Social Environment Influences the Progression of Atherosclerosis in the Watanabe Heritable Hyperlipidemic Rabbit

Philip M. McCabe, PhD; Julie A. Gonzales, PhD; Julia Zaias, DVM; Angela Szeto, BA; Mahendra Kumar, PhD; Alan J. Herron, DVM; Neil Schneiderman, PhD

Background—Although there is evidence that emotionally stressful behavior can accelerate the progression of atherosclerosis, there is less data to support the notion that affiliative social behavior can slow disease progression. The present study examines the influence of social environment on the progression of atherosclerosis in the Watanabe Heritable Hyperlipidemic (WHHL) rabbit, a model that spontaneously develops lesions because of a genetic defect in lipoprotein clearance.

Methods and Results—WHHL rabbits were assigned to 1 of 3 social or behavioral groups: an unstable group, in which unfamiliar rabbits were paired daily, with the pairing switched each week; a stable group, in which littermates were paired daily for the entire study; and an individually caged group. The stable group exhibited more affiliative social behavior and less agonistic behavior than the unstable group and significantly less aortic atherosclerosis than each of the other 2 groups. Although the unstable and individually caged groups had comparable aortic lesion areas, the severity of the disease progressed faster in the unstable group, as indexed by a larger area of calcification and increased fibrous cap thickness in complex lesions. The unstable group showed increased agonistic behavior and signs of chronic adrenocortical and gonadal activation, whereas the individually caged group was relatively sedentary, had low glucocorticoid levels, and was hyperinsulinemic compared with the other groups.

Conclusions—The present study demonstrates that social environment can slow, as well as accelerate, the progression of atherosclerosis. It also emphasizes the importance of behavioral factors in atherogenesis, even in a model of disease with strong genetic determinants. (Circulation. 2002;105:354-359.)

Key Words: behavior ■ social environment ■ atherosclerosis ■ rabbits

Behavioral factors have been implicated in the etiology of atherosclerosis and coronary heart disease (CHD).1 It has been established that cynomolgus macaques, fed an atherogenic diet and exposed to an emotionally stressful social environment, develop greater CHD than monkeys in a stable social environment or animals fed a low-fat diet.1–4 These studies suggest that behavioral factors can promote disease progression; however, there is far less evidence that manipulation of emotional behavior can slow the progression of atherosclerosis. Notably, it was demonstrated that repeated handling and petting of albino rabbits decreased aortic atherosclerosis relative to controls.5 The paucity of research relating to this topic may be attributable to the fact that in most of these models, atherosclerosis does not develop spontaneously, and, therefore, it is difficult to demonstrate an experimental attenuation of the disease process.

The Watanabe Heritable Hyperlipidemic (WHHL) rabbit is an inbred strain of rabbit that exhibits hypercholesterolemia, elevated plasma LDL levels, and severe atherosclerosis.6,7 This abnormality is inherited as a single-gene mutation, with a genotype and phenotype that is strikingly similar to human familial hypercholesterolemia.8 The WHHL rabbits spontaneously develop observable atherosclerotic lesions in the aortic arch beginning at 2 months of age, and by 6 months of age, 100% of WHHL rabbits have severe atherosclerotic lesions at all levels of the aorta.6 Although many diseases, such as familial hypercholesterolemia, have strong genetic determinants, the expression of disease can vary as a result of environmental factors. To date, there have been no published studies that have examined the relationship of behavioral factors to the progression of atherosclerosis in the WHHL rabbit. This may be attributable, in part, to the belief that genetic variables in this model are so predominant that behavior does not have an appreciable influence on disease progression.
The present study examines the role of behavioral factors in the progression of atherosclerosis in the WHHL rabbit. Specifically, animals were chronically exposed to 1 of 3 social conditions: (1) an unstable social environment, in which social pairings were rearranged weekly; (2) a stable social environment, in which social pairings were maintained throughout the study; and (3) a control environment, in which animals were individually caged. Because the WHHL rabbit spontaneously develops atherosclerosis, the experimental design allowed for an examination of the behavioral factors that slow the progression of atherosclerosis as well as the factors that accelerate disease progression. In addition, the present study provides a demonstration of the importance of behavioral factors in the pathophysiology of atherosclerosis, even in a model of disease where genetic determinants are prepotent.

Methods

Experimental Animals

All procedures were in accordance with protocols approved by the Animal Care and Use Committee of the University of Miami. Thirty-six male WHHL rabbits (2.5 months old, ~1.5 kg) were obtained from Covance Research Products, Inc (Denver, Penn). All animals were exposed to 12-hour light and dark conditions (lights on at 7 AM) and were housed in individual cages (6 sq ft). Animal were fed standard rabbit chow (Purina; 2.5% total fat, 0% cholesterol) and water ad libitum. The rabbits were weighed each week of the 4-month study. During the course of the experiment, 2 animals died (1 from the stable group and 1 from the individually caged group). Necropsy results were inconclusive; however, these animals did not exhibit remarkable heart disease or aortic atherosclerosis. Because the rabbit from the stable group died 1 month into the study, the animal that was socially paired with the dead rabbit was also excluded from the study.

Social-Behavioral Manipulation and Behavioral Scoring

After acclimation procedures, the rabbits were assigned to 1 of 3 social environments. In the unstable group (n=12), animals were paired together for 4 hours per day. Each week the pairings were switched within the groups, requiring rabbits to continually reestablish dominant-subordinate relationships. Because the pairings occurred in the home cages of the animals, each week one half of the rabbits remained in their home cage and one half of the rabbits were moved to different cages on the same daily schedule as the other groups (ie, one half of the animals in the home cage and one half were moved into a holding cage for 4 hours per day). All 3 groups of animals were drawn from the same pool of 11 litters that were born during the same week in the same colony. In an effort to enhance affiliative social interactions in the stable group, littermates from 5 different litters were identified and paired together for the entire study. Therefore, the stable group consisted of pairs of rabbits that were familiar with one another in addition to experiencing constant social pairings. The unstable group consisted of animals drawn from 7 litters, but these animals were never paired with a littermate, and the individually caged group was drawn from 4 litters. Because there was an overlapping distribution of animals from the 11 litters among the 3 groups, it is unlikely that any group differences in the dependent measures (see Results) were attributable to systematic influences of prenatal environment (ie, same litter). Preliminary ANOVA confirmed that there were no significant differences in total aortic lesion area, which was an important dependent variable, as a function of litter (F[10,22]=1.41, P=0.05).

When the rabbits were 3 months old, the social pairings were initiated and continued daily until the animals were 7 months old. The occurrence of specific behaviors was scored 3 times a week for each animal during the first 10 minutes of the social pairing for that day. Behaviors were classified into the following major categories: agonistic behavior, affiliative behavior, other nonagonistic behavior, and inactivity.9,10 Agonistic behaviors included approach behavior, chasing, mounting, copulation, pawing, marking, enuresis, biting, combat, immobility or freezing, escape, escape mount, squealing, and thumping. Affiliative behaviors included grooming the cage-mate, sniffing the cage-mate, and nuzzling.10 Other nonagonistic behaviors included self-grooming, cage exploration, and drinking or eating. Inactivity consisted of passive rest, ie, stretched out in a relaxed posture or sitting quietly in the cage. Because these behaviors consist of behavioral acts, which are momentary, and behavioral states, which persist over time, all behavioral activity was scored as the percentage of time that was spent in that behavior during the observation period. The total percent time of each behavior for each animal was then calculated for the week. The individual behavioral scores were then combined into the 4 main categories (ie, agonistic behavior, affiliative behavior, other nonagonistic behavior, and inactivity), and the weekly totals for each category were summed to provide a total behavioral score for the entire 4-month study.

Blood Sampling, Handling, and Biochemical Assays

Blood was drawn from all animals for the measurement of plasma corticosterone, cortisol, testosterone, and estradiol at the end of each week of the study. Rabbits produce both corticosterone and cortisol,11 and, therefore, it is necessary to measure both hormones to get a complete profile of glucocorticoid responses. Plasma lipids and insulin were measured from overnight fasted blood samples before and every 6 weeks during the behavioral pairings. All blood samples were taken between 7:00 and 9:00 AM. During loose restraint, blood was sampled from the marginal ear vein. The plasma was then separated by centrifugation at 4°C and drawn off and stored in aliquots at ~80°C until assay. The hormones were assayed using commercially available radioimmunoassay kits, and lipid and lipoprotein evaluation of plasma was performed on an automated analyzer (Roche Diagnostic Corp). Measurement of Cardiovascular Responses

Before the behavioral pairings and subsequently every 4 weeks, resting systolic, mean, and diastolic blood pressure and heart rate were measured while the animals were loosely restrained via an automated tail-cuff system (model 29SPP, IITC, Inc). Three separate measures of blood pressure and heart rate were taken, and the daily mean for each measure was computed.

Histomorphometric Methods and Measures

After euthanasia, the entire aorta, heart, adrenal glands, and testes were removed via a midline thoracic and abdominal incision, and the tissue was placed in a 10% solution of buffered formalin. Samples were coded, and all histomorphometric procedures were performed in a blind fashion. Under gross examination, the two-dimensional area of each aortic lesion from each animal was calculated by multiplying the length and width of each lesion in microns. The area of all of the lesions for each animal was summed for the calculation of total lesion area (mm²). For the entire extent of the aorta, each lesion or plaque that was identified by gross examination was sectioned at the point of maximum height, or midlesion. For sectioning, the tissue was paraffin-embedded, sectioned at 5 μm, and stained with H&E. Aortic lesions were observed under light microscopy and graded according to the present American Heart Association (AHA) histologic classification for atherosclerotic lesions.12,13
Once the social pairings were initiated, significant behavioral differences emerged among the groups (Table 2). The unstable group exhibited significantly more total agonistic behavior across the study than the stable group (F[1,20]=29.1, P<0.0001), whereas the stable group showed significantly more total affiliative behavior (F[1,20]=8.6, P<0.01) than the unstable group. Because the individually caged animals were never paired with a conspecific, by definition they did not exhibit agonistic or affiliative behavior and, therefore, were excluded from the analyses of agonistic and affiliative behavior. The individually caged group exhibited a larger percentage of other nonagonistic behavior than the stable group, who in turn showed more of these behaviors than the unstable group (F[2,30]=46.1, P<0.0001). The individually caged group also exhibited significantly more inactivity than either of the other groups (F[2,30]=288, P<0.0001), whereas the stable group was significantly more inactive than the unstable group (P<0.05). As can be seen from the mean values in Table 2, the individually caged animals were sedentary relative to the other groups over the entire experiment, spending >60% of the observed time in physical inactivity.

### Aortic Atherosclerosis

All animals exhibited some degree of aortic atherosclerosis, with the most prevalent lesions being types II, III, and V. Type II lesions consisted of stratified layers of foam cells (ie, lipid-laden macrophages) and lipid within smooth muscle cells. Type III lesions were considered preatheroma lesions and were characterized by the presence of extracellular lipid in addition to characteristics of type II lesions. Type V lesions were advanced, raised lesions characterized by a well-defined lipid core (atheroma) in association with a fibrous or smooth muscle cap (fibroatheroma, type Va; Figure 1) or focus of mineralization (calcified plaque, type Vb).

As seen in Table 3, there were significant group differences in the total aortic atherosclerotic lesion area (F[2,30]=4.4, P<0.025). Post-hoc tests revealed that the stable group exhibited significantly less lesion area than either the unstable (P<0.05) or individually caged (P<0.01) groups. The stable group also had a significantly smaller percentage of the total aorta covered by lesions than either of the other 2 groups (F[2,30]=4.4, P<0.025). In general, there were few significant differences among groups in the distribution of lesion volume throughout the aorta (ie, aortic arch, thoracic aorta, and abdominal aorta), suggesting that spatially within the aorta, the disease progresses similarly in all animals. One notable exception was that for the individually caged group, there was greater abdominal atherosclerosis than for either of the other 2 groups (F[2,30]=3.5, P<0.05). In terms of the severity of the lesions, there were no significant group differences among the groups in the distribution of lesion severity.
differences in the total area of AHA type III lesions (F[2,30]=1.7, P>0.05) or type Va lesions (F[2,30]=1.5, P>0.05); however, in the type Va lesions, the unstable group exhibited significantly greater cap thickness than either of the other groups (F[2,163]=7.68, P<0.001). In addition, there were significant group differences in the total area of type Vb lesions (log transformation, F[2,30]=4.8, P<0.025), such that the unstable group had more calcified lesion area than the other groups. Thus, although the unstable and individually caged groups showed statistically comparable total aortic lesion areas, the data suggest that the severity of disease progressed at a faster rate in the unstable group. Several physiological variables linked to behavior may have accounted for these group differences in atherosclerosis, including stress hormone responses, cardiovascular activity, lipid levels, body weight, and insulin levels. Each of these factors was evaluated as described below.

**Hormonal Responses**

There were significant group differences in the mean weekly corticosterone values averaged across the 4-month study (F[2,30]=5.87, P<0.01), such that the unstable group had significantly greater levels than the individually caged group (Figure 2). There were no significant group differences in mean cortisol values (F[2,30]=1.45, P=0.25). In terms of histopathological findings, the unstable group developed significantly heavier adrenal glands (corrected for body weight) than either of the other groups (F[2,30]=7.10, P<0.01), suggesting relative adrenal hypertrophy in the unstable group. Although there were no significant differences among the group for mean testosterone (F[2,30]=0.19, P=0.82) nor mean estradiol levels (F[2,30]=1.41, P=0.26), the unstable group exhibited greater testicular weight (corrected for body weight) than the individually caged group (F[2,30]=3.20, P=0.05). These data suggest that plasma gonadal steroids may be too variable to reflect chronic group differences; however, the testicular hypertrophy seen in the unstable group may reflect that increased aggression is accompanied by chronic activation of the hypothalamic-pituitary-gonadal axis.

**Figure 1.** Photomicrograph of an H&E-stained section through a lesion in the aortic arch (unstable-group animal). This is an example of an advanced, raised lesion with a well-defined lipid core (atheroma) in association with a fibrous cap (fibroatheroma, grade 5a). Scale bar=22 μm.

**TABLE 3. Histopathological Measures by Group (Mean±SEM)**

<table>
<thead>
<tr>
<th>Measure</th>
<th>Unstable (Mean±SEM)</th>
<th>Stable (Mean±SEM)</th>
<th>Individually Caged (Mean±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total aortic lesion area, mm²</td>
<td>522.0±90.9</td>
<td>258.6±92.6*</td>
<td>639.1±86.9</td>
</tr>
<tr>
<td>Aortic surface covered by lesions, %</td>
<td>37.8±6.6</td>
<td>18.8±6.7*</td>
<td>46.3±6.3</td>
</tr>
<tr>
<td>Lesion area by site, mm²</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Aortic arch</td>
<td>99.5±20.9</td>
<td>52.3±17.3</td>
<td>67.1±16.4</td>
</tr>
<tr>
<td>Thoracic arch</td>
<td>381.9±93.6</td>
<td>165.1±83.6</td>
<td>417.2±58.4</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>40.6±15.5</td>
<td>41.1±17.2</td>
<td>154.7±56.4†</td>
</tr>
<tr>
<td>Total area of type III lesions, mm²</td>
<td>185.4±72.6</td>
<td>26.7±10.1</td>
<td>189.3±90.3</td>
</tr>
<tr>
<td>Total area of type Va lesions, mm²</td>
<td>268.1±105.1</td>
<td>175.8±88.7</td>
<td>419.6±98.4</td>
</tr>
<tr>
<td>Total area of type Vb (calcified) lesions, mm²</td>
<td>68.5±27.8</td>
<td>48.4±43.5</td>
<td>30.1±14.5</td>
</tr>
<tr>
<td>Log transformation</td>
<td>1.5±0.2*</td>
<td>−0.3±0.6</td>
<td>0.1±0.5</td>
</tr>
<tr>
<td>Maximum cap thickness (type Va lesions)</td>
<td>80.4±7.3‡</td>
<td>49.3±5.0</td>
<td>55.4±5.1</td>
</tr>
</tbody>
</table>

*P<0.025.
†P<0.05.
‡P<0.001.
Cardiovascular Measures

All animals in this study remained normotensive throughout the experiment; however, there were significant group differences in some of the resting cardiovascular measurements at the end of the 4-month study (Table 4). Most importantly, the individually caged group had significantly elevated heart rate compared with the unstable group (F[2,30]=3.39, P<0.05; Fisher’s mean comparison, P<0.05). In addition, the unstable group exhibited significantly lower mean arterial pressure than either of the other groups (F[2,30]=4.13, P<0.05).

Lipids, Insulin, and Body Weight

As seen in Table 5, at the end of the study there were no significant differences among the groups in total cholesterol (F[2,29]=2.11, P=0.14), triglycerides (F[2,29]=0.56, P=0.58), or HDL cholesterol (F[2,29]=1.23, P=0.31). Additionally, there were no group differences in these measures as a function of time (ie, baseline to end of study). Although the plasma insulin levels of the 3 groups did not differ at baseline, the individually caged group became significantly hyperinsulinemic relative to the other groups by the end of the study (F[2,30]=6.35, P<0.01). Finally, although the 3 groups did not differ in body weight at baseline, the individually caged group weighed more at the end of the study than the other groups (F[2,30]=6.61, P<0.01). Taken together, it is apparent that the individually caged animals developed greater body weight and hyperinsulinemia and higher resting heart rates relative to the other groups.

Discussion

The major finding of this study was that a stable social environment, characterized by increased affiliative behavior and

<table>
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<th>TABLE 4. Final Cardiovascular Measures by Group (Mean±SEM)</th>
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<tr>
<td>HR, bpm</td>
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<td>SBP, mm Hg</td>
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<td>DBP, mm Hg</td>
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<td>MAP, mm Hg</td>
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Abbreviations as in Table 1.
*P<0.05 vs unstable.
†P<0.05 vs stable and individually caged.

<table>
<thead>
<tr>
<th>TABLE 5. Final Lipid, Insulin, and Body Weight Measurements by Group (Mean±SEM)</th>
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<tr>
<td>Total cholesterol, mg/dL</td>
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<tr>
<td>Triglycerides, mg/dL</td>
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<tr>
<td>HDL cholesterol, mg/dL</td>
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<tr>
<td>Insulin, μg/mL</td>
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<tr>
<td>Body weight, kg</td>
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*P<0.01 vs stable and unstable.
relatively less agonistic behavior, slows the progression of atherosclerosis relative to unstable social conditions or individual caging in the WHHL. These data suggest that the behavioral profile observed in the stable environment may be protective by slowing the progression of disease, even in animals genetically predisposed to disease. It has been proposed that central nervous system mechanisms involved in the expression of affiliative behavior buffer the organism from the harmful effects of emotional stress by modulating the hypothalamic-pituitary-adrenocortical (HPA) axis and sympathetic nervous system (SNS) outflow.14,15

Although the total lesion area of aortic atherosclerosis was comparable in the unstable and individually caged groups, the unstable group exhibited more severe disease, as indexed by increased lesion cap thickness and greater calcified lesion area. Because the unstable group, relative to the individually caged group, exhibited more agonistic behavior, greater corticosterone values, adrenal hypertrophy, and signs of increased pituitary-gonadal activation, it is proposed that the severe progression of disease in this group is related to exposure to chronic emotional stress. In humans, the tendency to experience emotions such as hostility or anger has been associated with the occurrence of CHD.16 It has been suggested that chronic SNS activation or HPA activity elicited during chronic emotional stress plays an important role in the progression of atherosclerosis.4 Although the present study found evidence for HPA activation in the unstable group, the role of the sympathetic nervous system was not evaluated systematically.

Individual housing, which is the social condition for almost all previous WHHL studies, led to significant aortic atherosclerosis despite the fact that these individually caged animals, by definition, exhibited no agonistic behavior (or other stressful behaviors, such as cowering, vocalizations, or sleep or feeding disturbances) and had low corticosterone levels. It has been reported that female cynomolgus monkeys housed in single cages develop significantly more coronary artery atherosclerosis than animals housed in social groupings.17 The authors suggested that the results might be attributable to the distress of social isolation and activation of the SNS; however, no systematic measurements of the individually caged animals’ behavior or physiological indices of distress were presented. In addition, changes in body weight and insulin values were not reported in that study. Therefore, although individually caged cynomolgus monkeys and WHHL rabbits both develop significant atherosclerosis, it is not presently clear if emotional distress plays a significant role in disease progression during this social condition in rabbits. In the present study, individually caged animals were relatively sedentary, gained more body weight, and developed profound hyperinsulinemia relative to the unstable and stable groups. Interestingly, it has been demonstrated that WHHL rabbits are insulin resistant and hyperinsulinemic relative to healthy Japanese White rabbits.18,19 It is tempting to speculate that in the WHHL model, the progression of disease in the individually caged group may be related to the insulin metabolic variables. The insulin metabolic syndrome in humans is associated with a clustering of metabolic variables, including obesity, insulin resistance, hyperinsulinemia, lipid abnormalities, and atherosclerotic disease.20

The present study provides evidence that the expression of genetic disease can vary as a result of environmental factors. The demonstration that social environment influences disease progression in an experimental model with such strong genetic determinants illustrates the importance of behavioral factors in atherogenesis. The WHHL behavioral model should also facilitate a systematic investigation of the central nervous system, cardiovascular, endocrine, and immunological mechanisms by which behavior mitigates or promotes the progression of atherosclerosis.

Acknowledgment

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