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TIME COURSE OF DIET-EXACERBATED CAROTID ARTERY ATHEROGENESIS IN THE
WHITE CARNEAU PIGEON

The Ohio State University

PH.D.

1980

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TIME COURSE OF DIET-EXACERBATED
CAROTID ARTERY ATHEROGENESIS
IN THE WHITE CARNEAU PIGEON

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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1980

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CHAPTER I

INTRODUCTION

Several classifications of arterial diseases exist. Of these, atherosclerosis is the most prevalent and ranks as the primary cause of death in the United States and Western Europe. Morphologically, atherosclerosis is characterized by focal cellular proliferation, increased production of formed and amorphous connective tissue components, and accumulation of intra- and extracellular lipid. The disease principally affects the intima of the elastic and large muscular arteries, including the aorta, coronary, renal and cerebral arteries, as well as large arteries of the extremities.

Initial atherosclerotic lesions are small, flat to slightly raised, yellow-white dots or streaks. These early lesions are found almost universally in children and young adults and do not produce clinical symptoms. Later lesions include larger, raised, fibro-fatty plaques that contain a core of lipid (primarily cholesterol and its esters) covered with a fibromuscular cap. Advanced or complicated plaques show one or more of the following characteristics: calcification, hemorrhage, thrombus formation, surface ulceration, and/or rupture of the plaque contents into the lumen. Thinning of the tunica media subjacent to a plaque also may occur. The more advanced lesions usually become apparent by the third decade of life, but are not

world-wide in distribution.

The clinical complications of atherosclerotic plaque lesions vary. The increase in plaque size may narrow or occlude the arterial lumen resulting in ischemia or infarction of the tissue supplied by the affected vessel. In addition plaque rupture can produce thrombosis or atheromatous emboli that can themselves produce ischemia or infarction. Aneurysm formation may also occur due to the associated medial damage.

The causes and pathogenesis of atherosclerosis remain unclear. One major difficulty is that atherosclerosis progresses insidiously for many years before symptoms become apparent, making it difficult to follow the early development of the disease in individual patients. An additional problem is that the identification of risk factors for the disease has depended on the relation of the risk factors to the clinical symptoms shown in the adult, rather than on the extent and severity of the initial arterial lesions.

Another problem in studying atherosclerosis progression concerns the causal relationship between the several types of lesions that have been described. The early lesions, i.e., the fatty streaks, are considered important in atherogenesis because there is evidence that some progress into plaques. The mechanism for this progression is unknown. However, not all researchers agree that progression to a plaque is the fate of the fatty streak since not all fatty streaks necessarily correspond in location to the more advanced lesions.

Many experiments have utilized various animal models to study the early manifestations of atherosclerosis and the effect of various risk factors on the disease progression. Some of the animal models that have been used include dog, rabbit, monkey, swine, chicken, turkey, and pigeon. No single animal model, however, has been found adequate to study all facets of the disease. Consequently, the development of suitable animal models remains an important research area.

The randomly-bred White Carneau pigeon (RBWC) has been developed as a model for the study of aortic and coronary artery atherosclerosis (1,2). Naturally occurring lipid accumulation in the aortic intima of this species begins during the first few weeks of life (3), and by four years of age, all birds have grossly visible lesions at the celiac bifurcation (4). Coronary artery atherosclerosis develops more slowly in this species with 70% of the birds having lesions at eight years of age. Significant sex differences have not been observed in either the prevalence or severity of atherosclerosis at either of these locations (5).

The RBWC lesions are similar in many respects to those found in human beings. Lesions contain both intra- and extracellular lipid. Cholesterol crystal accumulation and proliferation of smooth muscle cells and connective tissues are also observed. In addition, mineralization, hemorrhage, ulceration, and thrombus formation have been reported to occur in RBWC lesions (2). The resemblance to human lesions is an advantage of the RBWC pigeon model, since most

naturally-occurring arterial lesions in other species are composed almost solely of the connective tissue components.

Although atherosclerosis occurs naturally in the aorta and coronary arteries of the RBWC, lesion development can be exacerbated by feeding a cholesterol-containing diet (6). The more rapid development of the hypercholesterolemic-induced lesion is an advantage because of the time required for the spontaneous lesion to occur. Cholesterol-induced lesions are similar morphologically to those that develop naturally although the former contain more foam cells and have a less prominent fibromuscular cap (7).

At present, no animal model has been described for the development of severe extracranial carotid artery atherosclerosis, limiting studies of lesion progression at this location. The carotid arteries are important sites for study since for humans, there is general agreement that transient cerebral ischemia and cerebral infarction frequently are caused by emboli that originate from atherosclerotic lesions located at the carotid bifurcation. Based on the previously-assessed suitability of the hypercholesterolemic RBWC in atherosclerosis research, it was hypothesized for the present study that this animal model would be appropriate for the study of lesion progression in the extracranial carotid arteries. The specific aims of the present study are to:

- (1) determine if there is a predictable time when and a predictable location where atherosclerotic lesions begin to appear in the dorsal carotid artery, carotid bifurcation

and proximal internal carotid artery of the hypercholesterolemic RBWC pigeon.

- (2) characterize the microscopic appearance of these arteries during atherogenesis and atherosclerosis progression.
- (3) determine if fatty streaks at these locations progress to atherosclerotic plaques.

CHAPTER II

HISTORICAL REVIEW

More than 100 breeds of pigeons exist, and several varieties are known to develop atherosclerosis under normal conditions of diet and housing (4). Of these atherosclerosis-susceptible breeds, the randomly-bred White Carneau (RBWC) has been studied in considerable detail. The usefulness of this breed of pigeon for the study of atherosclerosis is based on several characteristics. This species develops naturally-occurring (or spontaneous) atherosclerotic lesions relatively quickly. Like man, the progression of the disease is age-related. All birds older than 4 years demonstrate grossly visible aortic lesions, and these lesions occur predictably just proximal to the celiac bifurcation. The birds show accelerated lesion development when fed a cholesterol-containing diet. Unlike the case with other hypercholesterolemic models (e.g. rabbit), the cholesterol-induced lesions in the White Carneau meet all of the criteria for the diagnosis of atherosclerosis as defined by the World Health study group (8). The White Carneau is easier to work with than other animal models for atherosclerotic disease (e.g. swine and monkeys) and is also less expensive. As in man, genetic factors in the White Carneau control the susceptibility of the different arterial vessels to atherosclerosis (9).

Since the RBWC was the species used in the present study, the following historical overview describes highlights of the research on lesion morphology and atherosclerosis progression done on this breed.

LESION MORPHOLOGY: The RBWC has been established as a model for the study of both aortic and coronary atherosclerosis, and the lesions that develop resemble those of the human disease both grossly and microscopically (1,2,4).

The early lesions in the RBWC consist of focal intimal thickenings containing spindle-shaped cells and both intra- and intercellular lipid. Using scanning electron microscopy, Lewis and Kottke (10) reported the occurrence of focal areas of endothelial damage during atherogenesis. Thrombocytes (platelets) and leukocytes were observed adherent to damaged endothelium and exposed subendothelial fibrils in aortic preparations from normocholesterolemic RBWC.

The more advanced atherosclerotic lesions grossly appear yellow or yellow-white, somewhat ovoid, with an occasional central depression. Microscopically, the lesions contain collagen, amorphous ground substance, and spindle-shaped cells. The presence of both intra- and intercellular lipid is a distinctive feature. The lipid usually accounts for approximately 40% of the plaque mass (4). Cholesterol clefts and calcium deposition are seen which are associated with the amount of plaque lipid. Plaque vascularization by vasa vasora, hemorrhage, hemosiderin deposits, medial elastic degeneration with medial thinning, adventitial lymphocyte aggregation, and ulceration

of the plaque with thrombus formation also have been observed in mature lesions (2). As lesions progress, the majority of the lipid becomes localized into a central core of atheronecrosis that is overlaid by a fibrous cap composed of collagen and spindle-shaped cells.

PROGRESSION OF NATURALLY-OCCURRING ATHEROSCLEROSIS: Aorta: By 1 week of age, about 30% of the White Carneausquabs have microscopic lesions in the aortic intima, and by 12 weeks of age, approximately 70% of birds show lesion involvement (3). The early incidence of fatty streaks is considered to be the result of moderate hypercholesterolemia. The newly-hatched squab has a plasma cholesterol concentration of 600-700 mg/dl since its food supply is derived from cholesterol-rich egg yolk. The squab remains hypercholesterolemic until it is weaned from its parents at approximately 5-6 weeks of age (11). The continued hypercholesterolemia is due to the diet of "pigeon milk". This "pigeon milk" is composed principally of desquamated epithelial cells of the parent's crop and contains approximately 30% lipid and 0.3% cholesterol (12).

Grossly-visible plaques are apparent in 30% of the 1-year-old birds, and by 4 years, the incidence of gross plaques is 100% (13). The raised yellow plaques cover approximately 10-15% of the intimal aortic surface and are consistently located at the origin of the celiac artery, although other plaques may occur in the elastic (proximal) segment of the aorta.

Additional studies have attempted to define more clearly the precise area of lesion development in the aorta. Lauper et al.(14) described 4 distinct zones of the normal RBWC aorta, each possessing different microscopic morphologies.

In order from proximal to distal aorta, these zones are the elastic, transitional, hybrid, and muscular. The transitional zone begins 0.5-1.0 cm cephalad to the origin of the celiac artery and includes the insertion of the ligamentous vestige of the left systemic arch, the orifice of the intercostal artery, and the origin of the celiac artery. The authors described the presence of longitudinal bundles of subendothelial smooth muscle cells in the transitional zone. These bundles are present from birth; the cell numbers increase with age. Early lipid accumulation begins in the smooth muscle cells of these areas, prompting the authors to conclude that atherosclerotic lesions initially develop from the same specific sites.

The most recent work defining precise lesion location in the transitional zone was reported by Tesar and Kottke (15). The wall directly across from the intercostal artery orifice was named the "lesion area" because it was here that the largest and fastest-growing typical atherosclerotic plaques initially developed. The "lesion area" lacked musculoelastic cushions. The intimal thickening that occurred was described as being the result of, or coincident with, lipid accumulation at this site.

Coronary Arteries: Coronary artery atherosclerosis in RBWC pigeons is rare before birds are 9 months of age but occurs in

approximately 70% of RBWC by 8 years of age. By 10 years, the incidence is increased to 85%. Coronary arteries of all diameters are involved, but the lesions are usually less severe and exhibit fewer complications than those occurring in the aorta. Overall, there is less lipid accumulation; calcification comprises the greater part of the plaque mass. The percent lumen stenosis itself is rarely greater than 5% (4).

During the first 2 years of life, the prevalence of atherosclerosis in the coronary arteries appears to be independent of the aortic manifestations of the disease (16). This lack of correlation between coronary and aortic atherosclerosis is quite similar to that observed in humans. Deaths resulting from myocardial infarctions have been observed in pigeons (17), but were due to embolization of atheromatous material originating from the aortic root and aorta rather than from thrombosis of atherosclerotic coronary arteries.

Other Vessels: In general, atherosclerosis in other major arterial segments of the pigeon has not been well documented or characterized. Pritchard (5, 13) described the occurrence of plaques in the brachiocephalic, iliac, renal, and carotid arteries of RBWC birds; however, neither the age at which these plaques appeared nor the prevalence of atherosclerosis was described. Atherosclerosis of the cerebral arteries was not observed.

PROGRESSION OF CHOLESTEROL-INDUCED ATHEROSCLEROSIS:

Hypercholesterolemic RBWC were shown to have an accelerated development of both aortic and coronary atherosclerosis. These lesions

closely resemble the naturally-occurring variety in gross appearance, lipid content, and percent lipid composition (18).

Clarkson and Lofland (18) described lesion progression in 6-week-old birds that were fed an atherogenic diet, containing 1% cholesterol, for 6-7 months. Aortic lesions were present in 100% of the birds by 7.5 months of age; coronary lesions occurred with an approximately 25% incidence. The mean serum cholesterol value was approximately 1425 ± 325 mg/dl for the cholesterol-fed birds. Control birds had an average serum cholesterol of 450 ± 25 mg/dl.

Another study (6) examined the relationship between the amount of dietary cholesterol, duration of feeding, and gross extent of atherosclerosis after birds had been fed an atherogenic diet for 6 and 12 months (aortic) and 12 months (coronary). Cholesterol comprising 0.05% of the diet did not affect aortic atherosclerosis but did increase coronary atherosclerosis at 12 months with an incidence of 6%. Atherosclerosis in both areas was increased as the amount of dietary cholesterol was increased (0.1; 0.25; 0.5; 1%). Those birds fed the diet for the longer periods had increased lesion involvement.

BIOCHEMICAL CHANGES ASSOCIATED WITH ATHEROSCLEROSIS: Lipids: The accumulation of lipid, notably cholesterol, is a characteristic feature of atherosclerotic lesions. In addition, high plasma cholesterol concentrations have long been recognized as a principal risk factor in the development of atherosclerosis in man as well as several animal models.

Middleton et al. (19) noted that, in 8-year-old normocholesterolemic birds, plasma cholesterol concentrations were correlated with aortic atherosclerosis among male birds but not among the females. Both plasma cholesterol and triglyceride levels correlated with coronary artery atherosclerosis among the female, but not among the male pigeons.

St. Clair et al. (20) compared the lipid composition (phospholipid, free cholesterol, cholesterol esters, free fatty acids, and triglycerides) between normal aortas, and aortas with fatty streaks and plaques from normocholesterolemic (1-year-old and 6-12-years-old) and hypercholesterolemic (1-year-old) White Carneaux. The concentration of all lipid classes was increased in fatty streaks when compared to the normal arteries from the hypercholesterolemic birds. In plaques from hypercholesterolemic birds, there was an additional increase in lipid concentration; however, this increase was not significantly different for free fatty acids and triglycerides. Fatty streaks and plaques from normocholesterolemic birds also showed significant increases in all lipid classes; however, there was relatively less phospholipid, triglyceride, and cholesterol ester in plaques from normocholesterolemic pigeons when compared to hypercholesterolemic birds.

Nicolosi et al. (21) analyzed various lipid classes that were present in the upper thoracic aortas and in the celiac cushions from 3-6-month-old normocholesterolemic RBWC and Show Racer (SR) birds. The lipid content in the upper thoracic aortas from both breeds of

pigeons was similar and less than that present in the celiac cushions. The authors concluded that hemodynamic stresses were responsible for the increased accumulation of lipid in the celiac area. The WC birds also had significantly greater lipid accumulation in their celiac regions when compared to the SR pigeons. Tissue culture studies of cells derived from the upper thoracic aortas of embryos also showed more lipid accumulation in the RBWC when compared to the atherosclerosis-resistant SR bird, suggesting that inherent factors in RBWC aortic cells were responsible for lipid accumulation. The nature of the inherent factors was not identified.

Subbiah et al. (22) have noted spontaneous sterol accumulation, marked by an increase in cholesterol esters, in the aorta of normo-cholesterolemic WC between 9 and 12 months of age. These changes were not present in age-matched SR birds. Morphologically, at this age, both breeds had intimal cushions composed almost entirely of smooth muscle cells with little interstitial material. At later time intervals, the WC cushions began to accumulate both intra- and extracellular lipid and connective tissue. The authors concluded that the critical intracellular changes associated with atherogenesis, i.e. cholesterol esterification, appeared prior to the manifestation of overt lesions.

The origin of cholesterol in the atherosclerotic lesion has been considered by several investigators. Lofland and Clarkson (23) have suggested that areas of the aorta that showed early lesion formation tended to accumulate cholesterol because of the decreased

ability of the cells to regulate both the influx of cholesterol from the plasma as well as the efflux of cholesterol from the arterial wall.

St. Clair et al. (24) noted that local synthesis might also play a role in cholesterol accumulation. Arterial segments from both normo- and hypercholesterolemic RBWC pigeons synthesized squalene, a precursor of cholesterol, and this synthesis was positively correlated with the severity of atherosclerosis. The predominance of squalene, as opposed to cholesterol itself, was thought to indicate an hypoxic condition in which the oxygen available to the arterial wall was inadequate for the conversion of squalene to cholesterol.

Stimulation of cholesterol esterification is an established feature of atherosclerotic tissue in man and animal models, and this stimulation is correlated with progression of the disease. St. Clair et al. (25) studied the stimulation of cholesterol esterification using cultures from 2-4-month-old RBWC aortas. The arterial segments were maintained in a medium containing either normo- or hypercholesterolemic serum, and stimulation of cholesterol esterification was seen as early as one day after culture with the hypercholesterolemic serum. Associated with the increased esterification was the appearance of both intra- and extracellular lipid droplets. No attempt was made to identify the specific components of the hypercholesterolemic serum responsible for the stimulation of the esterification process. The question of whether or not this

abnormality of cholesterol ester metabolism is an initial metabolic lesion or is the result of other early metabolic changes remains unanswered.

Enzymes: Although cholesterol esters may accumulate by increased synthesis, they also may increase by decreased hydrolysis. Cholesterol ester hydrolases are located in lysosomes, microsomes, and within the cytosol. The significance of these fractions in cholesterol metabolism is unknown, but the decreased activity of lysosomal hydrolase has been proposed to explain the accumulation of cholesterol esters that is associated with cholesterol-induced atherosclerosis. Subbiah (26) assayed the three subcellular hydrolase fractions in both normo- and hypercholesterolemic WC and SR pigeons. Birds were 10 months of age when assayed, and the hypercholesterolemic RBWC had been fed a cholesterol-containing diet for 4 months prior to the assay. Cholesterol ester accumulated in the WC aorta under both dietary regimens. The accumulation of cholesterol ester in normocholesterolemic birds was accompanied by a decrease in the microsomal hydrolase fraction when compared to the SR breed. An increase in the lysosomal hydrolase fraction was observed in the hypercholesterolemic WC.

Factors influencing cholesterol ester hydrolase activity include estrogens and prostaglandins. Subbiah (27) reported that equine estrogens given to 6-year-old normocholesterolemic male WC decreased cholesterol ester synthetase activity and increased microsomal hydrolase activity. Subbiah and Dicke (28) also noted that

prostaglandin E_1 (PGE_1) inhibited cytosol hydrolase activity. Preparations from 6-month-old normocholesterolemic WC showed increased synthesis of PGE_2 when compared to age-matched SR birds (29). The PGE_2 inhibited the action of cholesterol ester hydrolase of the cytosol fraction and may have a role, therefore, in the accumulation of cholesterol ester in atherosclerotic lesions.

Changes in mitochondrial morphology, accumulation of lipids, and the uncoupling of oxidative phosphorylation are characteristic of naturally-occurring atherogenesis. The mitochondrial involvement in initial lipid accumulation has been related to a loss of ATPase activity, suggesting oxidative uncoupling. Smith et al. (30) reported the lack of mitochondrial ATPase in intimal cells cultured from 17-day-old WC aortas. The observation was made that lipid vacuoles in the cells appeared to originate from enlarged, misshapen mitochondria that lacked ATPase activity.

Hajjar and Smith (31) measured the activity of mitochondrial ATPase in combined intimal and medial layers from upper thoracic aorta and celiac cushions from WC and SR breeds aged 1 day, 6 weeks, 12 weeks, and 6 months. Analysis of the thoracic aorta revealed that the WC had an initially high level of mitochondrial ATPase activity and that these levels decreased over time. The authors suggested that the initially high level of mitochondrial ATPase activity of the WC when compared to the SR might have resulted from a greater degree of mitochondrial maturation. Patterns at the celiac bifurcation were slightly different. The WC levels

of mitochondrial ATPase activity were initially low, increased at 6 weeks, and then decreased at subsequent time intervals. As with the thoracic aorta, the SR birds demonstrated a slow, steady, linear increase in enzyme activity. The rise in the WC ATPase activity at the celiac site at 6 weeks was thought to be due to the increased concentration of fatty acids which could act to stimulate enzyme activity through increased permeability of the inner mitochondrial membrane. The continuing lipid accumulation at the celiac region could produce structural or functional disruption of the mitochondria resulting in the decrease of mitochondrial ATPase observed in the later time periods.

Glycosaminoglycans: Several authors have suggested that extracellular glycosaminoglycans (GAGs) may play a major role in the lipid accumulation that is a characteristic feature of atherosclerotic lesions (32,33). Glycosaminoglycans bind lipoproteins in vitro (34,35), and in addition, GAG-lipoprotein complexes have been localized in fatty streaks and fibrous plaques (36). It has been hypothesized that the quantitative and qualitative changes in arterial GAGs may be initiating events in atherogenesis and that the lipid accumulation characteristic of fatty streaks and plaques may be an indicator of pathological alterations in the GAG fractions (37,38).

A few morphological papers on the RBWC have included stains for GAGs as part of their studies. Santerre et al. (39) observed that accumulations of GAGs were common in advanced plaques. Tesar and Kottke (15) noted that there was no focal accumulation of GAGs in

the celiac 'lesion area' of young WC, but abundant GAGs were present in focally-necrotic plaques from older birds.

Curwen and Smith (40) performed biochemical studies on thoracic and celiac samples from WC and SR birds aged 1 day, 6 weeks, 4-6 months, and 2-3 years. At the time intervals studied, the thoracic portions contained similar amounts and types of GAGs for both breeds. The total GAG concentration from the thoracic aorta was greater than that from the celiac portions for both breeds. The total GAG content as determined in the study was therefore the opposite of what one would expect if the primary role of GAGs is the enhancement of lipoprotein deposition within the vessel wall. The WC celiac bifurcations contained more total GAG than those from the SR birds at all 4 age groups studied. There was a progressive increase in total GAGs by 6 weeks, and this increase was also apparent at 6 months. There was a relative decline by 3 years of age. At 6 months, increased amounts of chondroitin-6-sulfate and decreased amounts of heparitin sulfate and chondroitin-4-sulfate were observed in the celiac specimens from the WC birds. The authors concluded that the metabolic capacities of the WC smooth muscle cells to synthesize and degrade GAGs may be responsible for lesion progression.

ADDITIONAL RISK FACTORS: Besides hypercholesterolemia, other primary risk factors for atherosclerosis in humans include cigarette smoking, hypertension, and aging.

Cigarette smoking has been implicated as a causative agent in coronary artery atherosclerosis in human and animal models of the

disease. The effects of intermittent exposure to carbon monoxide (in amounts similar to those inhaled by cigarette smokers) on the extent and severity of atherosclerosis were studied in 6-12-month-old normo- and hypercholesterolemic WC birds (41). There was no effect on the development of aortic atherosclerosis; however after 52 weeks of treatment (the initial sampling time) there was an increased incidence of coronary artery atherosclerosis observed in the hypercholesterolemic birds.

Hypertension is thought to exacerbate the development and accelerate the progression of atherosclerosis, notably within the coronary arteries. Some workers (42) have reported blood pressure increases in RBWC pigeons aged 9-17 months; these increases have been related to the augmented atherosclerosis development seen during this time period. However, Middleton et al. (19) described a lack of correlation between blood pressure and lesion development in 8-year-old normocholesterolemic WC pigeons. The studies of Wagner et al. (43) indicated that neither age nor sex influenced the blood pressures of RBWC birds and that blood pressure changes did not account for the susceptibility to atherosclerosis characteristic of the RBWC pigeon.

Age is a risk factor for atherosclerosis, and the life-shortening effect of radiation has often been interpreted as an acceleration of the normal aging process. Long-range effects of repeated doses of ionizing radiation were studied in WC and SR pigeons (44). Differences in aortic atherosclerosis were apparent after 10-13 months of

irradiation combined with a cholesterol-containing diet (1% dietary cholesterol for the SR; 0.1% for the WC). The irradiation also affected the development of coronary atherosclerosis in the two breeds. The higher cholesterol content of the SR diet made comparisons between the irradiated and non-irradiated SR birds difficult to make. The lower cholesterol content of the diet fed the WC permitted a significant difference in the incidence of coronary atherosclerosis to be observed in irradiated birds.

In conclusion, collective research has indicated that the RBWC pigeon is a good model for the study and manipulation of atherosclerosis progression in the aorta and coronary arteries. It is reasonable to expect that these birds also can be developed as models for the study of carotid artery atherosclerosis.

CHAPTER III

MATERIALS AND METHODS

Randomly bred White Carneau pigeons of either sex ($n = 99$) were obtained from the Specialized Center of Research on Arteriosclerosis, Bowman Gray School of Medicine, Winston-Salem, North Carolina. Birds were weaned at six weeks of age and fed a cholesterol-free, commercial pellet diet until seven weeks of age. Birds were then assigned randomly to one of nine groups. The baseline group was sacrificed at seven weeks of age after having been fed the cholesterol-free diet for one week. The remaining birds were fed an atherogenic diet containing (on a weight basis) 89.5% pigeon pellets, 10% lard, and 0.5% cholesterol for either 1, 2, 3, 4, 6, 7, 8, or 12 weeks (Table 1). At the end of each period, the group was killed for evaluation of the extent and severity of carotid artery atherosclerosis.

Total plasma cholesterol concentrations were determined in fasted blood samples of all birds just prior to the time of sacrifice using a modification of the method described by Rush et al. (45).

At the time of sacrifice, birds were anesthetized, and a midline abdominal incision was made. An 18-gauge needle was inserted into the left ventricle, the inferior vena cava incised, and the vascular system was flushed briefly with a 0.135 M sodium phosphate buffer (pH 7.4). Two percent glutaraldehyde in a 0.05 M sodium phosphate

buffer (pH 7.4) was then perfused through the vascular system. Both the 0.135 M buffer and the 0.05 M buffer with the 2% glutaraldehyde were found, with an Advanced Osmometer (Model 3-L; Advanced Instruments Inc.) to have an osmolality of 300 mOsm/Kg which is similar to the osmolality of pigeon plasma. The inferior vena cava was clamped and 130 ± 5 mmHg pressure maintained until fixation was complete, i.e. the entire bird was rigid with subsequent dissection revealing fixation of muscles as well as arteries. The standard time for the perfusion fixation was one hour. After this time, the neck was opened, rinsed in buffer, and the carotid arteries further fixed by immersion in 10% neutral buffered formalin for several weeks.

GROSS AND MICROSCOPIC TISSUE PREPARATION

The carotid arteries were examined with a dissecting microscope. The bifurcation area itself was recognized by its elliptical shape, wider external diameter, and the presence of the internal and external carotids on the cranial aspect. From each bird, three tissue blocks were taken for light microscopic evaluation. These sites included tissue from either the right or left carotid bifurcation at the point of maximal outside diameter; ipsilateral internal carotid artery from a point measured 1.5 mm superior to the midportion of the bifurcation site; ipsilateral dorsal carotid artery at a point measured 3 mm inferior to the midportion of the bifurcation site. A segment of rectus capitis ventralis muscle was retained on the posterior wall of each of the arterial segments and served as a marker for later identification of anterior and posterior walls on frozen section. On a random basis, half of the

birds studied from each of the groups (control and experimental) had the three vessel segments taken from the right side; the remaining birds in a given group had the three vessel segments taken from the left side.

Four serial frozen sections, 8 micrometers in thickness, were prepared from each of the three tissue blocks from each bird, and stained with hematoxylin-phloxine-saffron (HPS) (46), either Verhoeff-Van Gieson or resorcin fuchsin-Van Gieson (47) for demonstration of elastic tissue, collagen and muscle, and Oil red O-hematoxylin (48) for evaluation of lipid lesions.

The birefringence of the fat stained with Oil red O was evaluated using polarizing microscopy and the birefringence scored subjectively on a scale of 0 to +4. A score of 0 indicated the absence of birefringent properties; +1 indicated that up to 25% of the cross-sectional area of the lesion showed birefringence; +2: 26-50% birefringence; +3: 51-75% birefringence; and +4, that 76-100% of the lesion showed birefringence. A mean birefringence score for the dorsal carotid artery, carotid bifurcation and internal carotid artery from each experimental group was calculated by summing the individual vessel scores and dividing by the number of birds in that group.

The fourth slide sectioned was initially left unstained and, at a later point, samples were stained with either Carstairs Method for fibrin and platelets (49), Aldehyde Fuchsin pH 1.7 followed by Alcian Blue pH 2.5 for evaluation of sulfated and non-sulfated

glycosaminoglycan (GAG) content (50), non-specific esterase (51), or ATPase (52). According to the staining intensity, positive enzyme reactions were scored subjectively as trace (focal weak positivity); +1 (uniform weak positive); +2 (moderate); +3 (strong); +4 (very strong).

Specimens for transmission electron microscopy were washed in 0.15 M phosphate buffer containing 0.26 M sucrose (pH 7.4), post-fixed in Millonig's phosphate buffered osmium tetroxide fixative (pH 7.3), dehydrated through increasing concentrations of ethanol, and embedded in Spurr low-viscosity resin. Thick (1 μ m) and thin sections were cut on a Porter-Blum MT-1 Ultramicrotome. Thick sections were stained with toluidine blue. Thin sections were stained with a saturated solution of uranyl acetate followed by Reynold's lead citrate and examined in an Hitachi 12 electron microscope.

Lesion sites were confirmed by gross staining representative samples (n = 66) from the contralateral arteries using Oil red O (53).

EVALUATION OF ATHEROSCLEROSIS

The serial cross-sections of the three carotid artery segments were evaluated microscopically for the following characteristics of atherosclerosis: length of circumference of the lesion, mean intimal thickness of the lesion, cross-sectional area of involvement, percent luminal stenosis, and lesion type. Measurements were made with a Zeiss ocular micrometer eyepiece that was calibrated with a known standard for the objectives used in this study.

The arterial circumference was determined by measuring the major and minor diameters of the internal elastic lamina (IEL). The lengths of the major and minor radii were then calculated and these values substituted either in the formula for the circumference of a circle (when the radii were equal) or in the formula for determining the circumference of an ellipse, i.e.

$$C = 2\pi \sqrt{\frac{a^2 + b^2}{2}}$$

where a and b equal the radius of the major and minor axis, respectively. The latter formula (which is the same as that for a circle when a = b) was used to evaluate those sections which, although perfused in a consistent manner, had been slightly compressed when processed or that were from the bifurcation, a region that is normally elliptical in contour. The percent IEL that was subjacent to a lesion was visually estimated. The length of the circumference of the lesion was then calculated by multiplying this percentage figure by the circumference of the IEL.

The mean thickness of a lesion was calculated from three measurements taken from standard regions of each atherosclerotic lesion. The first thickness measurement was made at the midpoint of the lesion and included the distance between the endothelium and the abluminal surface of the underlying IEL. The second and third measurements were made on either side of the midpoint, equidistant to the edge of the lesion.

The total cross-sectional area of involvement by a lesion was calculated by multiplying the length by the mean thickness. Results

were expressed in square micrometers $\times 10^3$. The percent lumen stenosis was determined by first calculating the area within the IEL using either the formula for the area of a circle or an ellipse. ($A = \pi a b$, where a is the semi-major axis and b is the semi-minor axis.) This area was then divided into the calculated area of the lesion and multiplied by 100 to give percent lumen stenosis.

Atherosclerotic lesions were qualitatively classified as fatty streak, proliferative, or atheromatous lesions. Fatty streaks were seen as relatively thin, focal, or diffuse areas of extra- and/or intra-cellular lipid within the tunica intima. Proliferative lesions were thicker than fatty streaks and contained varying amounts of foam cells, spindle-shaped cells, collagen and ground substance. Atheromatous lesions were as thick or thicker than the proliferative lesions but were, in addition, characterized by a focus of necrotic debris at the base of the plaque. Overlying the necrotic focus were several layers of foam cells, spindle-shaped cells and varying amounts of collagen and ground substance.

Foam cell content was graded subjectively on a scale of 0 to +4. A score of 0 indicated a lack of foam cells; +1 was recorded if up to 25% of the lesion was composed of foam cells; +2 indicated 26-50% foam cell content; +3, 51-75% foam cell content; and +4, 76-100% foam cell content. A mean foam cell score for each of the vessel segments in each group was calculated in a manner identical to that for the mean birefringence score.

DATA ANALYSIS

Parameters of lesion progression, i.e., length of the lesion circumference, thickness, and cross-sectional area of involvement were determined by two-way analysis of variance with repeated measures on one factor (i.e., either length, thickness, or area) followed by a Student-Newman-Keuls post test (54). The three arterial segments for a group were compared for the preceding parameters using a one-way analysis of variance followed by the Student-Newman-Keuls post test. Parameter differences from arteries taken from the right side vs. the left side were compared using a three-way analysis of variance with repeated measures on one factor.

NATURALLY OCCURRING CAROTID ARTERY ATHEROSCLEROSIS STUDIES

Prior to beginning the previously-described study of the time course of atherogenesis in the carotid arteries of hypercholesterolemic White Carneaux, a pilot study was performed on normocholesterolemic birds. The primary purpose of this pilot was familiarization with details of working with the carotid vessels.

Carotid arteries of 7 randomly-bred White Carneaux (ranging in age from 6-12 months) and 4 WC-2 birds (ranging in age from 8-12 months) were dissected and tissue blocks taken from the vessel areas previously described. Those samples embedded in paraffin were stained with hematoxylin and eosin (47), Verhoeff Van Gieson (47) and Alcian Blue-PAS (47). Frozen sections were stained with Oil red O (48) and occasionally ATPase (52). Samples for TEM were processed as described previously.

CHAPTER IV

RESULTS

PLASMA CHOLESTEROL CONCENTRATIONS

Figure 1 shows the mean plasma cholesterol concentrations for each group determined at the time of sacrifice. Baseline values were 307 ± 14 mg/dl (Mean $\pm$ SEM); after four weeks of consuming an atherogenic diet, mean plasma cholesterol concentration was 1112 ± 201 mg/dl. At the conclusion of the study, the values averaged 2245 ± 235 mg/dl.

GROSS AND MICROSCOPIC ANATOMY OF THE CAROTID ARTERIES

The gross anatomy of the extra-cranial carotid arteries is similar to that described for the domestic pigeon (55). At the level of the fourth cervical vertebra, the paired dorsal carotid arteries diverge laterally, each toward the ipsilateral internal angular process of the mandible. The dorsal carotid artery at this position lies ventral to the rectus capitis ventralis muscle and bifurcates into the external and internal carotid arteries. Arteries from baseline birds had the following mean IEL circumferences: dorsal carotid artery, 2.2 mm; carotid bifurcation, 2.8 mm; internal carotid artery, 1.8 mm. Differences were not observed in the circumferences of these arteries during the time period of the study.

Histologically, arteries from baseline birds had a relatively thin subendothelial zone which contained ground substance and occasional fine elastic fibrils. The bifurcation area contained two prominent flow dividers composed of longitudinally oriented smooth muscle cells with occasional, non-continuous elastic fibers.

The tunica media contained variable numbers of lamellar units (6-8 in the dorsal carotid artery; 8-10 at the carotid bifurcation; 5-7 in the internal carotid artery) characterized by circularly oriented smooth muscle cells, ground substance, occasional collagen fibrils, and fine elastic fibrils between the elastic plates. The tunica adventitia contained cells that resembled fibroblasts, collagen fibers, vasa vasora, and nerve fibers.

LESION PROGRESSION

The length of the circumference that contained lesions is presented in Fig. 2. The length of the circumference involved with atherosclerotic lesions increased as the period of time the animals were fed the atherogenic diet increased. Lesions occupied about 10% of the circumference at the carotid bifurcation after the birds had been fed the diet for 1 week; this increased to about 70% after 6 weeks of the diet. Although lesions involved 100% of the circumference length in some birds, there was an overall tendency, as seen in Fig. 2, for this parameter to cease progression and level off when most, but not all, of the circumference was involved. In cases with less than 100% circumferential involvement, lesions were usually lacking over the posterior flow divider.

Statistically, the baseline group (Group 1) was significantly different from Groups 4 through 9 (i.e. those birds fed the diet for 3 through 12 weeks) ($p < 0.05$).

The bifurcation showed preferential and accelerated lesion involvement as the time fed the atherogenic diet increased. The dorsal and internal carotid arteries had less circumference which contained lesions at any given time when compared to the carotid bifurcation, but lesions at these sites did progress over the time course of the experiment. Dorsal and internal carotid arteries were not significantly different from each other at any time interval, but each of these arteries did differ significantly from the carotid bifurcation in Groups 5 through 9 (i.e. those birds fed the atherogenic diet for 4-12 weeks) ($p < 0.01$).

Mean lesion thickness for the three arterial segments is presented in Fig. 3. Increased intimal thickness at the carotid bifurcation was first observed in the Group 3 birds (2 weeks on diet) and progressed with time. Increases in intimal thickness of the dorsal and internal carotid arteries were initially observed in Group 4 (3 weeks on diet). The graphs of the lesions of these two vessels remained relatively flat through Group 8 (8 weeks on diet), but by 12 weeks (Group 9) began to show an upward tendency. Analysis of lesion thickness with respect to time showed that Groups 1 (baseline) through 6 (6 weeks on diet) were all significantly different from Group 9 ($p < 0.01$). Group 7 (7 weeks on diet) was also different from Group 9 (12 weeks on diet) for this variable ($p < 0.05$). Statistical

analysis showed that the dorsal and internal carotid arteries were not significantly different from each other for the thickness parameter at any of the time segments used in the study. The bifurcation was significantly different from the dorsal and internal carotid arteries among Groups 4 through 9 (i.e. 3 through 12 weeks on diet respectively) ($p < 0.05$).

The cross-sectional area of lesion involvement (Fig. 4) and the percent lumen stenosis (Fig. 5) for the three arteries correlated with the thickness parameter, and similar curves were produced.

There were no significant differences between right and left sides among the groups for any of the four parameters.

LESION TYPES AND LOCATIONS

The incidence of lesion types observed in this study is presented in Table 2. Fatty streaks were the earliest lesions observed and histologically were composed of focal accumulations of interstitial lipid within a relatively thin intima and in the sub-jacent media (Fig. 6). Later-stage fatty streaks, although still relatively thin, contained occasional foam cells, interstitial lipid, and a slight increase in ground substance when compared to the earliest lesions (Fig. 7).

Proliferative lesions were thicker than fatty streaks. Microscopically, early stages of this type of lesion were composed primarily of foam cells (Fig. 8). Mean foam cell content scores for the 3 vessel segments of each group are listed in Table 3. At the

bifurcation, atherosclerotic lesions contained the greatest amount of foam cells after birds had been fed the atherogenic diet for 6 weeks; thereafter, the foam cell content began to decrease.

With time, the proliferative lesions increased in thickness and area and were composed not only of foam cells but also of non-lipid-containing spindle-shaped cells with associated increases in collagen and ground substance. In the larger and later-stage proliferative lesions, early fibromuscular cap formation was observed in the superficial regions of the plaque (Fig. 9). Fully-developed fibromuscular caps were not observed. Evaluation of the HPS stain indicated that the cell types comprising the cap resembled fibroblasts as well as smooth muscle cells, with the latter cell type predominating. Cell types in the fibromuscular cap were verified using transmission electron microscopy (Fig. 10).

Atheromatous lesions were observed in the arteries of several birds fed the atherogenic diet for 12 weeks. These lesions were as thick or thicker than the late-stage proliferative lesions and contained a central core composed of necrotic cells and lipid (Fig. 11). Overlying the necrotic core, there were usually several layers of intact foam cells. Thin fibromuscular caps were observed overlying parts of the intact foam cell component; however, fully developed caps were not observed.

Birefringence was negative in all fatty streaks and early proliferative atherosclerotic lesions. During the experimental time course, the birefringence increased in both incidence and intensity

for the dorsal and internal carotid arteries (Table 3). Table 2 shows that for the carotid bifurcation, lesion birefringence reached a peak after the birds had been fed the diet for 7 weeks; thereafter the birefringence began to decrease. This decrease was attributed to the increasing content of non-birefringent substances such as ground substance in the lesion. On an individual basis, however, all proliferative and atheromatous lesions showed some degree of birefringence.

The temporal development of proliferative lesions compared well with the other descriptions of atherosclerosis, i.e., lesion thickness, area, and percent lumen stenosis. Fatty streaks began to progress into proliferative lesions at the lateral walls of the carotid bifurcation in birds fed the atherogenic diet for two weeks, and in the dorsal and internal carotid arteries after birds consumed the diet for three weeks. After four weeks of consuming the atherogenic diet, all birds had lesions at the carotid bifurcation, and after six weeks all of the lesions of the bifurcation were of the proliferative type. One hundred percent involvement with proliferative lesions was not observed for either the dorsal or internal carotid arteries even in those birds that were fed the atherogenic diet for the longer time periods.

Table 4 shows that the earliest lesions at the carotid bifurcation did not occur on the flow dividers. Instead, the earliest lesions were observed on the lateral walls that would, more distally, form the lateral wall of the external carotid artery and

the medial wall of the internal carotid artery (Fig. 12). There appeared to be an equal chance of lesions first appearing in either or both of these two areas.

Atherosclerotic involvement of the flow dividers occurred slightly later with the first lesions being noted on the lateral edges of the flow dividers. The early lesions associated with the flow dividers were fatty streaks. During the early time periods, when the flow divider had fatty streaks, the lateral walls of the bifurcation had either late-stage fatty streaks or proliferative lesions on either or both walls.

During atherogenesis, the anterior flow divider showed a predilection for atherosclerosis development when compared to the posterior flow divider. As time progressed, both areas usually developed lesions; however, lesions were invariably thicker overlying the anterior flow divider.

The majority of lesions within the dorsal carotid artery (67%) were located on the anterior and/or the anterior-medial wall. The distribution of lesions within the internal carotid artery was similar, i.e., of the lesions observed, 47% were present on the anterior and/or anterior medial wall.

Microscopically, the normocholesterolemic birds appeared to have the same trends of lesion location.

Gross staining of the contralateral carotid arteries of the hypercholesterolemic birds confirmed the microscopic observations of lesion location.

There was no focal accumulation of either sulfated or non-sulfated glycosaminoglycans (GAGs) preceding lesion development in the lesion-prone areas, i.e., the lateral walls of the carotid bifurcation extending to the lateral edges of the flow dividers. Both non-sulfated and sulfated GAGs were present in the proliferative and atheromatous lesions, with the latter variety predominating.

Samples of carotid arteries from the hypercholesterolemic pigeons showing either no lesions, fatty streaks, proliferative or atheromatous lesions were stained for ATPase activity. Results were uniformly negative.

Non-specific esterase reactions revealed that lesion-free intimal areas produced a +2 to +3 reaction. Intimal lesions, however, gave a negative to +1 reaction. Regardless of lesion involvement, the medial reaction ranged from trace to +1 in all birds, and the adventitial reaction ranged from +2 to +3.

A Carstairs stain for fibrin and platelets was performed on the carotid arteries from at least 2 birds per group to determine if fibrin and/or platelets were present on the endothelial surface. Slides were fibrin negative. Confirmation of platelet adherence could not be made with this stain because bird thrombocytes are nucleated and difficult to distinguish from other nucleated blood cells at the light microscopic level. Transmission electron microscopic studies showed that neither fibrin nor thrombocytes were adherent to the endothelial surface.

Examination of the Carstairs and hematoxylin-phloxine-saffron stains from birds that lacked distinct fatty streak development showed that some of the specimens had alterations of the tunica intima in those areas lateral to the flow dividers. The alterations included the following: (1) the presence of numerous cells in the subendothelium, (2) irregular shapes to the endothelial cell profile, (3) crescent-shaped (or otherwise bent and irregular) appearances of the endothelial cell nuclei, and (4) non-uniform spacing between the cells with some piling up of the cells. The tunica intima over the flow divider itself revealed normal morphology. The endothelial cells in this area appeared elongated with low-lying profiles; the nuclei were nearly parallel to the endothelial basement-membrane area. Transmission electron microscopy on representative samples confirmed that many of the altered cells of the tunica intima were endothelial in nature with an occasional adherent monocyte-like cell (Fig. 13). Monocyte-like cells thought to be in the beginning stages of transformation into foam cells were also observed in the subendothelial space (Fig. 14). It is possible that the cell piling up observed at the light microscopic level was partially due to the presence of these sub-endothelial monocyte-like cells.

Correlating the presence of the altered intimal layer with the Oil red O staining indicated that, in most cases, the appearance of intimal damage in the lesion-prone areas preceded the appearance of

lipid accumulation. There were a few cases, however, in which trace amounts of intimal lipid were present in areas that lacked manifestations of damage at the light microscopic level.

CHAPTER V

DISCUSSION

The present study has defined the time course of atherogenesis and atherosclerosis progression in the carotid bifurcation, distal dorsal carotid and proximal internal carotid arteries of randomly-bred White Carneau pigeons fed a cholesterol-containing diet. Atherosclerotic lesions at these locations progressed in terms of length of circumference involved, intimal thickness, cross-sectional area of lesion involvement, and percent lumen stenosis as the time the birds were fed the atherogenic diet increased. These were expected findings since an elevated plasma cholesterol concentration has long been recognized as a principal risk factor in the development of atherosclerosis in humans. Studies using experimental animals have also shown a positive correlation between the degree of hypercholesterolemia and the development of atherosclerosis.

A characteristic feature of the distribution of atherosclerotic lesions in animal models and in human beings is that neither all arteries nor sites within an artery become affected at the same time or to the same extent. Similar observations were noted in this study. Of the three arterial segments examined, the carotid bifurcation developed lesions prior to the other sites. Increase in lesion thickness also occurred preferentially at the bifurcation, and this trend

continued during the experimental period. The circumference of the internal elastic lamina of all vessels remained relatively constant among the different time periods, indicating that arteries were predisposed to stenosis rather than dilatation. Some medial thinning was observed in the vessels of birds that had been fed the atherogenic diets for 12 weeks indicating that arterial dilatation may perhaps be a subsequent development in lesion evolution. There was some within-group individual variation for lesion development at the three anatomical sites studied; however, this was an expected phenomenon of lesion induction in a randomly-bred population. Statistically significant differences between sites examined from the right and left carotid arteries were not observed, indicating bilateral involvement. In human beings, atherosclerotic lesions at the carotid bifurcation are also quite often bilateral.

In human beings, the lesions most likely to progress to significant atherosclerotic plaques are located near vessel branch points. As noted previously, the present study has shown that the carotid bifurcation is a predilective site for early lesion development in hypercholesterolemic White Carneau pigeons. Santerre et al. (39) have previously reported the development of naturally-occurring atherosclerosis at the celiac bifurcation of White Carneau pigeons and noted that, whereas the earliest lesions appeared over the left lateral subendothelial cushion, older birds showed greatest involvement on the right lateral cushion. The majority of the birds used in the celiac study ranged in age from one to six years, and it is

possible that observation of the carotid arteries of comparably-aged pigeons might show that the flow divider is a predilective site for initial lesion appearance. However, our observations of early lesion development of carotids of hypercholesterolemic randomly-bred White Carneau pigeons, 7-19 weeks of age, showed that the initial lesion appeared, not over the flow dividers, but rather to the sides, i.e., in those areas that would eventually form either the lateral wall of the external carotid or medial wall of the internal carotid artery. The results presented in this study, therefore, are more similar to those reported by Tesar and Kottke (15) who recently described the lack of association between naturally-occurring early lesion development and the presence of musculoelastic intimal cushions in the aortas of White Carneau pigeons. Flow divider involvement was noted in the present study as a slightly later feature. After birds had been fed the atherogenic diet for four weeks, the flow divider showed some degree of lesion involvement approximately equal to that found on the lateral walls at the carotid bifurcation. The anterior flow divider was invariably involved to a greater extent than the posterior flow divider. Hemodynamic conditions and subtle metabolic differences have been postulated to play a role in lesion localization in human beings and in animal models, and it is likely that similar factors would operate in lesion localization in the carotid arteries of the White Carneau pigeon.

One of the interesting observations made in this study was the appearance of an altered tunica intima at the lateral walls of the

bifurcation - the "lesion-prone" area. These alterations were present prior to fatty streaking in most cases, and were thought to indicate endothelial injury. An altered intimal layer was not present over the flow divider areas; here the endothelium appeared normal. Injury to the endothelium has long been implicated in the initiation and development of atherosclerotic lesions. However, experimental studies have indicated that no single agent is responsible for the endothelial cell damage. Hemodynamic forces and hypercholesterolemia are among those factors that have been implicated as injurious agents.

Fry (56) and others (57,58) have suggested that atherosclerotic lesion development at bifurcations may result from the hemodynamic effects of high shear stress. Increased velocity, as well as flow patterns at these sites, produces a "drag" effect on endothelial cells, which may result in altered endothelial cells. Features of hemodynamically altered cells include irregular shape, non-uniform spacing between cells, and cellular edema (56). Such descriptions are similar to those for the altered cells observed in the present study, and the alterations could therefore be due solely to hemodynamic factors. However, the alterations were minimal to non-existent in control birds and increased as the level of hypercholesterolemia increased. This indicates that hypercholesterolemia and/or cellular metabolic differences as a response to the cholesterol levels could also be injurious factors.

The specific role of hypercholesterolemia in initial injury to the endothelium has not been extensively studied, although discontinuities in endothelial lining have been described as resulting from hypercholesterolemia in the rabbit by Goode et al. (59) and in the monkey by Ross and Harker (60). It is not clear whether the endothelial injury results from a sustained increase in cholesterol or from complicated effects of either hyperlipidemia or altered lipoprotein levels upon the endothelial cell. Alterations in membrane fluidity due to the increased cholesterol content could be the crucial initiating event (61). Membrane fluidity is known to have an important role in maintaining cell functions, and in certain cases, cells may adapt to different environmental conditions by altering the composition of the membrane in such a way that proper fluidity is maintained. For example, when placed in an environment containing increased cholesterol concentration, the membrane may maintain fluidity up to a point by increasing the unsaturation of the phospholipids. When this adaptation fails, the membrane is altered, and this may result in alteration of metabolism and/or lipoprotein binding.

Ross (62) has proposed that regardless of the nature of the causative agent of the injury, endothelial cells desquamate and the underlying connective tissue elements are exposed to blood components. Platelets adhere to the exposed areas, aggregate, and release their constituents, including a mitogenic factor that stimulates smooth muscle cell proliferation. This platelet proliferative factor is

not long acting, but other substances, including low-density lipoproteins are thought to have a role in supplying an additional stimulus for continued smooth muscle cell proliferation (63).

Duguid (64) has postulated that the atherosclerotic lesion is the end result of organization of mural thrombi, and this is referred to as the encrustation or thrombogenic theory. The mechanism involves the deposition of fibrin on the endothelium, which in the course of organization is covered by new endothelium and thus becomes incorporated into the arterial wall as an atherosclerotic lesion. The source of the contained lipids within the lesion is presumed to be the entrapped red cells and platelets. In progressing lesions, mural thrombi are largely converted to connective tissues by traditional fibroblast action.

In the present study, fibrin deposition was not observed at any stage of lesion development. The lack of fibrin in early stages was expected since the thrombogenic theory is not considered convincing in the early stages. However, in certain cases of later-stage lesions in man, morphological evidence for the thrombogenic theory is available. The lack of fibrin deposition in the later-stage lesions observed in the present study may be explained by the fact that many atherosclerotic lesions, especially those induced by hypercholesterolemia in certain animal models, do not show evidence of thrombus formation.

Platelets were not observed adhering to the endothelium in the present study. This could be due to a deaggregation phenomenon.

Most platelet aggregates deaggregate and return to the circulation unless the binding effect of the fibrin acts, and in this study, fibrin was not observed. Also, avian platelets (thrombocytes) are nucleated, and as such are difficult to distinguish, on a light microscopic basis, from the other nucleated cells that may be observed at the blood-artery interface (i.e. altered endothelials and white blood cells). Smith (65) also has stated that thrombocytes have not been observed in his studies of the White Carneau pigeon. However, published reports by Lewis and Kottke (10) and Albert et al. (66), using scanning electron microscopy, report the occurrence of thrombocytes adhering to endothelium and postulate for the avian thrombocyte a role similar to that proposed by Ross for the mammalian platelet.

Monocyte-like cells were observed on the endothelial surfaces and in the subendothelial space in this study. Such monocyte adherence has been described in swine (67) and has also been observed with scanning electron microscopy techniques in hypercholesterolemic White Carneau pigeons (68). It has been suggested that these monocyte-like cells migrate into the arterial intima in response to the presence of lipid within the arterial wall and then are subsequently transformed into the classic foam cells. Monocytes may not be the precursors of foam cells, however. Other researchers (69) believe that the source of these lipid-laden cells are the smooth muscle cells of intimal or medial origin. Morphological evidence from the present study indicates that it is possible that the monocyte

and/or the smooth muscle cell could be implicated as the precursor of the foam cell.

Histologically, at the carotid bifurcation, there was a transition from relatively thin fatty streaks to proliferative lesions that were initially composed predominantly of foam cells. The exact relationship between the fatty streak and the evolution of a developing atherosclerotic plaque has long been controversial. Human studies, for example, have shown that in the aorta, fatty streaks may appear in areas that differ from those where fibrous plaques develop. In other sites, notably the coronary and extracranial cerebral arteries, fatty streaks appear earlier and in the same anatomic locations where the fibrous plaques are eventually observed. The present study suggests that the fatty streaks observed lateral to the flow dividers at the carotid bifurcation are the precursors of the more advanced lesions at these sites primarily for such temporal and spatial reasons.

The composition of late-stage proliferative lesions was an admixture of foam cells, spindle-shaped cells, collagen, and ground substance and showed the tendency to the early formation of a fibromuscular cap. Atheromatous lesions appeared only in a few of those birds fed the atherogenic diet for 12 weeks, and this type of lesion was localized, with one exception, to the carotid bifurcation. These lesions also had relatively thin fibromuscular caps.

Lesions in the dorsal and internal carotid arteries remained predominantly, although not exclusively, fatty streaks and

early-proliferative-stage lesions until the birds had been fed the atherogenic diet for eight weeks. By 12 weeks, the lesions at these sites began to increase in thickness in a manner similar to that observed at the carotid bifurcation in the earlier time periods.

The significant human atherosclerotic lesion, the fibrous plaque, consists of a fibrocellular cap usually with an underlying lipid-rich atheromatous core. The fibrocellular cap itself contains collagen, smooth muscle cells and/or fibroblast-like cells, and elastic fibers. More than half of the dry weight of human plaques is composed of connective tissue components.

Increased collagen synthesis in vitro has been reported to occur in cultures of atherosclerotic pigeon aorta (70), and it is thought that this increased collagen synthesis is an important factor in the conversion of predominantly lipid-containing lesions to fibrous plaques. Histologic observations from this study would suggest a beginning conversion from the primarily foam cell-containing lesions into fibrous plaques. Prichard et al. noted a correlation between fibromuscular cap formation and age in naturally occurring atherosclerosis of the aorta in White Carneau pigeons (5). It is possible, therefore, that more extensive fibrous cap formation would have developed in the birds used for this study if the atherogenic stimulus was continued for a longer period of time. Overall, it would appear that the trend at the carotid arteries under study is toward the evolution of fibrous plaques not unlike those observed in humans.

When examined using polarizing microscopy, the fatty streak and early proliferative lesions were monorefringent. Birefringent lipid deposits were present in late proliferative and atheromatous lesions. Although polarizing studies of birefringence do not yield specific biochemical information concerning lipid characterization, most triglycerides, phospholipids, unsaturated fats, and fatty acids are usually monorefringent. Cholesterol and cholesterol esters are usually birefringent. St. Clair et al. (25) have demonstrated that in normal arteries newly synthesized fatty acids are esterified primarily to phospholipid and triglyceride, but in atherosclerotic lesions, most synthesized fatty acids are esterified with cholesterol. Cholesterol esters are the principal lipid components to increase during atherogenesis in man (71), pigeon (20), as well as most animal models (8). The stimulation of cholesterol esterification correlates well with the progression of atherosclerosis in the White Carneau (72) and other animal models such as squirrel monkeys (73) and rabbits (74).

The increased incidence of birefringence observed in the carotid arteries from the present study is attributed to the increase in cholesterol and cholesterol esters in the plaque. The change of monorefringent to birefringent lipids suggests that the predominant type of lipid in the plaques changes over time.

Cholesterol esters in atherosclerotic arteries may accumulate either by increased synthesis and/or by decreased hydrolysis. Low levels of hydrolase activities have been shown to be responsible

for the accumulation of the esters seen in cholesterol-induced atherosclerosis (26). In addition, aortic preparations contain cholesterol ester hydrolases in the microsomal, supernatant, and lysosomal fractions. Subbiah has reported that the microsomal fraction also contains non-specific esterase activity and that the hydrolytic capacity of this fraction on cholesterol esters is high (29). Studies of non-specific esterase in the present study showed a decrease in non-specific esterase activity, indicating that decreased hydrolytic activity may be responsible for the cholesterol ester accumulation that occurs during atherosclerosis progression in the carotid arteries of White Carneau pigeons. Additional specimens would have to be examined to verify these observations. In addition, biochemical studies for the various hydrolytic fractions would be of research value.

The lack of ATPase activity as demonstrated in the carotid arteries of this study supports other research findings that report a lack of ATPase activity for both intimal and intimal-medial layers of White Carneau pigeon aortas (30,31). The lack of ATPase activity is thought to be an early manifestation of uncoupling of oxidative phosphorylation which in turn slows the Krebs citric acid cycle, produces a higher rate of anaerobic glycolysis, and promotes lipid accumulation.

Crawford et al. have proposed that atherosclerosis is correlated with a deficiency of essential fatty acids (EFAs) (75). EFAs are important for growth and maintenance of cell membranes.

If arterial membranes are not properly synthesized during growth due to EFA deficiencies, the cells may be predisposed to atherosclerosis at a later time period. One of the effects of EFA deficiency is uncoupling of oxidative phosphorylation. EFA deficiency could produce a decrease in ATPase activity that in turn would result in the uncoupling of oxidative phosphorylation with subsequent lipid accumulation. Interestingly, it has been stated (75) that an EFA deficiency exists in experimental atherogenic diets in which the fat is provided either as cholesterol or as saturated fat indicating that a synergism may be acting between the lack of EFAs and a hypercholesterolemic state to produce arterial lesions.

The results for the GAG staining in the present study are in agreement with those reported by others (15,39). Evaluations of appropriately-stained tissue sections did not show focal accumulations of GAGs prior to the appearance of atherosclerotic lesions. However, the amounts of sulfated and non-sulfated GAGs in lesion areas do increase as the lesions themselves develop at those sites, indicating that GAG accumulation is the result, rather than the cause of lesion progression.

Clearly, histochemical stains are not sufficiently specific or quantitative for the several types of GAGs. The increased staining intensities observed may be the result, not only of enhanced synthesis of GAG, but also of possible alterations in their macromolecular structure. In addition, degradation of the native

proteoglycans (i.e. GAG plus protein combinations) may release additional binding sites from the GAG that result in increased staining intensity.

Overall, the results of the morphological data do not agree with the results of biochemical studies (40,76) that describe alterations in the content and composition of GAGs. These alterations occur prior to plaque formation and are postulated to be involved in atherogenesis by trapping and interacting with low-density lipoproteins producing their precipitation within the arterial wall and thereby initiating a lipid lesion.

There are several possible explanations for this variance between the morphological observations and the biochemical data. One reason is sample size. The morphological studies were performed on 6-8- μ m-thick tissue sections; the biochemical studies required comparatively large sample sizes. Curwen and Smith (40), for example, reported that 40 thoracic aortas or 300 celiac foci constituted optimal pool sizes for GAG isolation from RBWC pigeons. Such a large sample volume could therefore reveal differences that were undetectable at the tissue-section level. A second reason for the discrepancies concerns the arterial layers examined. The morphological studies examined only intimal layers since it is here that the postulated GAG alterations are said to occur. The biochemical studies (40,76) used aliquots of whole aorta, i.e. intimal, medial, and adventitial layers. The GAG changes that are reported by these studies cannot, therefore, be attributed solely to intimal alterations.

CHAPTER VI

CONCLUSIONS

The present study has demonstrated that the hypercholesterolemic randomly-bred White Carneau pigeon is a good model for studying carotid artery atherosclerosis. Lesions induced by the atherogenic diet appeared at a definable time and in a definable location.

After consuming an atherogenic diet for two weeks, the three arterial segments studied, i.e., the dorsal carotid artery, carotid bifurcation, and internal carotid artery developed lesions. Not all birds showed involvement, but the individual variation in the lesion development at the three sites was an expected phenomenon of lesion induction in a randomly-bred population.

Early fatty streaks were the initial lesions seen and were thin, focal accumulations of interstitial lipid in the intimal space. Later-stage fatty streaks were still thin but contained, in addition to the interstitial lipid accumulation, occasional foam cells and increased amounts of intimal ground substance. In those birds fed the atherogenic diet for 4 weeks, the bifurcation areas of all birds contained some type of lesion, with the minimal lesion being the fatty streak.

Proliferative lesions were observed subsequent to fatty streaks. These lesions were thicker than fatty streaks and were composed of variable amounts of foam cells, spindle-shaped cells, collagen and ground substance. Proliferative lesions began to occur at the carotid bifurcation during the second week of hypercholesterolemia. After six weeks on the diet, all of the lesions at the bifurcation were of the proliferative type.

Atheromatous lesions began to appear after 12 weeks of hypercholesterolemia, and principally at the bifurcation site. The atheromatous lesions were characterized by a central core of necrotic cells and lipid material; overlying the core were several layers of foam cells, spindle shaped cells, collagen, and ground substance.

The dorsal carotid and internal carotid arteries showed similar trends with regard to lesion types. Both of these arterial segments lagged behind the bifurcation, however, in the development of all three lesion types.

Because the transition from early lesions to later lesions occurred temporally and in the same location, it was concluded that the fatty streaks observed at the bifurcation were the precursors of the more developed manifestations of the atherosclerotic process.

The length of the circumference involved with atherosclerotic lesions increased as the period of time the animals were fed the atherogenic diet increased. Lesions occupied about 10% of the circumference at the bifurcation after birds had been fed the

atherogenic diet for 1 week, and increased to about 70% after 6 weeks. The carotid bifurcation was more susceptible to lesion development when compared to either the internal or dorsal carotid artery, but lesions at all three sites tended to increase in length until the majority of the circumference was involved.

A similar trend was observed in lesion thickness except that lesions became thicker after 2 to 3 weeks of hypercholesterolemia. The mean lesion thickness at the bifurcation after 12 weeks of the atherogenic diet was 175 μm , compared to approximately 75 μm each for the dorsal and internal carotid arteries.

The mean cross-sectional area of the lesions at the bifurcation initially increased between 2 and 3 weeks on the diet and then increased gradually through the remainder of the time groups. As with the length and mean thickness characteristics, the internal carotid and dorsal carotid arteries lagged behind the bifurcation for the area parameter.

Beginning between the second and third week of hypercholesterolemia, there was a gradual increase in percent lumen stenosis. Birds fed the atherogenic diet for 12 weeks had approximately 55% stenosis at the bifurcation, compared to approximately 20% each for the dorsal and internal carotid arteries.

In man, the lesions most likely to progress to plaques are located near vessel branch points; the present study demonstrated that the carotid bifurcation was the predilective site for early lesion appearance. Early lesions did not occur first on flow

dividers at the bifurcation, but instead on the lateral wall. Only after this did lesions involve the edges of the flow divider, or the flow divider itself. Altered endothelial integrity due to hemodynamic factors acting in synergy with hypercholesterolemia was thought to be the primary cause of lesion induction.

As lesions progressed, both monocytes and smooth muscle cells appeared to be converted into foam cells. With time, however, lesions ceased to be principally foam cell in nature and began to acquire the beginnings of fibromuscular caps not unlike those observed in humans.

Different types of lipids were found at different time periods as indicated by the polarizing microscopy observations. The change from mono- to birefringent lipid was attributed to an increase in cholesterol and cholesterol esters in the lesion. Birefringence increased in the dorsal and internal carotid arteries throughout the time course. At the bifurcation, birefringence reached a peak at 7 weeks on the atherogenic diet and thereafter began to decrease. The decrease was attributed to the growing percent content of non-birefringent substances such as ground substance and collagen in the lesion.

With time, there was an increased staining intensity observed for both sulfated and non-sulfated glycosaminoglycans (GAGs). Focal increases in staining intensity for GAGs were not observed to occur prior to the induction of an atherosclerotic lesion. The indications were that GAG accumulation was a result, rather than a

cause, of atherogenesis.

This study has described the time course of initiation of atherosclerosis and progression of the disease in the carotid arteries of cholesterol-fed White Carneau pigeons. This animal model should prove useful for studying additional anatomic, metabolic, and hemodynamic events associated with atherogenesis and atherosclerosis progression at the carotid bifurcation.

TABLES

EXPERIMENTAL DESIGN

Group	Weeks fed atherogenic diet	Age at sacrifice (wks)
1 (n = 10)	0	7
2 (n = 10)	1	8
3 (n = 10)	2	9
4 (n = 10)	3	10
5 (n = 10)	4	11
6 (n = 9)	6	13
7 (n = 14)	7	14
8 (n = 10)	8	15
9 (n = 16)	12	19

LESION TYPES IN CAROTID ARTERIES

Group	Artery	Percent of birds with lesions		
		Fatty Streak	Proliferative	Atheromatous
1	D ¹	10%	0%	0%
	B ²	10	0	0
	IC ³	0	0	0
2	D	0	0	0
	B	60	0	0
	IC	20	0	0
3	D	40	0	0
	B	60	20	0
	IC	30	0	0
4	D	40	30	0
	B	30	60	0
	IC	50	20	0
5	D	40	30	0
	B	40	60	0
	IC	50	30	0
6	D	44	11	0
	B	0	100	0
	IC	22	33	0
7	D	0	43	0
	B	7	93	0
	IC	21	36	0
8	D	10	60	0
	B	0	100	0
	IC	20	30	0
9	D	6	38	6
	B	6	69	25
	IC	6	69	0

¹Dorsal Carotid Artery²Carotid Bifurcation³Internal Carotid Artery

TABLE 3

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FOAM CELL CONTENT AND BIREFRINGENCE SCORES

Group	Vessel	Mean Foam Cell Score	Mean Birefringence Score
1	D ¹	0	0
	B ²	0	0
	IC ³	0	0
2	D	0	0
	B	0	0
	IC	0	0
3	D	0	0
	B	0.8	0.2
	IC	0.1	0
4	D	1.1	0.2
	B	1.8	0.4
	IC	0.5	0
5	D	1.3	0.3
	B	1.5	0.6
	IC	0.2	0.1
6	D	0.11	0.11
	B	3.67	1.33
	IC	1.0	0.22
7	D	1.1	0.28
	B	2.86	2.5
	IC	0.86	0.14
8	D	2.2	0.3
	B	2.7	1.2
	IC	1.1	0.3
9	D	0.94	0.56
	B	2.56	2.06
	IC	2.3	0.75

¹Dorsal Carotid Artery²Carotid Bifurcation³Internal Carotid Artery

LESIONS INVOLVING FLOW DIVIDERS AT THE CAROTID BIFURCATION

Group	% of birds with lesions	Mean % of intimal lesions overlying flow dividers	Mean % of intimal lesions not overlying flow dividers
1	10	0	100
2	60	11	89
3	90	37	63
4	90	42	58
5	100	45	55
6	100	46	54
7	100	69	31
8	100	51	49
9	100	57	43

FIGURES

FIGURE 1. Mean Total Plasma Cholesterol Concentrations.

FIGURE 2. Mean Length of Circumference that Contained Lesions.

FIGURE 3. Mean Lesion Thickness.

FIGURE 4. Mean Lesion Area.

FIGURE 5. Mean Percent Lumen Stenosis.

FIGURE 6. Early Fatty Streak Lesion.

Lateral wall of the bifurcation of a Group 4 bird. Section was stained with oil red O-hematoxylin and photographed with a green filter to enhance areas of red staining. Lipid accumulations appear black. 220X magnification.

FIGURE 7. Later-Stage Fatty Streak Lesion.

Section taken from the lateral wall of the bifurcation of a Group 6 bird. Note the presence of a few foam cells as well as intimal thickening and intimal lipid deposits. Oil red O-hematoxylin; 220X magnification.

FIGURE 8. Foam Cell Proliferative Lesion.

Detail of a proliferative lesion from the bifurcation area of a Group 9 bird. Note predominance of foam cells. Oil red O-hematoxylin stain; photographed with a green filter. 250X magnification.

FIGURE 9. Later-Stage Proliferative Lesion.

Later-stage proliferative lesion from the bifurcation of a Group 7 bird. The lesion has nearly occluded the portion of the bifurcation that will extend to form the internal carotid artery. Note the predominance of foam cells at the base of the plaque; the beginnings of fibromuscular cap formation can be seen overlying these foam cells. Oil red O-hematoxylin stain; photographed with green filter. 85X magnification.

FIGURE 10. Cells Comprising the Fibrous Cap.

Electron micrograph of the cell types found in lesion areas showing beginning fibromuscular cap formation. Note the numerous smooth muscle cells; part of a fibroblastic-like cell with cytoplasmic extensions and abundant rough endoplasmic reticulum may be seen at the lower right portion of the photomicrograph. Uranyl acetate and lead citrate stain. 7600X magnification.

FIGURE 11. Atheromatous Lesion.

Atheromatous lesion from the bifurcation of a Group 9 bird. Intact foam cells and the beginning of fibrous cap formation are observed overlying the necrotic core of the lesion. One μ m thick section; toluidine blue stain; photographed with green filter. 85X magnification.

FIGURE 12. Lateral Wall Lesions.

These lesions are from the bifurcation of a Group 8 bird. Note the involvement of the lateral walls with proliferative lesions. The anterior flow divider is showing partial involvement with a fatty streak lesion (arrow); lesions are lacking over the posterior flow divider. Verhoeff-Van Gieson stain; photographed with green filter. 85X magnification.

FIGURE 13. Altered Cells of the Tunica Intima.

Electron micrograph of the altered lining cells of the tunica intima; lateral walls of the bifurcation region of a Group 2 bird. These cells appear to be an admixture of injured endothelials and monocytes. Uranyl acetate and lead citrate stain. 4800X magnification,

FIGURE 14. Subendothelial Monocyte-Derived Foam Cell.

Electron micrograph of an early foam cell. The morphological appearance indicates that this cell is most probably of monocyte origin. Note the ovoid shape, irregular cytoplasmic borders, round nucleus with prominent nucleolus. Uranyl acetate and lead citrate stain. 11,600X magnification.

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