Platelet and Megakaryocyte Changes in Cholesterol-Induced Experimental Atherosclerosis

John F. Martin, David N. Slater, Yehia T. Kishk, and Eric A. Trowbridge

Rabbits were fed either 2 g cholesterol in 10 ml olive oil daily with normal diet (n = 5) or normal diet alone (n = 5). After 12 weeks, the cholesterol-fed animals had developed fatty plaques involving 24% ± 4% of the surface area of the aorta; the control animals had none. Mean platelet volume was significantly smaller (p < 0.04) in the cholesterol-fed animals (4.1 ± 0.3 fl) compared with the controls (4.8 ± 0.4 fl). The heterogeneity of the average volume distributions of the two groups, characterized by the statistical parameters of the coefficient of variation, skewness, and kurtosis, was also significantly different. Platelet count was significantly higher (p < 0.001) in the cholesterol-fed group (7.48 ± 1.06 x 10^11 platelets/liter blood) compared to the control group (4.86 ± 0.60 x 10^11 platelets/liter blood). Mean megakaryocyte cytoplasmic volume was significantly larger (p < 0.001) in the cholesterol-fed rabbits (12,262 ± 1485 fl) compared with controls (6,814 ± 761 fl). The range of cytoplasmic volumes was also significantly increased in the cholesterol-fed rabbits. A significant (p < 0.01) increase in mean megakaryocyte nuclear volume in the cholesterol-fed animals was accompanied by a nonsignificant increase in mean nuclear DNA content: 30.2 ± 3.7 N compared with a control value of 23.6 ± 4.0 N. This evidence indicates that a high cholesterol diet in rabbits is associated with changes in platelet production from megakaryocytes as well as the development of atherosclerosis.

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Platelets in vascular disease have increased reactivity in both human and animal models. The cause of this increased reactivity may either be an effect of vessel wall changes on normal platelets or be related to an intrinsic change in the platelets themselves. In humans, the increased platelet reactivity has been associated with platelet size changes which have been confirmed in recent studies. This evidence suggests that there may be alterations in platelet production in vascular disease. Two further observations support this hypothesis. Men who suffered sudden cardiac death associated with atherosclerotic arteries had larger megakaryocytes than a control group who died unexpectedly from unnatural causes. Similar large megakaryocytes, which produced larger than normal platelets, were also observed in men with acute myocardial infarction.

It has been well documented that the administration of a high cholesterol diet to rabbits causes the formation of lipid-rich arterial lesions, which are similar to human arterial fatty streaks. However, atherosclerosis, together with its complications, can develop in humans without elevated cholesterol. Furthermore, there may be substantial differences between complicated atherosclerosis in humans and cholesterol-induced atherosclerosis in rabbits. Nevertheless, this experimental model does provide the opportunity for investigating whether changes in megakaryocyte and platelet size, seen in men with vascular disease, also occur in rabbits fed on a high-cholesterol diet.

Methods

Male New Zealand White rabbits (n = 5), mean weight 2.34 ± 1.00 kg (x ± sd), were fed for 12 weeks a diet of SG1 rabbit pellets (Argus PLC, Penistone Hall, Penistone, South Yorkshire, England) that also contained 2 g of cholesterol mixed with 10 ml of olive oil. Paired male litter mates (n = 5), mean weight 2.34 ± 1.00 kg (x ± sd), were fed a normal diet.
weight 2.28 ± 1.06 kg, that had been fed rabbit pellets alone for the same time period were used as experimental controls. Apart from diet, all animals were housed and treated identically for the entire experimental period. All procedures were performed in accordance with the Cruelty to Animals Act of 1876.

Venesection was performed on a marginal ear vein. Blood was taken from unanesthetized animals for platelet count and volume distribution measurement into 38.0 g/liter of sodium citrate and 400 µg/liter of prostaglandin E, (PGE,). Pilot studies demonstrated that the concentration of PGE, used here had no effect on platelet size compared to citrate alone. Plasma cholesterol and triglyceride levels were measured in plasma from blood anticoagulated with sodium citrate 38.0 g/liter alone.

**Platelet Volume and Count**

Platelets were separated from whole blood by using velocity centrifugation. The blood was diluted 1:1 in a solution containing 0.218 g/liter of KH2PO4, 1.22 g/liter of Na2HPO4, 6.832 g/liter of NaCl, 4.00 g/liter of Na2C6H5O7·2H2O, and 2 g/liter of glucose; the final pH was 7.0, osmolarity 290 ± 1 mOsm/liter. The mixture was then layered onto a 10 ml gradient of 50% (vol/vol) polyvinyl pyrrolidone-coated silica (Percoll, Pharmacia) and 50% of the solution described above, self-generated by centrifugation at 26,000 g for 20 minutes in a fixed angle rotor. The velocity gradient was centrifuged at 400 g for 10 minutes, leaving the platelets to be collected from near the top of the gradient.7 Percoll was adjusted to pH 6.5 and osmolarity to 290 ± 1 mOsm/liter for all gradients. Platelet recovery was 82% ± 16% in the cholesterol-fed group and 82% ± 10% in the control group. Platelet counts (PC) were performed with a Coulter ZB counter. White and red cell contamination in the platelets recovered from the gradient was negligible. The 100 µl aliquots of the platelet sample were placed in Isoton (Coulter Electronics), and platelet volumes were measured immediately.

The volume-measuring apparatus consisted of a Coulter ZB particle counter coupled to an Apple II microcomputer by an analogue-to-digital convertor. The latter was designed to exclude all bimodal signals, those that were longer than 20 μsec, and those that were in red-cell volume range8 (beyond 25 fl). The system was calibrated with latex particles of known modal volumes, 4.25 fl and 10.65 fl, and its linearity tested with a pulse generator.

The computer was programed to collect the raw data in a frequency histogram with class interval of 0.1 fl from which the mean platelet volume (MPV) and the distribution measures of dispersion (coefficient of variation), asymmetry (skewness), and convexity (kurtosis) were evaluated.9 (The computer program was written by Jonathan Adams.)

The platelet biomass was evaluated from the product of MPV and PC.

**Megakaryocyte Studies**

The animals were sacrificed by using intravenous sodium pentobarbitone. The right femur of each rabbit was removed immediately and split longitudinally. For light microscopy (LM), bone marrow fragments from the medullary cavity were placed in 10% phosphate-buffered formal-saline and fixed for 24 hours. Specimens were dehydrated in alcohol and embedded in glycol methacrylate. The 3 µ sections were cut on a Reichert Autocut and stained by hematoxylin and eosin. The specimen shrinkage was 4% to 5% with this method. Decalcification of the marrow was not necessary.

For transmission electron microscopy (TEM), 1 mm fragments of bone marrow were rapidly immersion-fixed in 3% glutaraldehyde at room temperature for 1 hour. The tissue was postfixed in osmium tetroxide and processed to glycol methacrylate. Ultrathin sections were stained with lead citrate and uranyl acetate and examined in a Phillips EM400 transmission electron microscope.

**Megakaryocyte Quantitation**

Total megakaryocyte (MK) and nuclear areas in the planimetric section were measured with a Kontron MOP AM03 system having a distribution analysis facility. At least 40 different random fields were examined for each specimen. The variation of different sets of results within the same animal was less than 5%. At least 200 MKs were measured for each rabbit. Measurements were restricted to MKs with a nuclear-cytoplasmic ratio of less than 1, classified at maturation stages III and IV,10 having at least two nuclear lobes. Small nuclear fragments apparently separated from the main nuclear mass were assumed to be connected by the shortest bridging distance. MK cytoplasmic devoid of a nucleus was not quantitated. The calibration constant was that stated by the manufacturers for the standard AM03 28 x 28 mm tablet. The equipment was assembled to measure absolute values by the magnification input (x 188). Thus, a measured value of 2 mm² was equivalent to a real area of 2 × 10⁶/(188)² = 56.6 µ².

The measured planimetric areas were converted into volumes by the method described in earlier studies.11,12 The correction factors used to allow for MKs not being sectioned through their midsection were those described in detail elsewhere.12 The computed corrections were of the same order of magnitude (approximately 95%) as those obtained by Harker13 from histological measurement of rabbit MKs. The number of platelets produced from a single MK (on average) was evaluated from the formula:

\[
\text{number of platelets} = \frac{\text{mean megakaryocyte cytoplasmic volume (MMKCV)}}{\text{mean platelet volume (MPV)}}
\]
Megakaryocyte Nuclear DNA Content

Bone marrow taken from the femoral shafts at the time of sacrifice was stained by Feulgen's method. MK nuclear DNA content was measured by using a Vickers M85A microdensitometer as described previously. The mean DNA content was evaluated by the method described by Bessman. N is used here as the unit of megakaryocyte nuclear ploidy.

Aorta

Quantitation of Lipid Accumulation

The thoracic and abdominal organs were removed in toto. The heart and aorta were dissected free, opened longitudinally, washed gently with 0.9% (wt/vol) saline, and fixed in 10% phosphate-buffered formal-saline for 6 hours. Each specimen of heart and aorta was photographed in an identical manner, and black and white prints were prepared at the same magnification. A measure of the percentage surface area of the aorta involved in lipid accumulation was made from the ratio of the weight of the photographic paper of the involved areas cut out from the photograph prints and the weight of the total area cut out from the aortic valves to the aortic bifurcation.

Histology

For the LM and TEM studies, four segments of aorta were excised from each specimen at the level of the mid-arch, the 1st and 12th intercostal arteries, and the superior mesenteric artery. Part of the tissue was sectioned on a cryostat for lipid examination (oil-red 0, polarized light, and Schultz reaction); part was processed routinely to paraffin wax for hematoxylin and eosin, Martius scarlet blue (fibrin), and Verhoeff's (elastic) stains. Part was also postfixed in osmium tetroxide and processed for TEM as described above.

For scanning electron microscopy (SEM) study, formal-fixed aortic segments were excised at the level of the second intercostal artery. Specimens were washed in sterile, particle-free distilled water. Critical-point drying was performed by using acetone (as the intermediate fluid) and liquid CO₂ in a Polaron E3000 drying apparatus. After the specimens were dried, they were mounted on SEM stubs using high conductivity paint. Coating was carried out in an Edwards 5150 sputter-coating unit with a gold-plated cathode. We examined the specimens with a Phillips 501 scanning electron microscope (15 kV) using a viewing angle of 45°.

Plasma Cholesterol and Triglyceride Concentration

Blood plasma was analyzed quantitatively for cholesterol and triglyceride by methods based on those described elsewhere.

Statistics

All data in the text are presented as means ± standard deviation (x ± sd). Since the animals were male paired litter mates, a paired t test was used as the test of statistical significance.

Table 1. Megakaryocyte Parameters In Rabbits Fed a Normal Diet (Control) and High Cholesterol Diet (Test) for 12 Weeks

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<tr>
<th>Rabbit group</th>
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<th>Mean nuclear vol (fl)</th>
<th>Mean nuclear DNA content (N)</th>
<th>Mean cytoplasmic vol (fl)</th>
<th>Range of cytoplasmic volumes (fl)</th>
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p
< 0.001
< 0.01
NS
< 0.001
NS
< 0.05
< 0.02
Results

Bone Marrow Histology

LM showed no difference in cellularity between control and cholesterol-fed rabbits, and all hematopoietic cell lines appeared normal. The cholesterol-fed rabbits showed occasional foam cells. EM showed no morphological difference between the MKs in control and cholesterol-fed rabbits (Figure 1).

Bone Marrow Megakaryocyte Measurements

Table 1 shows the measured MK parameters in the cholesterol-fed and control rabbits. The bone marrow MK volumes \( p < 0.001 \), nuclear volumes \( p < 0.01 \), and cytoplasmic volumes \( p < 0.001 \) were significantly larger in the cholesterol-fed rabbits than in the control rabbits. The control values were of the same order of magnitude as those reported previously. The range of cytoplasmic volumes was also significantly increased \( p < 0.05 \), with an increased percentage of larger MK cytoplasmic volumes in the measured distribution (Figure 2). There was a 25% increase in mean nuclear DNA content, compatible with the observed increase in nuclear size. However, this increase in DNA was just above the 5% significance level \( p < 0.08 \) (Figure 3).

Platelets

Table 2 shows the measured circulating platelet volume distribution and count parameters. There was a statistically significant increase in PC \( p < 0.001 \) in the cholesterol-fed rabbits compared with the controls, and a significant decrease in MPV \( p < 0.04 \).

The MPV was decreased in the cholesterol-fed rabbits because of an increased frequency of small platelets between 2 and 3 fl, although there was also an increased frequency of large platelets in the tail of

Figure 1. Transmission electron micrograph showing megakaryocyte in the bone marrow of a cholesterol-fed rabbit. The appearance is normal and indistinguishable from megakaryocytes in control rabbits. × 5000. Bar = 5 μ. 
Table 2. Platelet Parameters in Rabbits Fed a Normal Diet (Control) and High Cholesterol Diet (Test) for 12 Weeks

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the volume distribution (Figure 4). These characteristics produced a significant increase in dispersion ($p < 0.003$), measured by the coefficient of variation, a significant increase in skewness ($p < 0.02$) and convexity ($p < 0.05$) of the platelet volume distributions of the cholesterol-fed rabbits compared with the control rabbits. The 95% confidence limits (the shaded regions in Figure 4) show that the average volume distributions of the two groups are highly reproducible.

The scatter diagram of PC and MPV (Figure 5) shows that ranking of PC is maintained on the cho-

![](image1.png)

**Figure 2.** Average megakaryocyte cytoplasmic volume distributions in cholesterol-fed rabbits (○---○) and control rabbits fed a normal diet (●--●).

![](image2.png)

**Figure 3.** Average megakaryocyte nuclear DNA content distributions in cholesterol-fed (----) and control rabbits (---).
**Megakaryocyte Nuclear DNA Content**

Bone marrow taken from the femoral shafts at the time of sacrifice was stained by Feulgen's method. MK nuclear DNA content was measured by using a Vickers M85A microdensitometer as described previously. The mean DNA content was evaluated by the method described by Bessman. N is used here as the unit of megakaryocyte nuclear ploidy.

**Aorta**

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</table>
Figure 4. Average platelet volume distributions in cholesterol-fed (○---○) and control rabbits (●---●). The shaded regions represent the 95% confidence limits about the average distributions.

Figure 5. Scatter diagram of platelet count and mean volume in the cholesterol-fed (○) and control rabbits (●). The intersection of the perpendicular lines represents the mean values of platelet count and mean volume of the two groups. The letter C refers to the control rabbits, while T refers to cholesterol-fed animals.
sterol diet, although there is some alteration in the ranking of MPV.

The platelet biomass was significantly higher ($p < 0.01$) in the cholesterol-fed rabbits (30.8 ± 4.2 × $10^{11}$ fl platelets/liter blood) compared with the controls (23.3 ± 1.5 × $10^{11}$ fl platelets/liter blood). The number of platelets/MK (on average) was significantly increased in the cholesterol-fed animals (2983 ± 451 platelets/MK) compared to the controls (1421 ± 209 platelets/MK) ($p < 0.02$).

**Plasma Cholesterol and Triglyceride Concentration and Weight**

The plasma cholesterol concentration was significantly higher ($p < 0.03$) in the cholesterol-fed rabbits (46.1 ± 24.4 mmol/liter) compared with the controls (0.48 ± 0.19 mmol/liter). The plasma triglyceride concentration was also increased, but not significantly, in the cholesterol-fed rabbits (10.2 ± 15.9 mmol/liter) compared with the controls (0.6 ± 0.4 mmol/liter). All rabbits in the control group increased in weight in the 12-week experimental period, from 2.34 ± 1.00 kg to 3.24 ± 0.49 kg. In the cholesterol-fed group, there was a net increase in weight of 0.38 ± 1.24 kg from 2.28 ± 1.06 kg to 2.66 ± 0.50 kg over the 12-week experimental period.

**Aorta**

**Macroscopic Appearance**

Slightly raised (0.1 to 0.3 mm) yellow areas occupying 23.6% ± 4.2% of the total area were visible in the aorta of the cholesterol-fed rabbits. This occurred maximally in the upper part of the aortic arch and in relation to the exit of the major vessels. The severity of the involvement decreased distally, but was still apparent around the intercostal, coeliac, superior, and inferior mesenteric and renal arteries.

**Light Microscopy**

The aortas of cholesterol-fed rabbits showed severe intimal thickening due to the presence of numerous foam cells. Lipid was demonstrable in these thickened areas with the use of oil-red 0 stains. The presence of strong birefringence on polarized light and the positive Schultz reaction were indicative that these areas contained cholesterol and cholesteryl esters.

Fine elastic fibers were present between the foam cells, and apparent endothelial cell nuclei were present over most of the thickened intimal areas. The medias of both control and cholesterol-fed rabbits contained small foci of calcification on elastic fibers. However, these areas bore no relationship to overlying intimal lipid accumulation in the cholesterol-fed rabbits.

**Scanning Electron Microscopy**

Low power scanning confirmed the presence of elevated intimal plaques. Higher magnification showed cellular separation and numerous surface round bodies approximately 1 μ in diameter. These correlated with structures that resembled degenerate and partially degranulated platelets.

**Transmission Electron Microscopy**

Numerous foam cells in the intimal plaques were present. They were characterized by electronlucent cytoplasmic vacuoles. The cytoplasm also contained plentiful small electron-dense granules, suggesting a histiocytic or monocyteic origin for the cells. Medial smooth muscle cells were seen extending through the inner elastic laminas into the adjacent intimal area. These cells also contained small numbers of cytoplasmic vacuoles similar to those in the more superficial foam cells. The upper part of the plaques showed considerable degenerative change. Endothelial cells were still recognizable, but intercellular attachments were absent. The surface of these plaques showed cellular debris, and amorphous granular and electron-dense fibrillar material consistent with fibrin and the structures that resembled platelets described above.

Uninvolved areas of the intima showed normal surface endothelial cells. There was no evidence of cellular degeneration or ulceration.

**Discussion**

This study demonstrates that a high cholesterol diet in the rabbit produces changes in circulating platelets, bone marrow MKs, and the arterial vessel wall.

The MKs in cholesterol-fed animals were larger than the control MKs in both mean nuclear and cytoplasmic volume. In addition, the mean nuclear DNA content of the megakaryocytes was also increased (but not significantly). It has been demonstrated elsewhere that there is a positive correlation between bone marrow MK nuclear size and DNA content. However, it must be emphasized that the nuclear volumes measured here are from only those MKs at Stage III and IV maturation, while the mean DNA content is derived from a sample of all those MKs in the marrow with a nuclear DNA content of 8 N or greater, irrespective of their stage of maturity.

The range of MK cytoplasmic volumes, as well as mean MK cytoplasmic volume, was increased in cholesterol-fed rabbits (Table 1). Since it is likely that the observed changes in the MKs occurred over a time period that was substantially greater than the rabbit mean platelet lifetime, the MKs measured in both cholesterol-fed and control rabbits would be a representative sample of those cells that had earlier produced those circulating platelets sampled in the blood.

The platelets produced from the larger MKs in the cholesterol-fed animals had a smaller MPV than those observed in the control animals. Also, the PC was higher in the cholesterol-fed animals compared...
with the controls. In a similar way to the MK size parameters, the size differences between test and control platelets were not restricted to the mean values only; the heterogeneity of the platelet volume distribution was altered (Table 2 and Figure 3). If the platelet volume distribution is determined at thrombopoiesis,\textsuperscript{11,12,20} then these changes imply differences in platelet production between the cholesterol-fed and control animals. Such differences may be explained by the increase in MK cytoplasmic size and a difference in cytoplasmic fragmentation between the two groups of animals.\textsuperscript{12} The smaller platelets observed in this experimental model are in contrast to the larger platelets seen in myocardial infarction and sudden cardiac death.\textsuperscript{5-6} However, large megakaryocytes are commonly observed in both human and cholesterol-induced experimental atherosclerosis.

The increase in MK size in the cholesterol-fed rabbits is similar to the increase observed in rabbits after acute platelet depletion by antiplatelet serum (APS) administered daily for 6 days.\textsuperscript{19} However, in that study the platelets produced from the large stimulated MKs were larger than those observed in the control rabbits, whereas here the test platelets were smaller than the controls. This implies that the use of the different procedures of MK stimulation, namely, high cholesterol diet and APS, are each associated with the production of different sized platelets from MK cytoplasmic volumes of a similar size.

The PC in the cholesterol-fed animals was increased above that of the controls. This suggests that, on average, the platelet production rate was elevated above the platelet destruction rate during the experimental time period. This hypothesis is also supported by the increased average number of platelets produced from a MK in the cholesterol-fed rabbits (2983 ± 451) compared with the controls (1421 ± 209). However, this does not exclude the possibility, discussed later, that the platelet destruction rate itself is raised above normal levels. Although the platelets were smaller in the cholesterol-fed rabbits, the increased count gave a significant increase in platelet biomass in these animals (30.8 ± 4.2 × 10^11 platelets/liter blood) compared with the controls (23.3 ± 1.5 × 10^11 platelets/liter blood).

The arterial lipid accumulation in cholesterol-fed rabbits has similarities to that observed in human arterial fatty streaks. However, whether fatty streaks in humans are precursors of complicated atheroma
tous lesions has still to be resolved since atherosclerosis can develop without elevated cholesterol. The LM and EM findings in this study confirmed the significant accumulation of lipids in the raised intimal plaques accompanied by smooth muscle cells extending through the inner elastic laminae of the media into the adjacent intimal area. However, this was associated with considerable degenerative change in surface endothelial cells, superficial foam cells, concomitant fibrin, as well as possible platelet depo-
sition.

Isotopically labeled platelets have been used to demonstrate platelet consumption in the presence of atheromatous plaques in humans.\textsuperscript{21} Decrease in mean platelet lifetime has been observed in cholesterol-fed rabbits.\textsuperscript{22,23} Such evidence suggests an increased platelet consumption rate with a high cholesterol diet in rabbits and in the presence of atherosclerosis in humans. In our study, the migration of medial smooth muscle cells into the intima suggested the possible local release of growth factors from platelets or monocytes,\textsuperscript{24,25} and this was supported by the presence of platelet-like structures on the intimal surface. Although no direct evidence for an increase in platelet destruction rate was obtained, the increase in MK size and nuclear DNA content suggested a stimulation of these cells which may have been provoked by an increase in platelet destruction rate. Alternatively, the high cholesterol diet itself may have directly stimulated the bone marrow MKs or produced a response secondary to some unidentified mechanism that is unrelated to an increased platelet consumption. No ultrastructural evidence was obtained to suggest that high blood cholesterol levels had a toxic effect on the MKs. Indeed, no lipid accumulation was seen in these cells.

In spite of the acknowledged differences between complicated atherosclerosis in humans and the experimental model that we used here, there are similarities between the changes in MK volume in this experimental study and the changes observed in human atherosclerosis.\textsuperscript{6}

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