# *Early Atherogenesis in White Carneau Pigeons*

## *I. Leukocyte Margination and Endothelial Alterations at the Celiac Bifurcation*

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**The addition of 0.2% or more cholesterol to the diet of young White Carneau pigeons produced atherosclerotic lesions within 10 weeks in a 2-sq mm area of the lower thoracic aorta. Concurrent with lesion development, a shift in the shape of endothelial cells from fusiform to polygonal was noted. This shift changed the ratio of endothelial cell width to length from 0.34 in pigeons receiving a control diet to 0.50 in pigeons receiving cholesterol. In contrast, endothelial cells in an atherosclerosis-resistant region 6 cm superior to the test region retained their fusiform shape despite the addition of cholesterol to the pigeon's diet. Cholesterol diets also increased adherence of leukocytes to the luminal surface of the aorta. This was most prevalent at the edge of large lesions (2430 ± 180 cells/sq mm) and over small lesions (2240 ± 150 cells/sq mm). Leukocyte adherence was also increased in the central region of large lesions (960 ± 140 cells /sq mm). In addition, leukocyte activation, as evidenced by crawling or spreading cells, was increased almost twofold over small lesions and the edge of large lesions when compared with adherent cells over nonlesion areas. (Am J Pathol 1984, 116:56–68)**

THE WHITE CARNEAU (WC) pigeon is particularly useful as a model for studying aortic atherosclerosis since, by 4 years of age, nearly all WC pigeons have naturally occurring aortic lesions.[1] Histologically and biochemically the lesions resemble those of human atherosclerosis[2,3] and occur predictably in the lower thoracic aorta near the origin of the celiac artery.[3,4] In addition, the extent of aortic atherosclerosis can be significantly increased, and the timing of its occurrence accelerated by including cholesterol in the WC pigeon's diet.[5] Like the naturally occurring lesions, the cholesterol-induced lesions resemble those of human atherosclerosis[5] and occur predictably at the celiac bifurcation.[3,4]

A characteristic component of both naturally occurring[3,6] and cholesterol-exacerbated[5] atherosclerotic lesions in the WC pigeon is the presence of large, lipid-filled foam cells. The origin of these foam cells in the WC pigeon lesion is still undetermined, but evidence from other animal models indicates that foam cells can originate either intramurally from smooth muscle cells[7,8] or extramurally from blood monocytes.[9–11] With regard to the extramural origin of foam cells, blood leukocytes have been observed on the surface of atherosclerotic lesions in a number of species,[12–14] including the WC pigeon.[15] The exact relationship of these adherent leukocytes to atherogenesis is not known, but it has been suggested that the adherent cells represent cells which transiently move into and out of the artery wall.[16]

Recent studies in our laboratory have focused on early events in the cholesterol-accelerated atherosclerosis in the pigeon. The present report summarizes correlative scanning electron-microscopic

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(SEM) observations and morphometric analyses of lesion progression and leukocyte margination during the early stages of cholesterol-accelerated atherosclerosis in young WC pigeon aortas. In addition, comparisons are made between the cholesterol-accelerated lesions in young pigeons and lesions occurring naturally in older (6–12 years old) WC pigeons.

## Materials and Methods

The animals used in this study were randomly bred WC pigeons obtained from a closed colony at the Pigeon Resource at the Bowman Gray School of Medicine. Fifty pigeons were entered into the study at 8 weeks of age and assigned to one of five separate diets for 10 weeks before being sacrificed. The first group (control group) received a cholesterol-free pigeon pellet diet for 10 weeks, and the remaining groups received pellet diets supplemented with 0.1%, 0.2%, 0.3%, or 0.4% cholesterol for 10 weeks. An additional group of 10 older pigeons (6–12 years old) which had been maintained throughout life on a cholesterol-free pigeon pellet diet was included in the study. At necropsy the pigeons were given heparin (300 units) and pentobarbital anesthetic (0.5 ml) by intravenous injection. Following anesthesia the chest cavity was opened, and the animals were exsanguinated by cardiac puncture. The blood was drawn into 3.8% citrate (1 part sodium citrate to 9 parts blood) and analyzed for total plasma cholesterol with the use of the Autoanalyzer II method.[17] Subsequent to exsanguination the vascular system was flushed via intraventricular pressure perfusion (140 mmHg) with 0.1 M sodium cacodylate containing 0.1 M sucrose and then fixed by pressure perfusion with 500 ml of 4% (vol/vol) glutaraldehyde buffered to pH 7.2 with 0.1 M cacodylate–0.1 M sucrose. All perfusions were carried out at pigeon body temperature (41 C). Following perfusion, the thoracic aorta from the aortic arch to a point beyond the celiac bifurcation (with 5 mm of the celiac artery attached for orientation) was excised, opened longitudinally along the plane of the celiac and anterior mesenteric arteries, and pinned to balsa wood mounts in a configuration that preserved the vessel geometry. The mounted tissue was then stored overnight in the cacodylate–sucrose buffered 4% glutaraldehyde before being dehydrated through a graded series of ethanols to 100%. The 100% ethanol was followed by two changes of acetone, and finally the tissue was dried from $CO_2$ by the critical-point method. Dried specimens were attached to specimen stubs and sputter-coated with gold–palladium (60:40).

### Morphometrics

Care was taken throughout all microscopic evaluations to correct stage tilt and specimen orientation to compensate for image distortion caused by artery curvature. All observations were restricted to a predetermined 1 × 2-mm site (test region), which had a 1-mm inferior border located on the right laterial wall of the thoracic aorta. The segment was 1 mm directly superior to the origin of the celiac artery and 1 mm to the right of the plane of the intercostal arteries (Figure 3).

Quantitative data were obtained from SEM images of the test site in two ways. First, the extent of atherosclerosis (determined as raised areas visible by SEM), the magnitude of leukocyte adherence, and the number of adherent leukocytes with morphologic characteristics associated with spreading or migrating cells (lamellapodia, pseudopodia) in each diet group were evaluated with the use of standard point count stereologic techniques.[18] Ten scanning electron microscopic fields from the test region of each animal at a magnification of ×1800 were used for these measurements. At this magnification 1.2 sq mm of the test region from each animal was analyzed. In order to compare leukocyte adherence in aortas with different amounts of atherosclerosis, all adherence data were normalized to a square millimeter of tissue surface (surface density). As described in the text, leukocyte adherence was grouped for some analyses according to diet regimen and source of origin (lesion or nonlesion); and for other analyses the lesions were subdivided, without regard to diet regimen, into small and large lesions. For these latter analyses small lesions were defined as those with an area less than $6 \times 10^{-3}$ sq mm, and large lesions were those with an area greater than this value. The surface of large lesions was further subdivided into a central region and a superior edge. The superior edge of a large lesion was defined as an endothelial band 10 cells wide along the most superior border of the lesion.

The second set of analyses involved determination of the average cell width, cell length, the ratio of width to length, and the luminal surface area of endothelial cells in the test region and in a control region located 6 cm superior to the test region. The area 6 cm superior to the test region was chosen as a control region because the region was relatively free of atherosclerosis.[19] For endothelial morphometry, 10 micrographs from the test and control region of 3 randomly selected animals from each diet group were produced at a magnification of ×2500. Measurements were made on these micrographs with the use of a compu-

ter-assisted digitizer as previously described.[15] The analysis encompassed a total of 817 cells from test regions and 756 cells from the control regions, divided as equally as was feasible among aortas in the various diet groups.

### Statistics

Data were grouped according to the experimental conditions outlined in the text, and a group mean and standard error of the mean were computed. Following an analysis of variance, group comparisons were performed with the use of the Bonferroni multiple comparison method.[20] Where applicable, percentage data were first converted with the arcsin transformation.[21] Two means were considered significantly different if the probability of a Type I error was less than 0.05. Multiple regression and stepwise analysis were carried out at a 95% confidence level as described by Neter and Wasserman.[20]

## Results

The 1 × 2-mm test region of the thoracic aorta was always free of atherosclerosis in all the young birds receiving a cholesterol-free pellet diet (Table 1). However, 4 of the 10 young (8–20-week-old) WC pigeons receiving a normal pigeon pellet diet had atherosclerotic plaques in other areas of the thoracic aorta, particularly in the region around the ostia of the vestigial remnant of the left systemic arch. In contrast to the young birds described above, all of the mature 6–12-year-old pigeons had extensive atherosclerosis in the test region even though they had also been maintained on a cholesterol-free pellet diet (Table 1, Figure 1).

The prevalence of atherosclerosis among young WC pigeons receiving a diet containing 0.2–0.5% cholesterol was considerable: cholesterol-exacerbated lesions were observed in the test region of 28 of the 30 animals. In addition, plasma cholesterol levels were elevated in the animals. As summarized in Table 1, maintenance of animals for 10 weeks on a supplement of 0.1% cholesterol had little effect on either plasma cholesterol or lesion prevalence. In contrast, a 10-week cholesterol supplement of 0.2% elevated the plasma cholesterol to a mean level of 317 mg/dl, and 8 of the 10 pigeons receiving this diet had surface identifiable atherosclerosis in the test region. In animals maintained on 0.3% or 0.4% cholesterol diets the plasma cholesterol levels were elevated to 421 and 826 mg/dl, respectively. All of these animals had extensive atherosclerosis in the test region of the aorta.

Besides increases in the number of animals with atherosclerosis, the extent of involvement of the test

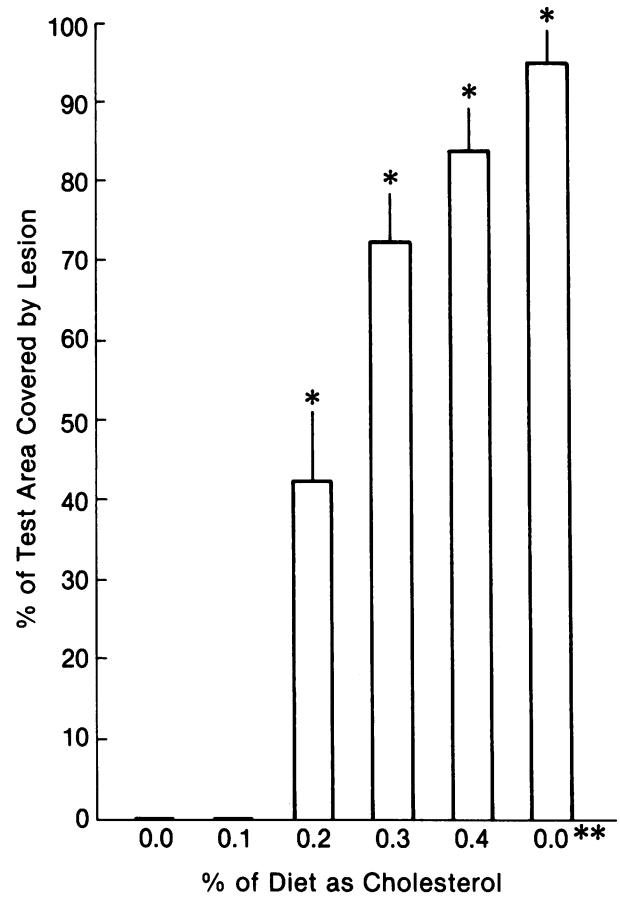


**Figure 1** — Effect of different levels of dietary cholesterol on the extent of raised atherosclerotic lesions (visualized as raised areas visible by scanning electron microscopy) in the test region of WC pigeon aortas. Except for one group of mature (6–12-year-old) pigeons (**), all pigeons were 18 weeks old at necropsy. Animals receiving a dietary cholesterol challenge were maintained on the cholesterol-supplemented diet for 10 weeks before being sacrificed. Values are presented as the mean (n = 10) ± 1 standard error. Asterisk (*) indicates a mean which differs significantly ($P < 0.05$) from the mean of the untreated control group.

Table 1 — Effect of Different Levels of Dietary Cholesterol on Aortic Lesions and Plasma Cholesterol

| % Diet as cholesterol | No. of aortas examined | No. of aortas with lesions in test region | Mean plasma cholesterol at necropsy (mg/dl) |
|---|---|---|---|
| 0* | 10 | 0 | 247 |
| 0.1*† | 10 | 0 | 224 |
| 0.2*† | 10 | 8 | 317 |
| 0.3*† | 10 | 10 | 421 |
| 0.4*† | 10 | 10 | 826 |
| 0‡ | 10 | 10 | 273 |

* Pigeons were 18 weeks old at necropsy.
† Dietary cholesterol challenge maintained for 10 weeks prior to necropsy.
‡ Pigeons were 6–12 years old at necropsy.

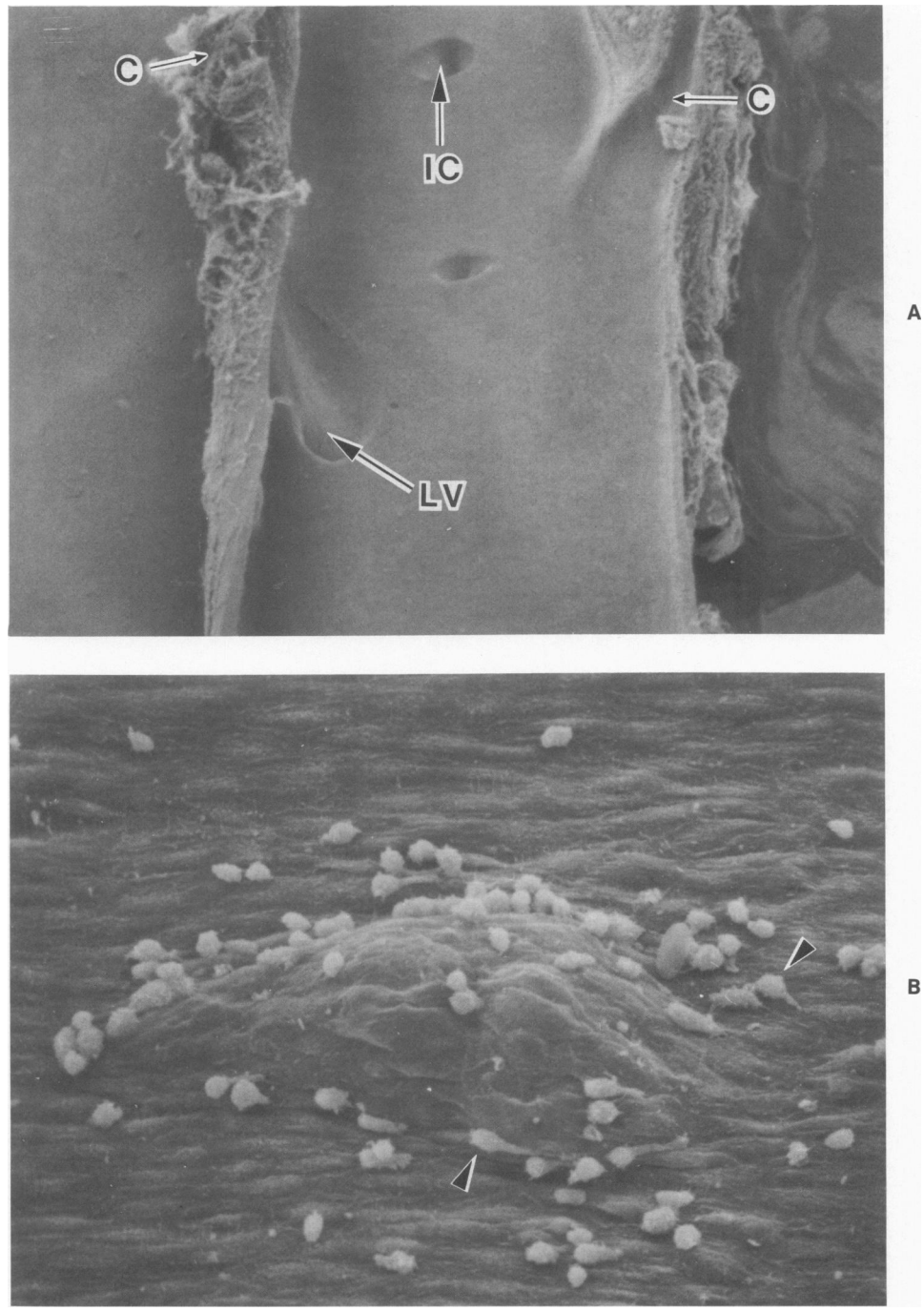


**Figure 2A** – Scanning electron micrograph of the thoracic aorta from a WC pigeon maintained on a 0.2% cholesterol-supplemented diet for 10 weeks. The aorta has been cut along the midventral line (bisecting the celiac artery) and reflected to expose the luminal surface. The two halves of the celiac artery (*C*) as well as the openings to intercostal arteries (*IC*) and the ligamentous vestige of the left systemic arch (*LV*) are clearly visible. Although (as demonstrated in **B**) small focal atherosclerotic lesions are present in the test region, they are not visible at this magnification (×15). **B** – Surface view of the small focal lesion in the test region of a WC pigeon maintained for 10 weeks on a 0.2% cholesterol-supplemented diet. The lesion is approximately 90 $\mu$ in diameter. Numerous leukocytes are adherent to the endothelium overlying the lesion. In addition, leukocytes are shown adherent to nonlesion areas directly adjacent to the lesion. Many of the cells overlying both lesion areas and nonlesion areas adjacent to lesions have surface alterations (lamellapodia, pseudopodia) suggestive of cells migrating or crawling (*arrowheads*). (×750)

region was also increased with increases in cholesterol supplementation above 0.2% (Figure 1). Thus, while atherosclerosis was absent in the test region of aortas from young pigeons fed a normal or 0.1% cholesterol-supplemented diet, an average of 43% of the surface of the test region of pigeons receiving 0.2% cholesterol displayed lesions. The extent of atherosclerosis in the test region increased to 73% and 85% in young animals receiving supplements of 0.3% and 0.4%, respectively (Figure 1). It should be noted, however, that the older pigeons (6–12 years old) fed a normal pigeon pellet diet had even more extensive atherosclerosis in the test region (an average of more than 95% involvement of the test region) than any of the cholesterol-fed groups (Figure 1).

In addition to differences in extent and severity of atherosclerosis, differences were also noted in the appearance of lesions in the various dietary groups.

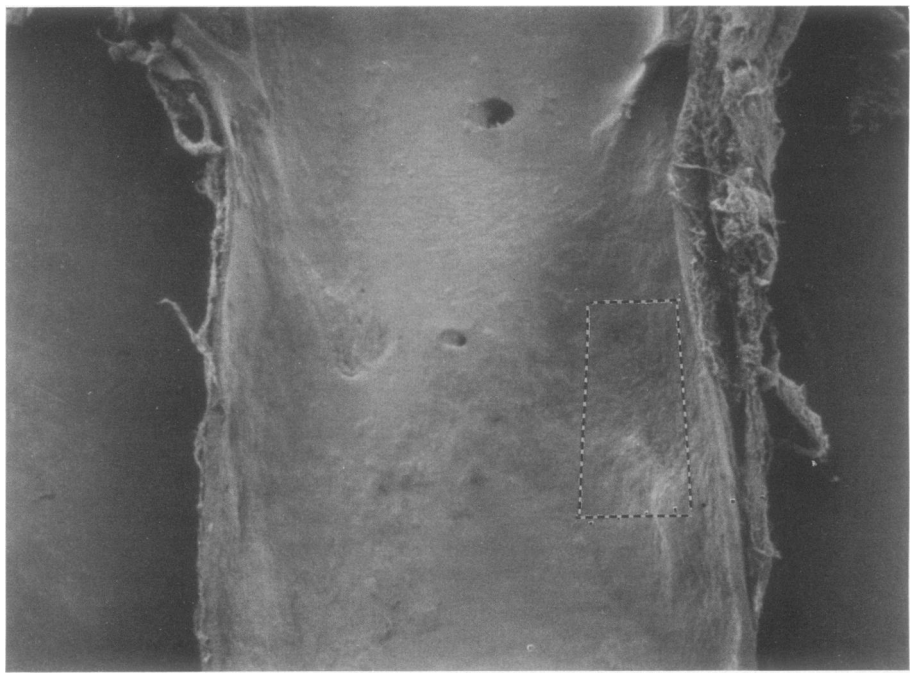

**Figure 3**—Scanning electron micrograph of the thoracic aorta from a WC pigeon maintained on a 0.4% cholesterol-supplemented diet for 10 weeks. The aorta has been prepared as in Figure 2A. The rough appearance of the aortic surface is indicative of the presence of atherosclerosis. The *dotted line* outlines the test region, which contains part of a large diffuse lesion. (×13)

Atherosclerosis in the pigeons receiving a 0.2% cholesterol supplement usually consisted of numerous, small focal raised areas on the luminal surface of the aorta (Figure 2B) which were generally too small to be seen at low magnification (Figure 2A). At the other extreme, the test regions of animals receiving a 0.4% cholesterol supplement more commonly had a single, extensive, atherosclerotic lesion whose boundary often lay outside of test region and which were clearly visible even at low magnification (Figure 3). In pigeons receiving 0.3% cholesterol, some aortas had only small focal lesions, and others had larger, more diffuse lesions occupying part or all of the test region. It is important to note that both 0.3% and 0.4% cholesterol-fed pigeons with large diffuse lesions often had small focal lesions located in close proximity to the expanding edge of the large lesions. The lesions found in old pigeons fed a cholesterol-free diet were very similar in appearance to those of young animals receiving a 0.4% cholesterol supplement, occurring ordinarily as large diffuse areas of atherosclerosis.

All of the lesions were covered by an intact endothelium, and evidence of gross endothelial damage or denudation was not found. The presence of subtle changes in the endothelium overlying atherosclerotic areas, however, was suggested by the altered appearance of the cells as observed by scanning electron microscopy. Normal endothelial cells, as observed in either the control regions of atherosclerotic aortas or in the test region of young birds receiving a cholesterol-free diet, were predominantly fusiform in shape, with a long axis paralleling the direction of blood flow (Figure 4A). In the test region of atherosclerotic aortas this uniformity of endothelial cell orientation was absent, and the cells were polygonal rather than fusiform (Figure 4B). Morphometric analysis confirmed this latter observation as an increase in the ratio of cell width to cell length for endothelial cells in the test region of pigeons receiving a cholesterol supplement. In these same animals the width-to-length ratio of endothelial cells in the control region, 6 cm superior to the test region, remained normal (Figure 5). The size of the endothelial cells, as measured by luminal surface area, was highly variable in the test region of cholesterol-fed animals, with sizes ranging from 122 sq mm, the smallest cell, to 700 sq mm, the largest. On the average, however, the endothelial cells in the test region of cholesterol-fed animals had a mean luminal surface area of 299 sq mm (±19.8). This value was significantly ($P < 0.05$) smaller than either the mean value for endothelial cells in test regions of normal pellet-fed young WC pigeons or control regions of cholesterol-fed young pigeons, which were 337 sq mm (±9.2) and 329 sq mm (±11.3), respectively.

A consistent feature of atherosclerotic lesions in the test region was the presence of leukocytes adhering to the surface of the lesion (Figures 2 and 8). While the number of leukocytes adhering to the luminal surface of the test region of normal young pigeons was generally very low (<100 cells per sq mm of aortic surface), the density of leukocytes specifically

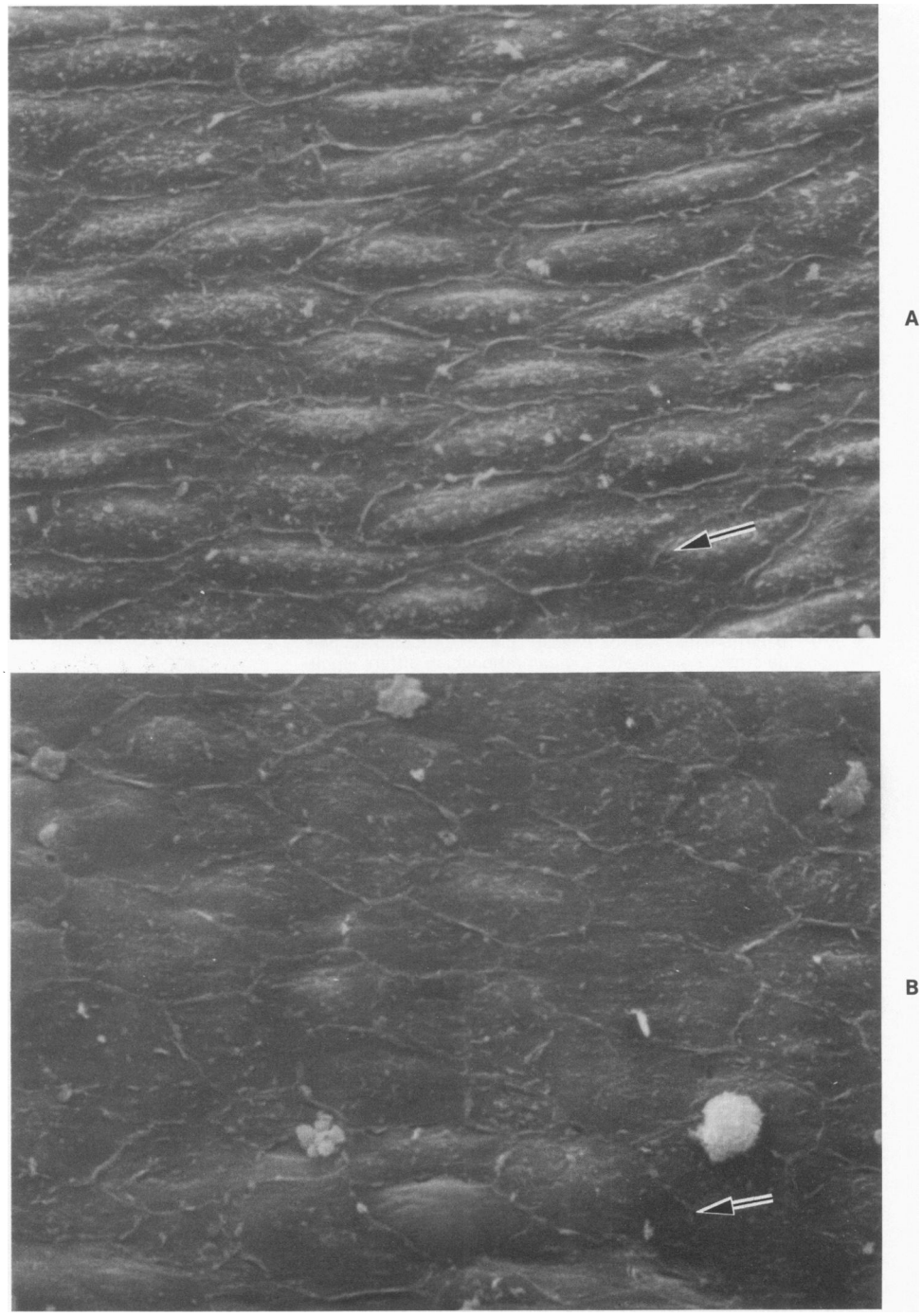


**Figure 4A** – Scanning electron micrograph of endothelial cells from the test region of the aorta of a WC pigeon fed a cholesterol-free diet. The cells are fusiform in shape and oriented in the direction of blood flow, as indicated by the *arrow*. (×1600) **B** – Scanning electron micrograph of endothelial cells overlying an atherosclerotic lesion in the test region of the aorta of a WC pigeon maintained on a 0.3% cholesterol-supplemented diet for 10 weeks. The endothelial cells are polygonal in shape and lack a uniform orientation. The *arrow* indicates the direction of blood flow. (×1600)

over lesions exceeded a mean of 1400 cells/sq mm in the pigeons receiving a 0.2% or higher cholesterol supplement. The greatest mean density of 2300 cells/sq mm was found in old animals with naturally occurring atherosclerosis (Figure 6). In contrast to the increases in leukocyte adherence over lesion areas following dietary cholesterol challenge of 0.2% or greater, leukocytes remained sparse in nonlesion areas of the test region when cholesterol was added to the diet (Figure 6).

In addition to the differences in leukocyte adherence between nonlesion and lesion areas, the pattern of leukocyte adherence to nonlesion areas was not uniform. This was particularly evident in test regions partially covered by atherosclerosis. In these cases leukocyte adherence to nonlesion areas appeared to

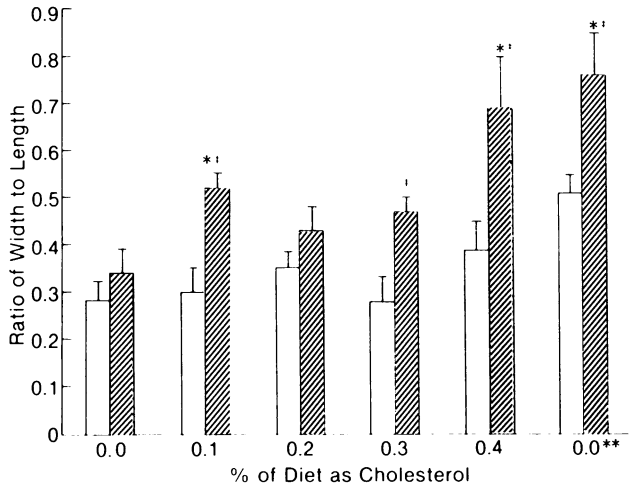


**Figure 5**—Effect of different levels of dietary cholesterol on the ratio of endothelial cell width to length in control (*open bars*) and test (*striated bars*) regions of WC pigeon aortas. Except for one group of mature (6–12 year-old) pigeons (**), all pigeons were 18 weeks old at necropsy. Animals receiving a dietary cholesterol challenge were maintained on the cholesterol-supplemented diet for 10 weeks before being sacrificed. Values are presented as the mean (n = 10) ± 1 standard error. Asterisk (*) indicates a mean which differs significantly ($P < 0.05$) from the mean of the test region of untreated animals. The Lorraine cross (‡) indicates a mean ratio which differs significantly ($P < 0.05$) from the mean ratio of control regions of the same aortas.

be greater near lesions than in areas further away from lesions (Figure 2B). A nonuniform pattern of leukocyte adherence was also observed over the lesions. When lesions from young, cholesterol-fed birds were analyzed on the basis of size, irrespective of dietary cholesterol levels, small lesions (those with a size less than $6 \times 10^3$ sq mm), and the superior edge of large diffuse lesions had leukocyte densities of 2240 cells/sq mm and 2430 cells/sq mm, respectively (Figure 7). These values were significantly ($P < 0.05$) higher than the 960 cells/sq mm computed for the more central regions of diffuse lesions. The 960 cells/mm over the lesion center, in turn, was significantly ($P < 0.05$) greater than the 200 cells/sq mm found in nonlesion areas (Figure 7).

With regard to the various factors affecting leukocyte adherence, Figure 6 shows that all of the diets which produce lesions result in enhanced adhesion and that no dramatic differences are found with diets greater than 0.2%. In addition, although both atherosclerosis severity (as measured by percent of the test area having lesions) and plasma cholesterol levels increased with increases in dietary cholesterol, there was a very wide variation in the values for each of these factors within each diet group. We performed a stepwise regression on the data obtained from young pigeons to ascertain which, if any, of these variables (dietary cholesterol, plasma cholesterol, atherosclerosis severity) were good predictors of the magnitude of leukocyte adherence. The stepwise regression indicated that atherosclerosis severity was the only variable which correlated ($P < 0.05$) with density of adherent leukocytes. This finding is consistent with observations in old WC pigeons with naturally occurring atherosclerosis in which both lesion severity and leukocyte adherence were quite high despite the fact that these animals had normal plasma cholesterol levels and had not received a dietary cholesterol supplement.

It should be noted that many of the leukocytes adhering to the test region had surface features such as lamellapodia and pseudopodia which are generally associated with cell spreading or migration (Figure 8). The percentage of total adherent leukocytes having these morphologic features was always higher ($P < 0.05$) in lesion-susceptible areas of both young cholesterol-fed and old pigeons with naturally occurring atherosclerosis than it was over these same areas of

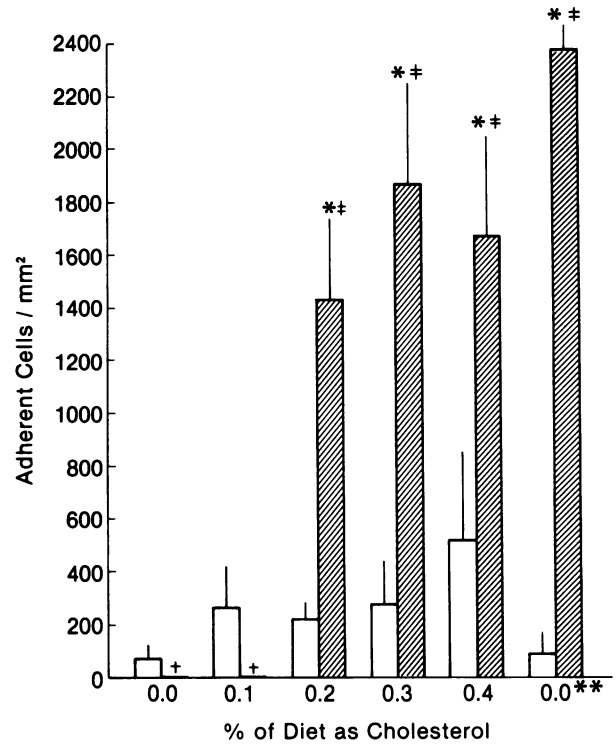


**Figure 6**—Effect of different levels of dietary cholesterol on the density of leukocytes over nonlesion (*open bars*) or lesion (*striated bars*) areas of the luminal surface of the test region of WC pigeon aortas. Except for one group of mature (6–12-year-old) pigeons (**), all pigeons were 18 weeks old at necropsy. Animals receiving a dietary cholesterol challenge were maintained on the cholesterol supplemented diet for 10 weeks before being sacrificed. Values presented are the mean (n = 10) ± 1 standard error. The asterisk (*) indicates a mean adherent leukocyte density which differs significantly ($P < 0.05$) from that of the nonlesion area of the untreated control group. The Lorraine cross (‡) indicates a mean adherent density in the lesion area which differs significantly ($P < 0.05$) from that of nonlesion areas of the same aortas. The Latin cross (+) indicates a condition where no aortic lesions were present.

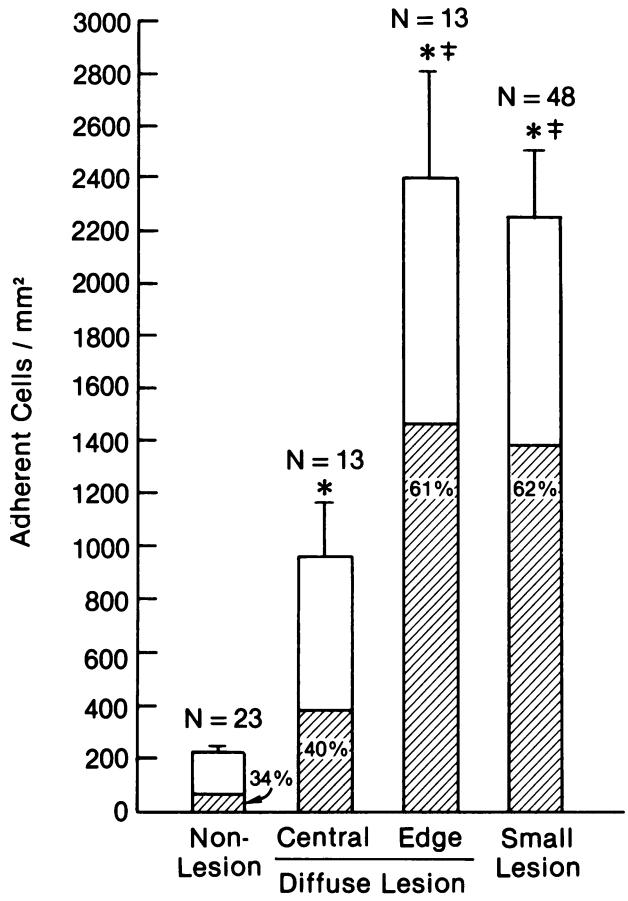


**Figure 7** — Correlation of leukocyte density and spreading or migrating morphologic features with location in the test region. All animals were 18 weeks old at necropsy. Values presented are the mean adherent leukocyte density ± 1 standard error for nonlesion areas, the central region of large diffuse lesions, and the edge of large diffuse lesions, and for small lesions. The *striated areas* represent the percentage of adherent leukocytes that had morphologic characteristics associated with spreading and migrating cells. Asterisk (*) indicates a mean adherent density which differs significantly ($P < 0.05$) from that for nonlesion areas. The Lorraine cross (‡) indicates a mean percentage of leukocytes with spreading and migrating morphologic characteristics which differs significantly ($P < 0.05$) from the percentage of cells with spreading and migrating morphologic characteristics in nonlesion areas.

young control birds (Figure 9). Furthermore, within animals with lesions in the test region, when areas with lesions were compared with lesion-resistant areas (6 cm superior to test region), the prevalence of spreading and migrating cells was always higher in lesion areas. Finally, when analyzed on the basis of lesion size, the percentage of total adherent cells with spreading or migrating morphologic characteristics differed in different regions, the values for small lesions (62%) and the superior edge of diffuse lesions (61%) being significantly ($P < 0.05$) higher than for nonlesion areas (34%) or the central portion of large diffuse lesions (40%) (Figure 7). Interestingly, leukocytes with spreading or migrating morphologic characteristics were also common in nonlesion areas directly adjacent to lesions (Figure 2B).

Most of the adherent cells with spreading or migrating morphologic characteristics were large (>10 $\mu$ in diameter), and their surfaces were folded into numerous extensive ruffles (Figure 8). Cells fitting this description were also seen in the unspread state, and together the spread and unspread cells with extensive surface ruffling accounted for greater than 80% of the cells adhering to the surface of atherosclerotic lesions (Figure 8). The majority of the remaining 20% were of two types: large cells with short microvilli on their surface and occasionally a thin hyalomeric skirt spreading across the aortic surface and small cells (4–7 $\mu$ in diameter) with short microvilli on their surface but always lacking surface alterations associated with migration or spreading (Figure 8).

## Discussion

The observations reported in this study were restricted to a specifically defined 1 × 2-mm region (test area) of the lower thoracic aorta. This region lay within the area near the celiac bifurcation, which has been identified as an area of high predilection for atherosclerosis in the adult WC pigeon.[3,4] Despite the high predilection for atherosclerosis in adult WC pigeons, the test area of 18-week-old WC pigeons was free of atherosclerosis. Thus, the test region of 18-week-old pigeons provides a control for changes associated with atherogenesis. Although absent from young animals, atherosclerotic lesions can be induced in the test region within 10 weeks if the pigeon's normal pellet diet is supplemented with 0.2% or more cholesterol. This rapid and predictable induction of atherosclerosis resulted in an excellent model for studying very early stages in atherosclerosis. Moreover, the prevalence of naturally occurring atherosclerosis in the test region of older (>6 years old) WC pigeons provided the basis for comparison of naturally occurring lesions with nascent cholesterol-induced lesions.

Comparison of cholesterol-induced nascent lesions in young pigeons with naturally occurring lesions in older pigeons revealed that the general scanning electron microscopic appearance, including the adherence of blood leukocytes, and the alteration in endothelial cell shape over lesion areas were similar. These scanning electron microscopic observations are consistent with earlier light and transmission electron microscopic comparisons of naturally occurring and cholesterol exacerbated atherosclerosis in the pigeon. Among the similarities previously reported are the presence of lipid-filled foam cells,[4-6] extracellular lipid,[19,22,23] and, in advanced lesions, cholesterol crys-

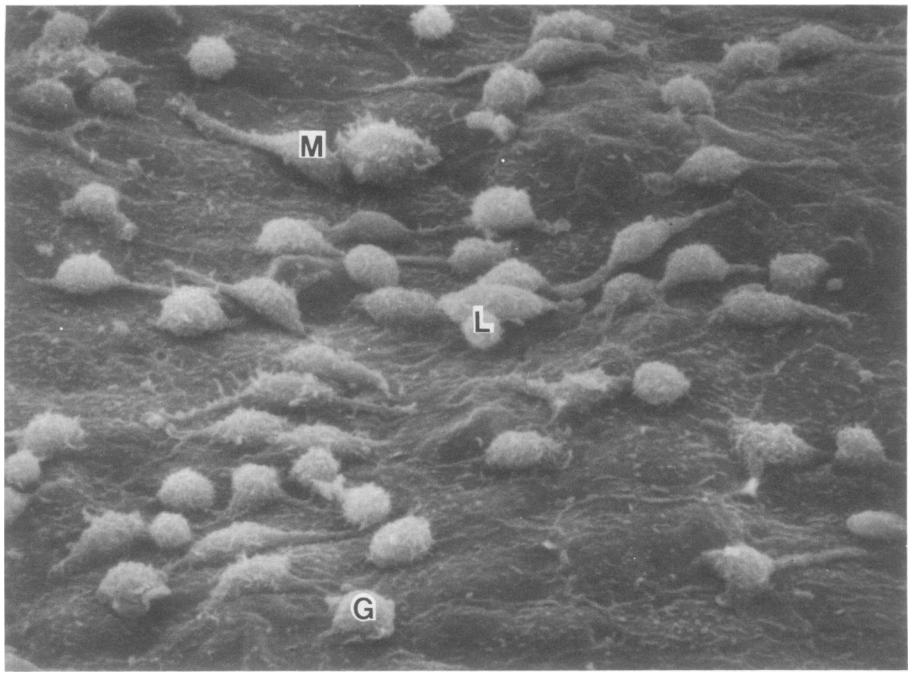


**Figure 8**—Scanning electron micrograph of surface of diffuse lesion in test region of WC pigeon maintained on a 0.4% cholesterol-supplemented diet for 10 weeks. Most of the adherent leukocytes have extensive surface ruffling characteristic of pigeon monocytes (*M*); however, granulocytes (*G*), distinguishable by short microvilli and occasionally a thin hyalomeric skirt, and small lymphocytes (*L*), distinguishable by their small size and short surface microvilli, are also present. Many of the monocytes have surface alterations (lamellapodia, pseudopodia) which are characteristic of cells spreading over a surface. (×1500)

tals and areas of intramural necrosis.[2,19] The exact factors mediating cholesterol-aggravated atherogenesis may be different from those affecting naturally occurring atherogenesis in the pigeon, but the marked similarities between cholesterol-aggravated and naturally occurring lesions suggest that the two processes have many features in common.

In the cholesterol-aggravated situation the prevalence and extent of atherosclerosis increased with increases in dietary cholesterol. However, an increase in dietary cholesterol from 0.2% to 0.3% produced the most dramatic change in the prevalence and extent of atherosclerosis. An increase in dietary cholesterol from 0.2% to 0.3% also raised the mean plasma cholesterol level above 400 mg/dl. Interestingly, St. Clair[24] has shown that at plasma cholesterol levels above 400 mg/dl, β-very low density lipoprotein (β-VLDL), an abnormal, cholesteryl ester-rich lipoprotein, can be detected in pigeon plasma. In dogs, β-VLDL has been shown to exacerbate aortic atherosclerosis,[25] presumably by increasing cholesterol ester accumulation in macrophages within the artery wall.[26] β-VLDL has also been shown to promote cholesteryl ester accumulation in pigeon macrophages.[24] It is possible that the increased incidence of atherosclerosis, at least in WC pigeons receiving a dietary supplement of 0.3% cholesterol or greater, noted in this study could occur in part through a β-VLDL–mediated increase in cholesteryl ester accumulation within macrophages residing in the artery wall. Clearly, though, other factors must also mediate lesion development in the test region of the WC pigeon, because 2 of the young pigeons on a 0.2% cholesterol diet and all of the old birds had atherosclerotic lesions even though their plasma cholesterol levels were well below 400 mg/dl.

It is not known precisely what factors mediate arterial susceptibility to atherosclerosis. Experimental removal of the endothelium by either mechanical[27–29] or immunologic[30] injury will produce atherosclerotic lesions at the site of the denuding injury. Denudation of the arterial surface is not a common feature of naturally occurring or slowly developing atherosclerosis,[31] but the correlation of endothelial removal with atherosclerosis suggests a cause-and-effect relationship between endothelial injury and lesion development. This relationship may also be present in less severely injured arteries. In this regard, alterations in the endothelium which are subtler and more focal than overt denudation have been identified in atherosclerotic arteries. These subtle alterations include increased permeability to plasma proteins such as fibrinogen,[32] albumin,[33] and, most notably, cholesterol[34] and alterations in the luminal glycocalyx.[15,35,36] The specific localization of subtle endothelial alterations to atherosclerotic lesions or areas of high predilection has led several authors to suggest that these alterations in the endothelium directly contribute to the development of atherosclerosis in a manner analogous to overt endothelial denudation.[37,38] In the present report endothelial injury was not directly investigated, but atypical endothelial cells with poly-

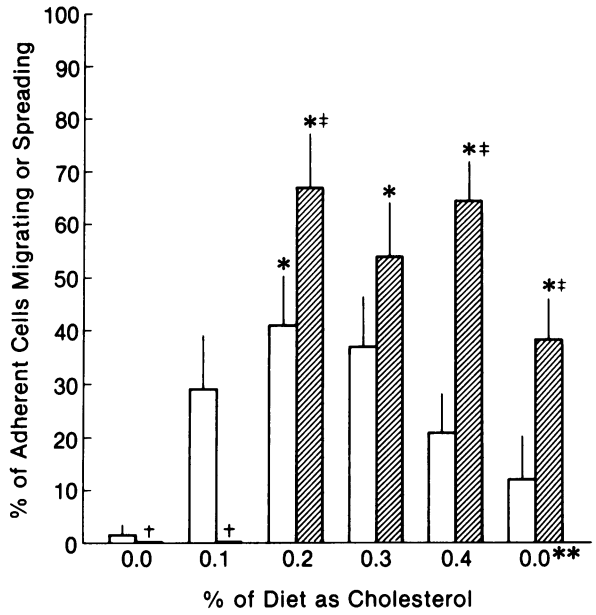


**Figure 9** — Effect of different levels of dietary cholesterol on the percentage of adherent leukocytes migrating or spreading. Data is presented for nonlesion (*open bars*) and lesion (*striated bars*) areas of the luminal surface of the test region of WC pigeon aortas. Except for one group of mature (6–12-year-old) pigeons (**), all pigeons were 18 weeks old at necropsy. Animals receiving a dietary cholesterol challenge were maintained on the cholesterol-supplemented diet for 10 weeks before being sacrifled. Values are presented as the mean (n = 10) ± 1 standard error. The asterisk (*) indicates a mean which differs significantly ($P < 0.05$) from the mean for the nonlesion area of the untreated control group. The Lorraine cross (‡) indicates a mean for the lesion areas which differs significantly ($P < 0.05$) from the mean of the nonlesion areas of the same aortas. The Latin cross (+) indicates a condition where no aortic lesions were present.

gonal shapes and significantly larger than normal width-to-length ratios were observed in the test region of all pigeons receiving a cholesterol supplement. Similar atypical endothelial shape has been reported for high predilection areas of coronary arteries in the pigeon; it has been suggested, on the basis of morphologic criteria, that these aberrant cells may represent endothelium which is either spreading or dividing to compensate for a focal loss of the endothelium due to subtle injury.[15] In partial support of this suggestion, a loss of both endothelial cell orientation and fusiform shape has been directly correlated with areas of increased endothelial cell replication in atherosclerotic rabbit aortas[39] and preatherosclerotic areas of pig aortas.[40] Since endothelial replication is also enhanced in atherosclerotic areas of the WC pigeon aorta,[41] it is conceivable that the alterations in endothelial cell shape and orientation noted in this report represent a response to subtle endothelial injury, which may play a role in determining the sites of atherosclerotic lesion formation.

It could, of course, be argued that the atypical appearance of the endothelial cells in the test region is merely a generalized response to dietary cholesterol unrelated to atherosclerosis. We think that this is unlikely, however, for three reasons. First, a dietary supplement of 0.1% had no effect on plasma cholesterol levels but did produce alterations in the endothelial cell width-to-length ratio. Second, the alteration in the endothelial cell width-to-length ratio was not observed in the control regions of pigeons on a cholesterol-supplemented diet. Third, the alteration in the endothelial cell width-to-length ratio was seen in the test region of old pigeons that had received no dietary supplement.

Although the exact relationship of abnormal endothelial morphologic features to atherosclerosis is not known, it is clear from this study that endothelial alterations consistently occur in the atherosclerosis-susceptible test region but not in nonsusceptible regions of the pigeon aortas. One possible explanation for the site-specific nature of endothelial alterations is that endothelial cells in the test region have a unique genotype which is different from that of endothelial cells in nonsusceptible regions. Although there is no data to support the presence of specific endothelial genotypes in atherosclerosis-susceptible regions, it has been clearly demonstrated by Wagner et al[42] that susceptibility to atherosclerosis can be enhanced by selective breeding. An alternate explanation for the site-specific nature of endothelial alterations is that rheologic factors acting at the high predilection site may alter the endothelium because of mechanical stresses due to vortexing[43] or because of localized blood stasis.[44] Typically, however, changes due to blood flow have been described at points distal to flow dividers,[45,46] whereas the endothelial alterations described in this report occur directly proximal to the celiac bifurcation. The lack of detailed knowledge of pigeon aortic rheology or endothelial cell genotype, however, precludes us from dismissing either hemodynamic factors or genetic factors as determiners of endothelial alterations.

Adherent leukocytes were a constant feature of all lesions observed in this study. The stimulus for leukocyte adherence is not known, but *in vitro* studies of everted arteries indicate that monocytes preferentially adhere to areas of the endothelium which are injured.[47] Moreover, autoradiographic studies have localized increases in endothelial cell replication to the growing edge of atherosclerotic lesions in the WC pigeon aorta.[41] This may be another indicator of endothelial cell injury. In the present studies the superior edge of large atherosclerotic lesions was an area of increased leukocyte density. Thus, leukocyte adherence appears intimately linked with endothelial cell injury and lesion development.

In addition to the superior edge of large lesions,

leukocyte adherence was also high over small lesions. Atherosclerotic lesions in WC pigeons typically begin as small focal nodules which expand laterally with time.[19,23] This lateral expansion at the celiac bifurcation occurs primarily in a superior direction.[23] The presence of many adherent leukocytes over small focal lesions and the superior edge of larger lesions indicates that the greatest adherence occurs over areas of the aorta which have most recently become atherosclerotic.

Increases in leukocyte density were not limited to small lesions or the superior edge of larger lesions. Leukocyte adherence was also increased over the central regions of large diffuse lesions (albeit not as dramatically as over small lesions or the superior edge of large lesions) when compared with nonlesion areas. The specific localization of leukocytes to atherosclerotic lesions suggests that adherence is directly related to the development of atherosclerosis rather than a generalized effect of hypercholesterolemia. This is corroborated by the statistically significant correlation of leukocyte density with the severity of atherosclerosis computed for nascent lesions of young cholesterol-fed pigeons. This correlation was also noted for lesions in the test region of old normocholesterolemic WC pigeons.

Lipid-filled foam cells are a hallmark of both naturally occurring and cholesterol-induced atherosclerosis.[5,6,23,41] The origin of foam cells is undetermined, but evidence from other studies indicates that monocytes can accumulate lipid within the arterial wall.[9,11,12] In addition, Gerrity[16] has observed monocytes adhering to the surface of atherosclerotic pig aortas, within intimal lesions, and in the junctional spaces between endothelial cells. Ongoing studies in our own laboratory have determined that the principal cell composing the early pigeon lesion is the macrophage foam cell.[41] Another recent report from our laboratory has established that blood leukocytes do enter the artery wall and become foam cells.[48] This suggests that in atherosclerosis, adherent monocytes can invade the subendothelial space by migrating through the endothelium. In the present study of nascent cholesterol-aggravated atherosclerosis, greatly increased numbers of leukocytes were observed adhering to the atherosclerotic regions of the test area, and many of these adherent cells had the appearance of cells migrating over a surface. We estimate that more than 80% of the identifiable leukocytes adhering to lesion surfaces had extensive surface ruffling. White and her colleagues,[49] in a correlative study using light microscopic study of Wright-stained sections and scanning electron microscopy, have identified extensive surface ruffling as an identifying feature of WC pigeon monocytes. Thus, the atherosclerosis-specific leukocyte adherence described in this report primarily involves monocytes, which conceivably contribute to the intimal foam cell population.

Finally, mention should be made of the timing of both leukocyte adherence and endothelial cell shape alterations. The present study of nascent atherosclerosis has established that both leukocyte adherence and endothelial alteration are early events in the formation of cholesterol-induced lesions. Their exact temporal position in atherogenesis, however, has yet to be established. Several of the observations in this study suggest that both of these phenomena may occur prior to the appearance of visible lesions. For example, since it is known that a diet containing 0.1% cholesterol is atherogenic for pigeons,[50] the significant increase in the width-to-length ratio of endothelial cells in the test region of young WC pigeons on a 0.1% cholesterol diet in the absence of atherosclerotic lesions in the test region suggests that endothelial shape alterations precede lesion development. However, definitive proof that endothelial cell shape alterations are a prelesion condition will require further studies. The conclusion that leukocyte adherence may also precede lesion development is primarily based on the finding of increased leukocyte adherence in seemingly normal areas of the test region directly adjacent to atherosclerotic lesions. This conclusion assumes that, with time, the lesions in the test region will expand to encompass adjacent nonlesion areas. Although the present experiment did not investigate this point, it is well documented that atherosclerotic lesions will progressively expand when challenged with a cholesterol-containing diet.[5,19,24] Further evidence that leukocyte adherence may precede lesion development comes from the observation of leukocyte adherence in atherosclerotic-susceptible areas of both the pig[12] and rabbit[39] prior to the occurrence of visible lesions. When viewed as a prelesion condition, the increases in leukocytes with morphologic characteristics suggestive of migration seen in nonlesion areas directly adjacent to atherosclerotic lesions suggest that these cells may migrate into the artery wall and become foam cells.

Atherosclerosis is undoubtedly a disease with multiple causes. The studies reported here demonstrate that leukocyte adherence and endothelial alterations are prevalent in early atherosclerotic lesions and suggest that they may be among the factors contributing to the progression of the disease.

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