Cholesterol-Induced Atherosclerosis

Clonal Characteristics of Arterial Lesions in the Hybrid Hare

Thomas A. Pearson, John Dillman, Haqvin Malmros, Niils Sternby, and Robert H. Heptinstall

Utilizing the observation that a majority of human atherosclerotic fibrous plaques show monoclonal characteristics, we carried out this study to determine the clonal characteristics of cholesterol-induced atherosclerosis in the hybrid hare. If this is a valid model for human atherosclerosis, the lesions produced in the aorta should be monoclonal. Glucose-6-phosphate dehydrogenase (G-6-PD) was used as an X-linked cellular marker in the female hybrid hare (Lepus timidus × Lepus europaeus), which is heterozygous for electrophoretically separable isoenzymes of G-6-PD. Hares were fed cholesterol over either a 6-month or a 16-month period, and the easily dissectable lesions in the aorta and common iliac arteries were assayed for isoenzyme activity at these times. Of the 93 lesions assayed, all had polyclonal characteristics except a single monoclonal lesion found in an animal fed cholesterol over a 16-month period. Hares fed over the 16-month period showed lesions with isoenzyme patterns having a significantly higher contribution of L. timidus isoenzyme than those found in underlying media. This suggested that a selection of cells with the L. timidus X-chromosome had taken place, but the degree of this selection was not great enough to allow any of the lesions to be defined as monoclonal.

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Glucose-6-phosphate dehydrogenase (G-6-PD) has been used as a cellular marker to demonstrate the clonal origins of a number of human diseases.1-2 G-6-PD is an X-linked enzyme in which electrophoretically separable isoenzymes frequently exist in black Americans. Accordingly, in black females heterozygous for G-6-PD isoenzymes, monoclonal lesions would contain only a single isoenzyme of G-6-PD. This cellular marker has been used to study human atherosclerotic lesions, and a majority of fibrous plaques have been found to contain a single isoenzyme, suggesting that they have monoclonal characteristics.3-5 In contrast, such monoclonal characteristics are found in only a minority of fatty streaks,4,5 the lesions regarded by many as precursors of the plaques. If, therefore, the monoclonal character of the fibrous plaque is regarded as the defining characteristic of the disease, it seems reasonable to require that lesions produced in animal models should have monoclonal characteristics in order to be considered analogous to human atherosclerosis.

The model used most extensively in experimental studies on atherosclerosis is that of the rabbit which has been fed cholesterol. Unfortunately, the rabbit cannot be tested using G-6-PD as a cellular marker since it does not possess two X-linked electrophoretically separable isoenzymes of G-6-PD. However, within the closely related hare species, a hybrid hare does exist in which the female possesses two separable isoenzymes for G-6-PD. This hare is the product of the mating of the European hare (Lepus europaeus) with the Scandinavian snowshoe hare (Lepus timidus). There is a 7% difference between the two isoenzyme bands on electrophoresis, and in a preliminary study we were able to demonstrate the feasibility of assaying induced “atherosclerotic” lesions for G-6-PD isoenzymes.7 In it aortic lesions were produced by repeatedly injuring the intimal surface of the aorta with a balloon catheter and feeding...
the hares cholesterol for a short time (2 months); all lesions were shown to contain both G-6-PD isoenzymes.

The present investigation was carried out as part of a number of studies designed to test the appropriateness of the various experimental models of atherosclerosis. It describes: 1) the production of intimal aortic lesions suitable for G-6-PD assay in the hybrid hare by feeding cholesterol over two different periods — 6 months and 16 months; 2) the G-6-PD characteristics of these lesions; 3) a comparison of the results obtained in the 6-month and 16-month cholesterol feeding regimens; and 4) a comparison of the findings in this study with those obtained in our previous experiment using balloon catheter injury and short-term cholesterol feeding. In particular, this study considers the clonal characteristics of lesions produced by cholesterol feeding alone, tests whether clonal selection on the basis of genes from one X-chromosome occurs in this animal model, and determines whether the magnitude of this selection is large enough to account for the variation in G-6-PD isoenzymes observed in human lesions. These data provide an important basis for comparison with other methods of production of experimental atherosclerotic lesions.

Methods

**Hybrid Hares**

Ten female hybrid hares were bred in Sweden by the mating of *Lepus europaeus* males with *Lepus timidus* females. The animals were 6 to 8 months of age and were fully mature at the time that the cholesterol feeding was begun. The average weight of the animals was approximately 3 kg.

**Lesion Production and Dissection**

Lesions were produced in six animals by feeding them rabbit chow containing 1.0% cholesterol by weight for 6 months. The diet was produced by soaking the pellets in cholesterol dissolved in ether/ethanol (2:1) with subsequent evaporation of all traces of the solvent. In an additional four animals, lesions were produced by feeding the animals rabbit chow containing 0.25% cholesterol and 10% cocofat by weight. These animals received this diet for 8 months, were given a regular diet for 4 months, and were then fed the cholesterol-cocofat diet for an additional 4 months, for a total feeding period of 16 months. The reason for this interrupted feeding was the fear of liver damage without a "rest" period. However, subsequent data suggest that these animals continued to be hypercholesterolemic for at least one-half the period of normal diet. Also, the lesions formed during the first 8 months were present for the entire 16 months. Thus, although cholesterol feeding per se occurred for only 12 months, the lesions assayed were 16-months-old, and for this reason, the group will be identified as the 16-month-feeding group. Serum cholesterol levels were determined using an enzymatic method (Abbott/A-Gent).

At the end of the feeding periods, all animals were sacrificed by inducing general anesthesia with intramuscular injection of ketamine and chlorpromazine followed by air embolization. The entire aorta and iliac arteries were dissected intact from each animal, opened lengthwise, and cleaned of all adventitia and intraluminal blood. A plastic overlay was placed over the aorta and an outline of the aorta and of each atherosclerotic lesion was made. The percentage of surface area involved by lesions in each aorta was then calculated by cutting and weighing on a Mettler balance. The weight of the tracing of the arterial lesions was divided by the weight of the tracing of the entire aorta and iliac arteries. Other tissues in the animal, including the spleen, liver, and heart, were also examined grossly.

Discrete lesions in the aorta and iliac arteries were then separated from underlying media using a No. 11 scalpel blade and a dissecting microscope (15–25×). One-half of each lesion was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin. Histologic sections were examined for the presence of underlying media. Lesions contaminated by media were excluded from analysis since the presence of media, which has polyclonal characteristics, would interfere with the isoenzyme patterns of the lesions. The other one-half of the lesion (surface area of 1 to 4 mm²) was assayed for G-6-PD isoenzymes. One G-6-PD assay was performed per lesion. Samples of media lying immediately below the lesion were also dissected free of adventitia for G-6-PD assay.

**G-6-PD Assay**

Small samples of lesions and of the underlying wall were assayed for G-6-PD using standardized techniques. The tissue was minced and immediately frozen on dry ice. The samples were then subjected to repeated thawing and freezing before being ground up to provide an extract. Small amounts of the extract were labelled onto plastic-backed cellulose acetate membranes (Helena Laboratories, Beaumont, Texas), and subjected to electrophoresis using Tris-EDTA-glycine buffer (pH 9.2, Gelman Sciences, Ann Arbor, Michigan). Twelve samples were applied to each membrane; lesions and their underlying wall were juxtaposed during assay. Electrophoresis was carried out at 400 V for 15 minutes at 4°C in a Helena electrophoresis chamber. The membranes were then specifically stained for G-6-PD. A 7% difference in electrophoretic mobility between the two hare isoenzyme bands was obtained, allowing an adequate, but not complete, separation of the bands. The amount of enzyme activity in each isoenzyme band was quantitated at 520 nm with the use of a high-resolution densitometer (Helena R&D). The results of electrophoresis were expressed as the percentage contribution of enzyme activity in the...
slower \((L. \text{timidus})\) isoenzyme band to the total G-6-PD activity.

**Statistical Analysis**

Means and standard deviations were calculated for the lesions and the underlying wall from each aorta. Definitions of monoclonality used in previous publications were applied to this material. Lesions whose isoenzyme values fell outside \(\pm 3\) standard deviations from the mean of values from the underlying wall were considered to have monoclonal characteristics. Correlation coefficients between the percentage of G-6-PD \((L. \text{timidus})\) in lesions and the arterial wall underlying them were also calculated. Differences between the means were tested for significance using Student's \(t\) test.

**Results**

The 10 female hares readily consumed the cholesterol-containing feed for the entire feeding period. Serum samples for cholesterol determinations were obtained when the animals were killed. The range of levels was 445 to 703 mg/dl, with a mean of 610 mg/dl. The prefeeding levels of serum cholesterol in the hare range from approximately 20 to 40 mg/dl. The elevated levels confirmed the adequate dietary intake by the animals. At autopsy, the animals revealed a number of stigmata of sustained hypercholesterolemia; these included cholesterol deposition in the sclera, liver, spleen, pleura, gut, and soft tissues.

The amount of disease, as measured by the percentage of aortic and iliac surface area involved by the lesions, varied greatly from animal to animal, and ranged from 0 to 78.2% of total aortic area (Table 1). The smaller percentage of surface area involved with lesions in the 16-month feeding group was attributed to the lower levels of cholesterol administered and the generally less intense hypercholesterolemia. The iliac arteries and the aorta distal to the renal arteries were heavily involved with discrete intimal lesions. The proximal aorta and aortic arch were also involved with multiple lesions, particularly at the branch points of the carotid and subclavian arteries, but not so severely as the abdominal aorta. In several animals, the epicardial coronary arteries were markedly stenosed with lesions similar to those seen in the aorta.

The lesions themselves were raised, whitish, and could be easily stripped off the intimal surface. Histologic examination showed the lesions to consist of large numbers of lipid-containing cells in the intima, clearly defined from the underlying media (Figure 1). A fibrous cap was noted to be forming in some of the lesions, and a breakdown of the deeper part of the fatty lesions was present in others. There was little difference between the lesions seen at 6 months and at 16 months, although the latter lesions tended to be larger and to have more easily identifiable fibrous caps.

A total of 106 samples of underlying wall and 65 samples of lesions was assayed from the six hares fed cholesterol for 6 months. The percentage of G-6-PD \((L. \text{timidus})\) in samples of both underlying arterial wall and lesions clustered in the central part of the range (Figure 2). The range and means of sample values were similar for both underlying wall and lesions (Table 2). None of the values for lesions fell outside the \(\pm 3\) so confidence limits around the mean value of underlying wall samples. Therefore, none could be defined as monoclonal.

### Table 1. Number of Samples and Percentage of Arterial Surface Area Involved with Intimal Lesions from Ten Hybrid Hares

<table>
<thead>
<tr>
<th>Hare</th>
<th>Duration of cholesterol feeding (mos)</th>
<th>Samples of underlying arterial wall (no.)</th>
<th>Samples of intimal lesions (no.)</th>
<th>Surface area involved (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>28</td>
<td>24</td>
<td>34.3</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>24</td>
<td>15</td>
<td>45.6</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>18</td>
<td>14</td>
<td>73.5</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>12</td>
<td>2</td>
<td>2.4</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>15</td>
<td>10</td>
<td>78.2</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>9</td>
<td>0</td>
<td>67.1</td>
</tr>
<tr>
<td>Subtotal</td>
<td>6</td>
<td>106</td>
<td>65</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>16</td>
<td>16</td>
<td>11</td>
<td>27.4</td>
</tr>
<tr>
<td>8</td>
<td>16</td>
<td>19</td>
<td>14</td>
<td>18.7</td>
</tr>
<tr>
<td>9</td>
<td>16</td>
<td>19</td>
<td>3</td>
<td>4.5</td>
</tr>
<tr>
<td>10</td>
<td>16</td>
<td>15</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>Subtotal</td>
<td>16</td>
<td>69</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>175</td>
<td>93</td>
<td>Mean = 35.2</td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Number of Samples and Mean Percentage of Total G-6-PD Activity in the Timidus G-6-PD Band (% Timidus) for Samples of Underlying Arterial Wall and Lesions from 10 Hybrid Hares

<table>
<thead>
<tr>
<th>Hare No.</th>
<th>Underlying media (Mean % ± 1 SD)</th>
<th>Lesions (Mean % ± 1 SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72.0 (±4.0)</td>
<td>71.9 (±4.5)</td>
</tr>
<tr>
<td>2 24</td>
<td>62.3 (±3.6)</td>
<td>58.1 (±3.3)</td>
</tr>
<tr>
<td>3 18</td>
<td>47.2 (±3.6)</td>
<td>42.8 (±1.7)</td>
</tr>
<tr>
<td>4 12</td>
<td>59.8 (±3.4)</td>
<td>56.2 (±2.3)</td>
</tr>
<tr>
<td>5 15</td>
<td>59.8 (±4.3)</td>
<td>58.6 (±6.2)</td>
</tr>
<tr>
<td>6 9</td>
<td>55.8 (±3.4)</td>
<td>0</td>
</tr>
<tr>
<td>Subtotal</td>
<td>61.1 (±9.0)*</td>
<td>59.9 (±11.6)*</td>
</tr>
<tr>
<td>7 16</td>
<td>60.4 (±4.3)</td>
<td>62.8 (±9.1)</td>
</tr>
<tr>
<td>8 19</td>
<td>59.9 (±6.7)</td>
<td>62.0 (±6.8)</td>
</tr>
<tr>
<td>9 19</td>
<td>56.0 (±3.1)</td>
<td>55.2 (±8.8)</td>
</tr>
<tr>
<td>10 15</td>
<td>53.9 (±6.9)</td>
<td>0</td>
</tr>
<tr>
<td>Subtotal</td>
<td>57.6 (±6.0)*†</td>
<td>61.6 (±8.0)</td>
</tr>
<tr>
<td>Total</td>
<td>59.7 (±8.1)†</td>
<td>60.4 (±10.6)</td>
</tr>
</tbody>
</table>

* test for difference between underlying wall and lesion
† test for difference between underlying wall and lesion
A total of 69 samples of underlying wall and 28 samples of lesions was studied from the four hares fed cholesterol and cocofat for 16 months. The percentage of G-6-PD timidus for lesions and samples of underlying wall again clustered in the central part of the range (Figure 3), although samples of lesions tended to have values higher than those of underlying wall from the same animals. The mean percent-

**Figure 1.** Section of aorta showing normal media and the thick, easily distinguishable intimal lesion. The thickened intima is made up of lipid-containing cells. Hematoxylin and eosin, x 320. Bar = 100 μ.

**Figure 2.** Distribution of the percentage of G-6-PD timidus isoenzyme values for 65 samples of intimal lesions and 106 samples of underlying wall from six hares fed cholesterol for 6 months.

**Figure 3.** Distribution of the percentage of G-6-PD timidus isoenzyme values for 28 samples of intimal lesions and 69 samples of underlying wall from four hares fed cholesterol and cocofat over a 16-month period.
age of G-6-PD "timidus" values of samples of lesions was significantly higher ($p = 0.008$) than that of samples of underlying wall (Table 2). Only one lesion had a value that fell outside the $\pm 3$ SD confidence limits around the mean value of underlying wall samples. This lesion had an isoenzyme value of 45% G-6-PD "timidus" and thus had significantly more G-6-PD "europaeus" isoenzyme than did the underlying aortic wall.

To better determine if any systematic deviation in the percentage of G-6-PD "timidus" distribution occurred in either group of hares, the percentage of G-6-PD "timidus" in lesions was plotted against the percentage of G-6-PD "timidus" in the samples of underlying wall from which the lesion was dissected. For the group fed 6 months, a very high correlation ($r = 0.950$, $p < 0.001$) was observed between the lesions and underlying wall (Figure 4). The G-6-PD isoenzyme values of lesions were virtually identical to those of the arterial wall lying underneath; not only was there a very high correlation coefficient, but also little variation between the lesion and the underlying wall. However, a similar analysis of lesions and underlying wall from the group fed 16 months showed a poorer correlation ($r = 0.347$, $p < 0.05$) (Figure 5). The G-6-PD isoenzyme values of lesions were no longer identical to those of underlying wall, and values of lesions showed considerable variation from the values of underlying wall. As reflected in the mean values of lesions and underlying wall, there was an overall trend of higher G-6-PD "timidus" values in the lesions.

Finally, the results from these two groups were compared with those for the female hybrid hares which were subjected to balloon catheterization and short-term cholesterol feeding (2 months, 1% cholesterol) described in our previous publication. The mean value for samples of underlying wall in the balloon catheterized animals (57.3% G-6-PD "timidus") was similar to the means for underlying wall in the present study (61.1% for 6-month feeding, 57.6% for 16-month feeding). The mean percentage of G-6-PD "timidus" in the lesions from balloon-catheterized animals was no different from the mean of underlying wall samples in the same group and showed a significant correlation with it ($r = 0.564$, $p < 0.025$). However, the mean percentage of G-6-PD "timidus" of the lesions in the balloon catheterized animals was lower than that of the 6-month feeding group (56.8% vs 59.9%, $p = 0.302$) and very much lower than the mean of the 16-month feeding group (61.6%, $p = 0.038$). The isoenzyme patterns of the animals in the balloon catheterization study resembled those of the 6-month feeding group in the present study more closely than they resembled those of the 16-month feeding group.

**Discussion**

Advances in our knowledge of the biology of human atherosclerosis have been hindered by a lack of appropriate animal models. Most studies in animal models have relied on gross morphologic or histologic comparisons with human lesions. Multiple techniques have been used to produce atherosclerotic lesions in a variety of animal species, with cholesterol feeding or mechanical trauma to the intima of the artery being the most widely used. On the basis of studies using these lesions, numerous conclusions about the natural history, cellular biology, and biochemistry of the atherosclerotic plaque have been drawn. However, the experimental lesions in the widely used rabbit differ from human lesions in...
several important ways. These include lack of pauci-
ty of ulceration, hemorrhage, or fibrous cap in the
lesion; location of the lesion in the proximal parts of
the aorta rather than the distal; predominant involve-
mint of intramyocardial branches of the coronary
arteries rather than of epicardial arteries; and the
widespread deposition of cholesterol in such organs
as the liver, spleen, skin, and eye.13,19

The present study was designed to test the validity
of the cholesterol feeding model using monoclonality
of the lesion produced as the definitive criterion for
atherosclerosis. The hybrid hare used in the exper-
mints showed lesions similar to those in the rabbit,
although it should be pointed out that there were
certain differences. These included the development
in certain of the aortic lesions of a fibrous cap with
some degree of breakdown of the core, a distribution
of lesions predominantly in the distal part of the aorta
and common iliac arteries, and involvement of the
epicardial portions of coronary arteries. However, it
continues to resemble the rabbit model especially in
the generalized visceral deposition of cholesterol.

Despite the long feeding period, almost no lesions
had monoclonal characteristics. All 65 lesions from
hares fed 6 months and 27 of 28 lesions from hares
fed 16 months contained isoenzyme patterns similar
to those of the underlying arterial wall. Even the sin-
gle lesion which met this study’s criterion for mon-
clonality contained both isoenzymes. The isoen-
zyme value of this lesion, 45% G-6-PD timidus, was
very different from the G-6-PD isoenzyme values
found in human fibrous plaques, which usually have
values of the B isoenzyme of less than 25% or great-
er than 88%.5 Thus, although it met the criteria for
monoclonality used in this study, it would probably
not have met our present requirements for mono-
clonality in man and would have been placed in an
intermediate position. Therefore, little or no evidence
was present in these data to suggest that the lesions
were becoming monoclonal and, in this way, they
remain fundamentally different from those of man.

The results are similar to those of the only other
study of G-6-PD isoenzymes in diet-induced athero-
sclerosis in the hybrid hare. Lee et al.20 produced
lesions in 12 female hybrid hares with an intermittent
feeding of diet containing 1% cholesterol and 6%
peanut oil. The diet was fed for 4 to 12 months.
Results were obtained from six animals and these
showed no monoclonal lesions among the 10 large
lesions and 36 small lesions produced. Unfortunate-
ly, the results were not analyzed according to dura-
tion of feeding, and only two animals were fed for
more than 6 months. However, these results coinci-
ded with those of the present study in that dietary
cholesterol alone did not produce any monoclonal
lesions.

A number of theories have been proposed to ex-
plain the observed monoclonality of fibrous plaques
in man. Since most monoclonal lesions described to
date have been tumors,1,2 mutation has been sug-
gested as the initial event in the formation of the
fibrous plaque. However, another theory has been
proposed,2 namely, that of clonal selection. Cycles
of cell proliferation and cell death may select clones of
cells with a proliferative advantage. Genes on the X-
chromosome may provide some of this selective ad-
vantage, resulting in monoclonal lesions with the
same G-6-PD isoenzymes. Evidence for this theory
has been derived from tissue culture experiments
using cultured skin fibroblasts from black females
heterozygous for G-6-PD.21,22 Cells passed serially
in tissue culture often lost one of the two isoen-
zymes. This suggested a selection of one or a few
clones of cells during cellular proliferation. Skin fi-
broblasts from the hybrid hare have also been stud-
ied in tissue culture.23 After serial passes in tissue
culture, these cells also exhibited only one or the
other isoenzyme types. It was again suggested that
certain cells had a selective proliferative advantage.
This same proliferative advantage could then explain
any formation of lesions with a single isoenzyme
type.

However, the results of in vitro studies are difficult
to interpret as predictors of in vivo behavior. The
selective stimuli may be much more intense than
would occur in vivo. Also, different conditions in the
tissue culture may select clones of cells with certain
characteristics. Evidence for this can be found within
tissue culture experiments using hybrid hare fibro-
blasts.24 The addition of 25-hydroxycholesterol to the
medium resulted in cells with the Lepus europaeus
X-chromosome (exhibiting G-6-PD europaeus) be-
coming predominant. Thus, the G-6-PD isoenzyme
type of cell population can be changed merely by
alteration of the tissue culture medium.

In vivo evidence for clonal selection has been
more difficult to obtain. We have documented that
this phenomenon occurs during the healing process
within human scars in the skin,20 a process that con-
sists of intense cellular proliferation without muta-
tion. However, the magnitude of the selection was
not comparable to the extreme variations in the G-6-
PD isoenzymes patterns observed in human athero-
sclerotic fibrous plaques. The aortic lesions pro-
duced by cholesterol feeding over 16 months
showed a significant trend toward higher values of
the percentage of G-6-PD timidus, suggesting that
the prolonged proliferative stimulus provided by the
dietary cholesterol may have preferentially selected
cells containing the timidus X-chromosome. Howev-
er, as was the case with the human cutaneous scars,
the magnitude of the selection was far less than in
human fibrous plaques, in spite of the statistically
significant differences in enzyme patterns. It is of
note that the only monoclonal lesion produced in the
present study had an isoenzyme value with a lower,
rather than higher, value of the percentage of G-6-
PD timidus than samples of underlying wall.

In conclusion, it has been shown that discrete and
easily dissectable lesions, amenable to assay for
isoenzymes of G-6-PD, can be produced by the
feeding of cholesterol. Although there was a sugges-
tion that a slight drift towards monoclonality was tak-
ing place in the lesions of hares fed over 16 months,
and that a single lesion was monoclonal using prees-
tablished criteria, the isoenzyme patterns indicated
an essentially polyclonal cell population. In spite of
the fact that the distribution and histologic features in
the cholesterol-fed hare more closely resemble hu-
man atherosclerosis than do those in the  similarly
produced lesion in the rabbit, the clonal characteris-
tics are not those of human fibrous plaques.

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characteristics of cutaneous scars and implications for ath-

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