

## Experimental Models of Hyperlipoproteinemia and Atherosclerosis

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### Summary

The first experimental model of atherosclerosis (in rabbits) is more than hundred years old. Several animal species have been used to produce hyperlipoproteinemia and possible atherosclerosis. The gene manipulation produced the most used models recently. This review acknowledges the extensive study of atherosclerotic changes in experimental models of hyperlipoproteinemia and atherosclerosis to come to light thus far and the purpose here is not only to summarize the published data but also to try to add some details of our experience in using these models. In addition to rabbit (the old but also improved model by reno-vascular hypertension) dog, birds, pig, hamster, mice, rat and non-human primate's animal models are described. The gene manipulation produced the most used models two decades ago. Germline genetically engineered (without apoE or LDL receptor genes) animals have become the most used models producing atherosclerotic changes in the aorta. Recent new models also producing atherosclerotic changes but without germline genetic manipulation are also described.

### Key words

Lipoproteins • Atherosclerosis-models

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Although cardiovascular disease mortality has decreased over recent decades in industrially developed countries, it still represents almost half of all causes of death. Although the general risk factors of atherosclerosis development are now known (thanks to epidemiological data going back to the end of Second World War) (Keys

1952), some details of atherosclerosis pathology are not yet fully understood. Despite the detailed documentation of the molecular principals of hypercholesterolemia (Goldstein and Brown 1973), the role of monocytes/macrophages in the subendothelial space of large and medium-sized arteries is still inconclusive. Their crucial roles in increasing cardiovascular disease death have been repeatedly documented, but the molecular principle behind the pro-inflammatory stimulation of atherosclerosis and the role of adipose tissue and insulin sensitivity are a matter of some debate.

Experimental models in biomedical research principally involve the analysis of physiological and pathophysiological topics learned from epidemiological and clinical data. Although there is substantial pressure from different groups of animal activists, several type of biomedical question still require using of animal models for their analysis of molecular principles.

In the majority of animal models of hyperlipoproteinemia, increase in the concentration of pro-atherogenic lipoproteins in the blood is induced by dietary changes. In animal species sensitive to dietary cholesterol (rabbits, hamsters, pigs), supplementation alone of crystalline cholesterol dissolved in different types of animal fat is sufficient to produce a several-fold increase in cholesterol concentration. In other species, a substitution of the diet using bile acid and/or toxic influence of the thyroid gland (dog, rat) is required to produce hypercholesterolemia. In this insensitive species, long-term inbreeding (Koletsky 1973, Poledne 1986, Vrana and Kazdova 1990) stimulates sensitivity similar to sensitive species. Also, gene manipulation has been used to increase sensitivity to cholesterol in mice and rat.

Two decades ago, application of molecular genetic manipulation produced ApoE-lacking and LDLr-lacking genes (apoE KO and LDLr KO) mice. Both these models have been frequently used in laboratories over the entire world. Recently, after the discovery of proprotein convertase subtilisin/kexin-type, 9 (PCSK9) (Cohen *et al.* 2005, Seidah *et al.* 2003), transgenic mice with PCSK9 were produced, creating atherosclerotic changes in the aorta resembling human disease.

## Rabbits

The first experimental model of hypercholesterolemia was published more than one century ago in a well-known experiment (Anitschkov and Chelatorov 1913) in which the diet with high proportion of animal protein and fat produced liver steatosis as well as changes in the arterial wall. Since Second World War this model has been employed repeatedly using crystalline cholesterol dissolved in different animal fats. Cholesterol supplementation usually represents 1-3 % of the diet. We tried to use this model to study the potential for atherosclerosis regression and lipid metabolism of the arterial wall. A diet containing 1 % of cholesterol dissolved in warm beef lard was shown to produce a high concentration of total blood cholesterol (50-100 mmol/l) with microscopic changes appearing in the aorta within several weeks. Unfortunately this change did not resemble human atherosclerosis (Stary *et al.* 1995) but only a robust type of fatty streak. Furthermore, the localization of the lesion was mainly in the thoracic aorta, but did not appear in the abdominal aorta or coronary and renal arteries. We applied an alternative model which combined a much lower amount of alimentary cholesterol (0.2 % in the diet) with renovascular hypertension (using a silver clamp to decrease the size of one of the renal arteries to 50 %). This model with cholesterolemia, which is similar to human with familial hypercholesterolemia (around 20 mmol/l) and increased blood pressure by 30-50 %, resulted in atherosclerotic changes resembling combined atherosclerotic lesions in men localized in the thoracic aorta and coronary arteries.

The best rabbit model of atherosclerosis was developed in Japan as a result of brother-sister inbreeding and back-breeding of animals with unusual mutation. These Watanabe rabbits developed complicated atherosclerotic lesions, xanthelasma (Tanzawa *et al.* 1980), and a deficient LDL-receptor (Buja *et al.* 1983). Unfortunately, the animals later lost fertility.

## Dogs

Because of the ease of communication and handling, these animals' species has also been used to produce some arterial wall changes (Poledne *et al.* 1978). Dogs do not develop atherosclerotic changes and are not sensitive to the production of hypercholesterolemia (Mahley and Weisgraber 1974). Hypercholesterolemia is induced in this species only upon suppression of thyroid gland activity (Geer and Guitry 1978).

## Birds

Different avian species were first used in the sixties and seventies. Although a cholesterol diet is able to produce hypercholesterolemia in pigeons, chickens and Japanese quail (for a review, see Vessilionovitch 1988), these models are only interesting from a historical point of view and have not been used recently. Artery size and extreme variability are probable reasons for their lack of use.

## Pigs

Pigs have been used as an experimental model in different studies (Poledne 1982), as their physiology and pathology closely resemble those of humans. Hypercholesterolemia is easily produced by supplementing an experimental diet with cholesterol alone (2-3 %). The popularity of this animal model increased in the eighties when a different type of miniature pig was obtained by breeding with a much smaller Vietnamese pig. The hypercholesterolemia limit can be reached within several days and similar patterns of lipoprotein are exhibited as in humans (Poledne *et al.* 1981a,b) with atherosclerotic changes appearing within 2 months due to accelerated inflow of LDL cholesterol to the arterial wall (Poledne *et al.* 1983). On the other hand, one limitation of using this experimental model is the very high variation in the sizes of atherosclerotic lesions in the aorta.

## Non-human primates

This animal species represents an attractive model of atherosclerosis because of its close similarity to *Homo sapiens sapiens* in terms of anatomy and physiology of the cardiovascular system. The rhesus macaque monkey has been most notably used in

atherosclerosis research. We studied the induction of atherosclerosis using a high-fat diet in baboons for six months in order to analyze the potential hypocholesterolemic effect of partial small intestine resection. Although a cholesterol diet containing 2 % crystalline cholesterol produced a long-lasting increase in blood cholesterol, we also documented an effect of the resection. We also documented a very high variability in the size of atherosclerotic lesions in the aorta as well as in the coronary artery, which resembles a similarly high variability of atherosclerosis in humans. A higher number (around 10-15) of experimental animals in compared groups would be required to obtain significant effects.

### **Hamsters**

These small animals have also been frequently used for the study of hyperlipoproteinemia and atherosclerosis. We analyzed the risk of postmenopausal status by comparing ovariectomized Syrian golden hamsters (Pitha *et al.* 2010) with using controls. This model is very suitable primarily because its dietary-stimulated hyperlipoproteinemia resembles that of humans. Substantial data on cholesterol metabolism in mammals have been obtained using hamsters (Turley *et al.* 1997).

### **Mice**

As the mouse is the most frequently used animal model in biochemical research it is not surprising that the first attempt to adapt it for atherosclerosis research began half a century ago. Piedrahita *et al.* (1992) described apolipoproteinE (apoE) deficient mice obtained by inactivation of the apoE gene. Chimeric mice (obtained by blastocyst injection within the targeted line) transmitted the disrupted gene to this progeny. These apoE KO animals display a substantially different spectrum of lipoproteins compared to control animals. Whereas normal mice has majority of cholesterol in the HDL fraction, apoE KO mice has 70-80 % of cholesterol in VLDL and LDL fractions with a slight increase in triglyceride concentration (Jawień *et al.* 2004). When fed a control diet ApoE KO mice develop fatty streak lesions within 10 weeks and complex fibrous lesions after one year. When fed Western diet (around 20 % of fat and 0.2 % of cholesterol), blood cholesterol concentration increases from 10 mmol/l to 20-25 mmol/l. This experimental diet decreases the time required to produce

complex lesions to one half. Earlier a morphological approach of section of aortic sinus was used for lesion characterization and determination, but later a surface area covered by lesions of aorta has been used with different mathematical program for analysis. ApoE KO mice are the most frequently used model of atherosclerosis. From our experience of breeding apoE KO animals for several years, we have been able to document the partial disappearance of a hypercholesterolemic pattern of lipoproteins.

Another experimental model of hypercholesterolemia (also related to a change in the apoE gene) was developed in the Netherlands by transferring a defective apoE3-Leiden (Van den Maagdenberg *et al.* 1993).

At the same time when apoE KO mice were described also another mice model – LDL receptor KO was obtained in the Goldstein and Brown laboratories. These defective animals were obtained by a similar gene targeting to embryonic stem cells (Ishibashi *et al.* 1993) used also for apoE KO model. This model is very sensitive to dietary cholesterol and using similar type of western diet is able to increase blood cholesterol around 20 mmol/l.

Atherosclerotic lesions form within several months but their pattern is less similar to human lesions compared to the apoE KO model. Ishibashi *et al.* (1994) later developed a double knock-out model lacking both the apoE gene and the LDL-receptor. Atherosclerotic changes in the aorta appeared earlier compared to the apoE KO model (Witting *et al.* 1999). This experimental model is currently most frequently used to analyze complex atherosclerotic lesions.

### **Rats**

The first hyperlipoproteinemia model of this animal species (the Zucker fatty rat) is more than half century old (Zucker and Zucker 1961). This obese hypertriglyceridemic hypertensive animal model is obtained by breeding two lines with a recessive mutation. Twenty-five years later this mutation was identified (Chua *et al.* 1996) in the leptin receptor locus. Although this model exhibits increased triglyceride concentration, it does not produce an increase in LDL cholesterol concentrations or vascular changes (de Artinãno and Castro 2009). Application of this mutation in the gene for leptin to other lines of spontaneous hypertensive and obese (SHROB) was described by Koletsky (1973). This

experimental model is frequently used for cardiovascular disease study as it develops also arterial changes (Russell 2009). Rat display as well as mice low LDL cholesterol concentration with majority of cholesterol in HDL fraction because neither species possess cholesteryl ester transfer protein (CETP). This protein is responsible for the transfer of cholesteryl esters from HDL to cholesterol-rich VLDL and the reverse exchange of triglycerides to HDL. Human CETP transgenic rat (Russell and Proctor 2006, Barter *et al.* 2003) exhibits hypercholesterolemia similar to humans. It has been shown that when this gene is transferred to spontaneously hypertensive animals certain atherosclerotic changes occur (Barter *et al.* 2003). Hereditary hypertriglyceridemic (non-obese) rat was developed by Vrana after the long-term inbreeding of rats sensitive to a high-sucrose diet (Vrana and Kazdova 1990). This experimental model was shown to have a blood triglyceride concentration five times higher than that of the Wistar rat control (used for parent generation 0). Hereditary hypertriglyceridemic rats are also insulin-resistant (Vrana *et al.* 1993) and hypertensive (Zicha *et al.* 2006).

The Prague hereditary hypercholesterolemic rat was developed in the 1980s at the Institute for Clinical and Experimental Medicine in Prague. Fifty male and fifty female Wistar rats were selected (parent generation 0) based on sensitivity to high cholesterol (a diet containing 2 % of crystalline cholesterol dissolved in 5 % of melted beef lard). Six pairs were used as parent generation 0. The concentration of stimulated cholesterolemia was used to select their offspring to follow cousin-cousin breeding for 10 generations. The same selection and brother-brother inbreeding was used for another 10 generations (Poledne 1986). Stimulated cholesterol concentration gradually increased in the third generation reaching 3 mmol/l of cholesterolemia in males and 4 mmol/l in females. No stimulated cholesterolemia appeared until the seventh generation, after which there was a substantial increase to 6 mmol/l in males and 10 mmol/l in females (appearing in two pairs). This level was sustained for another 10 generations using strict brother-sister inbreeding. This surprising jump in stimulated cholesterol was then repeated three years later in the following B line.

The main part of cholesterol in the PHHC rat is localized in the VLDL and IDL fractions with a slight increase in LDL particles. It exhibits substantially lower

HDL cholesterol in comparison to the Wistar rat. In this line of hypercholesterolemic animals we detected no effect of cholesterol synthesis feedback in the liver, with a decrease in the disappearance of LDL particles from the blood. Unfortunately this strain of rat does not develop any atherosclerotic changes in cases of long-term hypercholesterolemia (Kovář *et al.* 2009).

Although the frequency with which experimental atherosclerosis animal models are applied has decreased over recent years, it will probably rise again in the near future to reflect the introduction of newer forms of hypercholesterolemia treatment.

A certain disadvantage of the apoE KO and LDLr KO mice is their slightly decreased concentration of apoB-containing lipoproteins in the circulation. A new experimental model of atherosclerosis developed by a pharmaceutical company (Tadin-Strapps *et al.* 2011) overcomes this problem by human CETP transgene insertion to mice lacking LDL receptors, producing hemizygous mice with a similar pattern of lipoproteins, to humans.

Recently, a new type of experimental models with hypercholesterolemia and atherosclerosis was developed early after the discovery of PCSK9 (Cohen *et al.* 2005). The first type of this transgenic mice expressing human PCSK9 was produced in Anne Southar's laboratory (Bronwen *et al.* 2010). Later, a more stable model of transgenic PCSK9 was described by Bjørklund *et al.* (2014). Intravenous administration of recombinant adeno-associated viral vector – PCSK9 – increases PCSK9 concentration in the liver and, subsequently, that of cholesterol in the circulation. On the basis of LDLr KO mice, plasma concentration of these PCSK9 transgenic animals when fed a high-cholesterol diet show an increase of cholesterol to 100 mmol/l, producing complex fibromuscular atherosclerotic lesions in the aorta.

A single injection of gain of function PCSK9 is impressive model as it is able to produce cardiovascular calcification also in mice without previous genetic modification (Goettsch *et al.* 2016). An alternative experimental model was described by Sasaki *et al.* (2014) using i.v. administration of adenoviral vector expressing IDOL (inducible degradation of LDL receptor). This model has been shown to produce atherosclerosis not only in mice but, also in hamsters. Fast development of molecular genetic manipulations is opening a possibility of new models of atherosclerosis in the near future.

## Conflict of Interest

There is no conflict of interest.

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