in 3-month-old C3H wild-type mice

Sex	No.	Range	Mean	SD	Mean ± 2 SD	95% Range[a]
Male	470	78–171	132	15	102–162	100–161
Female	407	56–180	109	17	75–143	76–141

SD, standard deviation.
[a] Cutoff level: male: 160 mg/dl; female: 140 mg/dl.

TABLE 2. Screen and analysis of the inheritance for hypercholesterolemia in ENU mutagenized mice

Screen	Sample	Pedigrees	Variants[a]	Variants Mated	Without Offspring	Analysis of Inheritance[b]	
						Negative Offspring	Mutants[c]
	no.				*no. (m/f)*		
Dominant (G1)[d]	15,624	nd	60 (57/3)	50 (47/3)	22 (19/3)	22 (22/0)	6 (6/0)
Recessive (G3)	6,531	233	46 (35/11)	32 (21/11)	17 (7/10)	12 (11/1)	3 (3/0)
Total	22,155		106 (92/14)	82 (68/14)	39 (26/13)	34 (33/1)	9 (9/0)

m/f, males/females; nd, not determined.

[a] G1 and G3 offspring of ENU-treated mice showing hypercholesterolemia (male: ⩾160 mg/dl; female: ⩾140 mg/dl) in two measurements of a 3-week interval.

[b] A mutation as cause for the appearance of the abnormal phenotype was diagnosed in phenotypic variants with offspring showing cholesterol levels above the cutoff level (male: 160 mg/dl; female: 140 mg/dl) in two measurements of a 3 week interval. Offspring were produced by mating the G1 phenotypic variants to wild-type mice (screen for dominant mutations) and from G4 intercross or G4 × G3 backcross breedings after mating the G3 phenotypic variants to wild-type mice (screen for recessive mutations).

[c] Phenotypic variants inheriting hypercholesterolemia to the subsequent generations.

[d] Sixty percent of the G1 mice screened for dominant mutations were male animals.

specific compensation mechanisms of mutations affecting the plasma cholesterol metabolism and/or by the selective early loss of female phenotypic variants due to the pathologic consequences of hypercholesterolemia (**Table 2**).

Transmission of hypercholesterolemia to subsequent generations

The analysis of the inheritance of the hypercholesterolemic phenotype was performed on G2 animals from the mating of 50 G1 phenotypic variants to wild-type mice in the screen for dominant mutations and on G4 intercross or G4 × G3 backcross offspring after breeding 32 G3 phenotypic variants to wild-type mice in the screen for recessive mutations (see Materials and Methods). In the case no offspring occurred, the phenotypic variants were mated to another wild-type animal. The remaining 24 male phenotypic variants (10 from the screen for dominant mutations and 14 from the screen for recessive mutations) showing plasma cholesterol levels between 163 mg/dl and 215 mg/dl were not mated, but sperm of these mice was frozen for long-term storage.

From the 82 phenotypic variants mated, 39 (48%) produced no offspring due to severe illness and/or sterility in repeated breeding approaches (Table 2). The lack of offspring occurred in 13 of the 14 female phenotypic variants mated irrespective of the extent of the pathologic plasma total cholesterol values (141–351 mg/dl) and, as a tendency, to a higher degree in males showing plasma total cholesterol levels above 220 mg/dl (data not shown).

TABLE 3. Phenotypic penetrance and extent of hypercholesterolemia in the mutant lines

Line[a]	Mean PTC, Founder[b]	Offspring Tested,[c] m/f	Hypercholesterolemia[d]			
			Penetrance		Mean	
			m	f	m	f
	mg/dl	*no.*	*% (no.)*		*mg/dl (SD)*	
CHOHD1	171	85/65	82 (35)	62 (20)	208 (24)	204 (40)
CHOHD2	172	41/49	29 (6)	12 (3)	182 (15)	157 (16)
CHOHD3	178	26/31	38 (5)	0 (0)	228 (55)	—
CHOHD4	189	6/10	100 (3)	80 (4)	180 (14)	153 (19)
CHOHD5	208	29/33	41 (6)	30 (5)	186 (13)	162 (30)
CHOHD6	214	7/nd	100 (7)	nd (3)	219 (6)	202 (63)
CHOHR1	185	22/9	100 (7)	89 (2)	192 (14)	185 (—)[e]
CHOHR2	265	5/5	100 (2)	100 (3)	170 (8)[f]	190 (12)
CHOHR3	275	9/12	100 (5)	100 (3)	212 (51)[f]	202/22[f]

m/f, males/females; nd, not determined; PTC, plasma total cholesterol; SD, standard deviation; —, no values.

[a] CHOH, hypercholesterolemic line; D1-D6, dominant mutation; R1-R3, recessive mutation.

[b] The mutant lines are listed according to the mean plasma total cholesterol level (mg/dl) of both measurements of the founder G1/G3 mutant of the line.

[c] Offspring after mating heterozygous mutants to wild-type mice (dominant mutations) and offspring after mating heterozygous mutant mice (recessive mutations).

[d] Hypercholesterolemia in the offspring was diagnosed by the appearance of total cholesterol levels above the cutoff level (male: 160 mg/dl; female: 140 mg/dl). 100% phenotypic penetrance is defined in our breeding scheme by the appearance of 50% and 25% hypercholesterolemic offspring in the lines harboring a dominant and recessive mutation, respectively. The mean plasma total cholesterol level of the second measurements of the analyzed hypercholesterolemic mutants is shown.

[e] Value of only one of the two animals was included.

[f] Mean plasma total cholesterol level of the first measurements of the hypercholesterolemic mutants.

This may be due to pleiotropic effects of the mutations and/or to negative consequences of the elevated cholesterol levels on the reproductive system of the affected mice.

Of the 82 mated mice showing hypercholesterolemia, 43 (52%) produced offspring. A mutation as cause for the appearance of hypercholesterolemia was diagnosed in the phenotypic variants when plasma total cholesterol values above the cutoff level (male: 160 mg/dl; female: 140 mg/dl) were detected in the offspring in two measurements at a 3 week interval. Of the 43 offspring producing phenotypic variants, 34 (79%) did not transmit the abnormal phenotype (Table 2). In these breeding approaches, offspring showing hypercholesterolemia in the first measurement (male: 160–220 mg/dl; female: 144–358 mg/dl) were observed preferentially from phenotypic variants with higher hypercholesterolemia levels, but these results were not reproducible in the second test after 3 weeks (not shown). Therefore, inheritance of the abnormal phenotype did not occur in these cases.

In summary, nine male mutants transmitting hypercholesterolemia to the subsequent generations were revealed (Table 2). The extent of the pathologic plasma total cholesterol values in the phenotypic variants was not indicative of the successful establishment of a mutant line (data not shown). In all, breeding of 10 phenotypic variants was necessary to establish one mutant line with hypercholesterolemia in our project.

The further breeding of the descendants of the nine confirmed mutants resulted in the establishment of the lines CHOHD1 to -D6 and CHOHR1 to -R3 harboring dominant and recessive mutations, respectively. Mutant offspring showing hypercholesterolemia above the cutoff level (male: 160 mg/dl; female: 140 mg/dl) were produced by mating heterozygous mutants to wild-type animals (lines CHOHD1 to -D6) and by breeding heterozygous mutant mice (lines CHOHR1 to -R3). The male and female phenotypic mutants of the nine lines showed mean plasma total cholesterol levels between 170 mg/dl and 230 mg/dl and between 150 mg/dl and 200 mg/dl, respectively (**Table 3**).

The phenotypic penetrance of the increased total cholesterol levels in the nine lines was analyzed by defining complete phenotypic penetrance in the case of the appearance of 50% and 25% hypercholesterolemic offspring after mating heterozygous phenotypic mutants to wild-type mice and after crossing heterozygous mutant mice in the lines with dominant and recessive mutations, respectively. In five of the nine lines (CHOHD4, CHOHD6, CHOHR1, CHOHR2, CHOHR3), a high phenotypic penetrance of hypercholesterolemia was observed, which facilitates the effective subsequent phenotypic and molecular genetic analyses. The other four lines (CHOHD1, CHOHD2, CHOHD3, CHOHD5) showed an incomplete penetrance of various degrees (Table 3).

Additional phenotypic alterations in the mutant lines

For the further analysis of the hypercholesterolemic alteration, in six of the nine lines (CHOHD1, CHOHD5, CHOHD6, and CHOHR1 to -R3), the LDL-C and HDL-C levels were measured in the phenotypic mutants. The male and female physiologic ranges (mean value ± 2 standard deviations) were analyzed in wild-type C3H controls and resulted in 3–11 mg/dl and 8–20 mg/dl for LDL-C, and in 106–150 mg/dl and 85–125 mg/dl for HDL-C, respectively. In the lines analyzed, most hypercholesterolemic mutants showed LDL-C and HDL-C values above the physiologic range. Compared with normal levels, the increase of the pathologic LDL-C values was higher than the increase of the pathologic HDL-C values, which indicates the disproportional increase of LDL-C and HDL-C (**Table 4**). However, the exact analysis of the proportion of LDL-C and HDL-C in the particular mutant lines requires the examination of additional mutants and the use of the respective wild-type littermates as controls.

According to published results (19) (http://www.jax.org/phenome), our wild-type HDL-C values seemed to be elevated, compared with the physiologic total cholesterol levels. This may be due to species-specific inaccuracies of the detection method, which works with reagents for human samples and includes immunoinhibition steps (Olympus, Hamburg, Germany). However, overestimation of HDL-C in the hypercholesterolemic mutants due to inaccuracies of the detection method used even underestimated the real disproportional increase of LDL-C and HDL-C.

The overall clinical examination and the analysis of additional 21 clinical chemistry plasma parameters (including the substrates glucose, total protein, triglycerides, and urea and 13 hematologic parameters; see Materials and Methods) showed obvious deviations in the hypercholesterolemic mutants only for line CHOHR3, where the hy-

TABLE 4. LDL cholesterol and HDL cholesterol values in the hypercholesterolemic mutants

Line[a]	Mutants tested,[b] m/f	Mean LDL-C[c]		Mean HDL-C[c]	
		m	f	m	f
	no.	*mg/dl (SD)*			
CHOHD1	13/11	34(7)	31(5)	176(17)	147(13)
CHOHD5	2/1	21(3)	22(—)	162(13)	128(—)
CHOHD6	3/3	35(6)	42(21)	170(8)	152(71)
CHOHR1	4/0	27(10)	—	145(22)	—
CHOHR2	2[d]/3	28(7)	16(1)	141(9)	154(6)
CHOHR3[d]	5/3	24(8)	35(3)	167(35)	152(18)
Wild-type[e]	20/20	7(2)	14(3)	128(11)	105(10)

m/f, males/females; SD, standard deviation; —, no values.

[a] CHOH, hypercholesterolemic line; D1, D5-D6, dominant mutation; R1-R3, recessive mutation.

[b] The hypercholesterolemic mutants were produced by mating heterozygous mutants to wild-type animals (dominant mutation) and by breeding heterozygous mutant mice (recessive mutation).

[c] Mean plasma LDL/HDL cholesterol level of the second measurements of the hypercholesterolemic mice.

[d] Mean plasma LDL/HDL cholesterol level of the first measurements.

[e] As controls, the physiologic plasma LDL and HDL cholesterol values were analyzed in 20 male and female wild-type C3H mice each. The plasma total cholesterol values for these mice were as follows (mean/SD): male 143/13; female 118/17.

percholesterolemic mice had a decreased lifetime leading to death after about 5 months. In addition, they exhibited increased plasma urea and creatinine values, decreased urine urea and creatinine concentrations, severe albuminuria, and microcytic anemia. The hypercholesterolemic mutants of the other eight lines were inconspicuous, indicating that hypercholesterolemia most likely was not caused as a secondary effect due to preceding alterations in other metabolic pathways. Thus, the lines harbor mutations in genes that primarily and selectively influence the plasma cholesterol metabolism.

DISCUSSION

High-throughput screening of a great number of randomly mutant mice for hypercholesterolemia resulted in the establishment of nine lines harboring dominant and recessive mutations. Eight of the lines showed selectively increased plasma total cholesterol levels.

Apart from deviations in the plasma cholesterol level, the clinical chemistry screen of more than 15,000 G1 animals and of G3 mice from more than 230 pedigrees in the Munich ENU Mouse Mutagenesis Program (6, 7) detected mutants with deviations of the plasma substrates ferritin, glucose, total protein, urea, and uric acid. More than half of all mutants found with plasma substrate abnormalities in our screen showed a pathologic total cholesterol level (http://www.lmb.uni-muenchen.de/groups/mt/mouse.htm; Rathkolb et al., unpublished results). This may indicate the involvement of a high number of genes in the homeostasis of the blood total cholesterol level.

Reproduction of the hypercholesterolemic state succeeded in the random reanalysis of the affected animals. Under our breeding approaches for the establishment of mutant lines, nearly 80% of the fertile phenotypic variants did not transmit hypercholesterolemia to the offspring. A high ratio in the failure of the transmission of the defect to the offspring was also found for other phenotypic deviations in our clinical chemistry screen (Rathkolb et al., unpublished results). One reason may be the occurrence of non-genetically fixed hypercholesterolemia is due to the inherent impossibility of performing complete standardization of husbandry and experiment. In addition, loss of the additive phenotypic effect by segregation of multiple mutations in the subsequent generations also leads to the failure in the transmission of the aberrant phenotype.

Nearly half of the established lines showed an incomplete penetrance of the mutant phenotype of various degrees. Appearance of hypercholesterolemia due to the interaction of multiple unlinked mutations leads to lower numbers of affected offspring. Future linkage experiments in the lines will reveal the number and the chromosomal positions of the mutations involved in the development of hypercholesterolemia. We did not examine deviations in the number of offspring per litter and/or in the sex ratio of the offspring indicating early loss of mutants as an alternative cause for the decreased frequency of mice showing hypercholesterolemia. The phenotypic analysis of the mutation may be improved by analyzing the animals more frequently and/or by inducing the altered phenotype specifically in the mutants through appropriate diet challenges. Extensive variations in the plasma cholesterol concentrations have been found in different genetic backgrounds (20) (http://www.jax.org/phenome; http://www.eumorphia.org). Therefore, generation and subsequent analysis of hybrids and/or congenic strains will give rise to variations in the appearance of hypercholesterolemia.

In addition to the clinical chemistry screen in the Munich ENU Mouse Mutagenesis Project, a systematic clinical chemistry analysis has been described in another ENU mouse mutagenesis program (3). The appearance of dislipidemic lines was reported on a different genetic background (BALB/c × C3H), in which additional mutations may occur to cause hypercholesterolemia (19).

Due to the triggering of random mutations by ENU, discovery of genes not yet known to be involved in the plasma cholesterol metabolism may occur in our mouse mutants. Chromosomal linkage analysis will give a fast overview of the affected genomic sites. The subsequent identification of the causative mutations including the analysis of their functional relevance by reverse genetics methods, as well as the examination of the pathologic consequences of the mutations, will contribute to the analysis of the polygenic causes of human hypercholesterolemia.

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