Monocyte Chemoattractants in Pigeon Aortic Atherosclerosis

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Atherosclerosis occurs in the aorta of White Carneau pigeons proximal to the celiac bifurcation, where monocyte adhesion and migration into lesions have been demonstrated. This study documents chemoattractants that might be responsible for monocyte adherence and migration. Ten-week-old pigeons were fed either a cholesterol-free (normal) diet or a 0.4% cholesterol diet for 12 or 24 weeks. Birds with a normal diet did not have lesions in the lesion-prone area of the aorta, whereas birds fed a cholesterol-containing diet had simple intimal foam-cell lesions (12 weeks) or foam-cell lesions complicated with extracellular lipid and fibrillar matrix material (24 weeks). Plasma cholesterol levels in birds on the cholesterol-containing diet were 780–1080 mg/dl versus 140–240 mg/dl in the normal diet control group(s) at necropsy. To assay for chemoattractants, tissue was collected from lesion-prone and nonsusceptible (nonlesion) areas of the aortas. Samples from the two types of regions were separately pooled, then homogenized and tested for chemoattractant activity for pigeon peripheral blood monocytes. Monocyte chemoattractants were demonstrated in lesion area homogenates from pigeons fed cholesterol for 12 or 24 weeks and also in analogous homogenates from pigeons fed a normal diet. Monocyte migration to lesion-prone homogenates was significantly greater than that to nonlesion area homogenates. The chemoattractants in homogenates were monocyte-specific. The chemoattractant activity in the birds fed cholesterol for 12 weeks was confined to the aqueous phase of lipid extracts. This activity was abolished by pronase but unaffected by heat (100 C, 30 minutes), which indicated that the chemoattractant(s) in these homogenates was heat-stable protein(s). Activity in lipid extracts of lesion area homogenates from birds fed a cholesterol-containing diet for 24 weeks was found in both the aqueous and organic phases, suggesting that these samples contained lipid as well as proteinaceous chemoattractants. (Am J Pathol 1987, 126:464–475)

THE EARLY atherosclerotic lesion is characterized by the presence of lipid-filled foam cells in the intima of the artery wall. The current consensus, based upon evidence from studies of human and animal model disease,[1–12] is that these foam cells are derived from both smooth muscle cells and monocyte-derived macrophages. The monocytic origin of macrophage foam cells has been suggested by morphologic and cytochemical studies of foam cells, both *in situ* and after isolation from lesions. This origin is also favored by the demonstration of monocytes adhering to lesions and migrating through the endothelium, as noted in several species, including the pigeon,[13–16] pig,[17,18] rabbit,[12,19] rat,[20] and nonhuman primates.[21] The relationship between adhering/migrating monocytes and the atherosclerotic lesion foam cells has recently been demonstrated in an autoradiographic study[22] in which ^{3}H-labeled pigeon monocytes adhered to and migrated into lesions becoming foam cells. Leukocyte adhesion to the arterial surface has been quantitated in the pig[23] and pigeon,[15] and it has been shown that the density of leukocytes is dramatically and specifically increased over developing lesions. The observations of focal margination suggest that there are substances present in the arterial wall at sites of lesion formation that act as chemoattractants for monocytes. This suggestion has been substantiated by recent work on aortic extracts and cell cultures. Gerrity et al[24] have demonstrated that extracts

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of atherosclerosis susceptible regions from the aortas or hypercholesterolemic pigs contain monocyte chemoattractants. It also has been shown that smooth muscle cells cultured from baboon[25,26] and human[27] aortas release substances into the medium that induce monocyte migration.

In the White Carneau pigeon the area of the aorta immediately proximal to the bifurcation of the celiac artery is the site at which atherosclerotic lesions initially develop.[28,29] Lesions develop at this site in young birds within 10–12 weeks after initiation of a cholesterol-supplemented diet. Lesions also occur naturally at this site, and by 3 years of age the celiac/aortic site is atherosclerotic in virtually all birds maintained on cholesterol-free diets.[15,29] The occurrence of atherosclerotic lesions in this predictable manner at a well-defined area of the aorta makes the pigeon an excellent model for determining the role of monocyte chemoattractants in early atherosclerosis. The present report summarizes studies of monocyte chemoattractants in the lesion-prone area of the pigeon aorta. Our studies show that chemoattractants were present prior to lesion formation in the lesion-prone area of aortas from pigeons fed a cholesterol-free diet and in foam-cell lesions in the aortas from birds fed a cholesterol-supplemented diet for 12 and 24 weeks.

Materials and Methods

The animals used were randomly bred White Carneau pigeons obtained from a closed colony at the Pigeon Resource at the Bowman Gray School of Medicine. At the start of this study 10-week-old birds were divided into three groups based on the diet they were fed and the length of time on the diet: cholesterol-free pigeon pellet diet for 12 weeks, Group 1; pigeon pellets supplemented with 0.4% cholesterol and 10% lard for 12 weeks, Group 2; same supplementation for 24 weeks, Group 3.

Arterial homogenates were prepared from the pooled tissue samples of 20–40 pigeons from one of the groups defined above. Prior to necropsy, the birds were given a lethal intravenous injection of pentobarbital (0.45 ml/bird). The chest was opened, and the animals were quickly exsanguinated by cardiac puncture to reduce clotting in the aorta and to obtain blood for analysis of total plasma cholesterol by the use of the Autoanalyzer II method.[30] After exsanguination, the aortas were removed and rinsed with ice-cold Puck's G saline (PS) (137 mM NaCl, 0.14 mM $CaCl_2$, 1.28 mM $MgSO_4$, 1.1 mM KH_2PO_4, 1.1 mM Na_2HPO_4, 5.3 mM KCl, and 6.1 mM glucose, pH 7.0). Adventitial connective tissue was cleaned from aortas, which were then slit open ventrally for gross examination for lesions. Samples from lesions and nonlesion regions were taken from each aorta. The lesion samples were 5–8-mm segments proximal to and including the celiac bifurcation; the celiac artery itself was not included. Samples from nonlesion regions were 5–8-mm segments of the aorta immediately distal to the arch. These two types of samples were separately pooled and weighed. Pooled tissue samples were homogenized in ice-cold Puck's G saline (100 mg tissue/ml) with a Polytron (Brinkmann Instruments), then centrifuged at 1600*g* for 20 minutes at 4 C. The protein concentration of the supernatants was determined by the method of Lowry.[31] Homogenates were stored at −70 C without apparent effect upon chemoattractant activity. Based on the protein concentrations, lesion and nonlesion homogenates were diluted with Puck's G saline containing 2% (wt/vol) bovine serum albumin (PBSA) and tested for chemoattractant activity. The final dilutions of the homogenates varied as specified in the figure legends.

Cell Preparations

Mononuclear Cells

Ten milliliters of citrated pigeon blood was centrifuged at 150*g* for 10 minutes. The resulting buffy coat at the red cell/plasma interface was swirled into the overlying plasma with a plastic transfer pipette. Leukocyte-rich plasma was then diluted 1:1 with PS, layered over 9 ml of Isolymph (Gallard-Schleshinger Inc.), and then centrifuged at 400*g* for 40 minutes. Mononuclear cells located at the plasma/Isolymph interface were removed, washed twice in PS, and finally resuspended at a concentration of 4×10^6 cells/ml in PBSA.

Heterophils

The heterophils used in this study were elicited from the peritoneal cavity by the following procedure. Pigeons were anesthetized with methoxyflurane and given an intraperitoneal injection of 5 ml of 3% thioglycollate (g/100ml). Five hours after injection, the birds were given a lethal dose of pentabarbital, and the peritoneal cavity was opened and flushed with 30 ml of PS. The cell suspension collected from the peritoneal cavity was centrifuged at 150*g*, washed twice in PS, and finally resuspended at a concentration of 2×10^6 cells/ml in PBSA. This cell suspension was 90–99% heterophils as evaluated on Wright's-stained preparations. All pigeons used as donors for monocytes and heterophils were fed a cholesterol-free diet. Both monocytes and heterophil preparations were 95–99% viable, as assessed by exclusion of trypan blue.

Assay of chemoattractant activity was carried out with the use of a 48-well chemotaxis chamber (Neuroprobe Inc.) consisting of 25-μl capacity bottom wells and 50-μl capacity top wells. A polycarbonate filter (PVP-free) with 5-μ pores (monocytes) or 3-μ pores (heterophils) separated the attractants in the bottom wells from the cells in the top wells. In each assay, paired homogenates from lesion and nonlesion areas, prepared from the same diet group of birds, were assayed with cells from the same donor bird. PBSA was used as a negative control, and a 1% solution of zymosan-activated normocholesterolemic pigeon serum[32] in PBSA was used as a positive control. Chambers were incubated for 2 hours (monocytes) or 1 hour (heterophils) in a moist atmosphere of 95% air and 5% CO_2 at 37 C. After incubation the filter was taken from the chamber, and the cells remaining on the top of the filter was removed. The filter was then fixed in 100% methanol, stained 12 minutes in hematoxylin and 10 minutes in Wright's stain (Fisher Scientific), and rinsed in distilled H_2O. Stained filters were dried and mounted on glass slides with coverslips. Migration was evaluated as the number of cells counted in ten 1000× fields/well. Each dilution of the attractants was assayed in triplicate.

Lipid Extraction

Lipids were extracted from homogenates by a modification of the method of Bligh and Dyer.[33,34] Briefly, after the addition of $CHCl_3$:CH_3OH, the pH of extracts was adjusted to 3.5 with formic acid for maximizing the recovery of acidic and polar lipids in the organic phase. The organic phase of these extracts was dried on a rotary evaporator, resuspended in chloroform/methanol (1 : 2), placed in sealed glass vials containing N_2 gas, and stored under liquid N_2. The aqueous phase of these extracts was dried overnight to a powder by speed vacuum centrifugation (Speed Vacuum Concentrator, Savant). The resulting powder was redissolved in PS and stored at −70 C. The volume of the redissolved extracts was equal to that of the original starting volume of homogenate. In order to be assayed for chemoattractant activity, aqueous extracts were diluted with PBSA. Organic extracts were dried with a stream of N_2 gas and resuspended in PBSA.

Heat Treatment

Homogenates were diluted to 1 mg protein/ml and zymosan-activated serum (ZAS) to 10% (vol/vol) with PS. One tube each of homogenate and one of ZAS was heated for 30 minutes at 22, 56, 80, or 100 C. Heated samples were cooled to room temperature then diluted with PBSA and assayed for chemoattractant activity.

Protease Treatment

Homogenates and ZAS were diluted as described for heat treatment. Pronase used in these experiments was immobilized on agarose beads (Pronase-CB, Pierce Chemical Co.), which had been washed three times in PS before use to remove azide. Washed beads were added to tubes containing homogenate, ZAS, or PS for obtaining a final concentration of 2.8%, 5.6%, or 11% pronase (micrograms pronase/100 μg protein). Tubes were incubated for 1 hour at 37 C, after which they were centrifuged to pellet the pronase beads. Supernatants were removed, diluted with PBSA, and assayed for chemoattractant activity.

Molecular Weight Determination

A 0.5-ml sample homogenate from lesion-prone regions containing two milligrams of protein was applied to a 1 × 30-cm column of Biogel P-100, 50-100 mesh (Bio-Rad Laboratories) and fractions were collected by gravitational flow with PS as the eluent. The relative protein concentration of each fraction was determined by absorbance at 280 nm. Column fractions were diluted in PBSA, then assayed for chemoattractant activity.

Scanning Electron Microscopy

The lesion areas of the aortas of pigeons fed a 0.4% cholesterol-supplemented diet for 12 weeks were prepared for scanning electron microscopy as previously described.[15,16] All vessels were fixed *in situ* by perfusion under controlled pressure (120 mm Hg) with 0.1 M cacodylate-buffered 2.5% glutaraldehyde. Following fixation the vessels were excised, mounted on Teflon supports, dehydrated through an ethanol series, and dried from CO_2 with the critical-point method.[16]

Results

Previous studies[13-15] have shown that leukocyte adhesion to the lesion-prone area of the aorta was maximal in young birds fed a cholesterol-supplemented diet for 10–12 weeks. Based upon this observation, it was hypothesized that if monocyte chemoattractants were responsible for the specific adhesion and migration of leukocytes to lesions, the chemoattractants would be demonstrable at the time of maximal leukocyte adhesion. For this reason, the majority of the experiments in this study were con-

ducted with the use of homogenates prepared from 12-week-old pigeons that were entered in the study at 10 weeks of age and were fed a 0.4% cholesterol diet for 12 weeks. As noted in the methods section, studies were conducted on birds in groups of 20–40; throughout the course of this study 15 such groups of pigeons fed a cholesterol diet for 12 weeks were studied.

The mean total plasma cholesterol of all the pigeons after 12 weeks of the cholesterol diet was 776 ± 118 mg/dl (mean ± SD); this was in contrast to a baseline value below 250 mg/dl. The individual group means ranged from 142 to 241 mg/dl for baseline birds over 3 years of these studies. When aortas were opened and examined for grossly visible lesions at the celiac bifurcation, 60% of the animals had lesions. Most of the lesions (74%) were flat to slightly raised and were circular in shape, with sizes ranging from 0.5 to 2 mm in diameter. The remaining lesions appeared as faint, flat, yellow streaks. Lesions were not observed in the aorta near the arch, the region used as the nonlesion standard. Twelve-week lesions, similar to those used in the present study, when examined by transmission electron microscopy (TEM), were found to be composed principally of foam cells confined to the intima.[35] Consistent with previous observations, arterial lesions from the present study when examined by scanning electron microscopy were characterized by the adherence of leukocytes to the endothelium.[12–14] Many of the adherent leukocytes had lamellåpodia, which extended toward the lesion. This morphologic feature was typical of cells responding to chemotactic stimuli (Figure 1).

The mononuclear cell preparations used in assays for chemoattractant activity of arterial homogenates were 60% monocytes, 30% lymphocytes, and 10% thrombocytes, as evaluated by applying criteria of Lucas[36] to Wright's-stained cells. The presence of nonspecific esterase and peroxidase is commonly used with mammalian leukocytes for identifying monocytes and distinguishing them from lymphocytes; however, neither of these enzymes was found by cytochemical assay in pigeon monocytes. Monocytes of other avian species have also been reported to lack these enzymes.[37,38]

In the assays of chemoattractants, cells that had migrated through the filter in response to ZAS or arterial homogenates were 12–20 μ in diameter; the cytoplasm of these cells was often highly vacuolated and had the appearance of activated monocyte-macrophages. Monocyte migration toward homogenates from lesions in 12-week cholesterol-fed birds was greater than to homogenates from paired nonlesion regions (Figure 2), and at concentrations of 1, 31.6, and 100 μg protein/ml (log 0, 1.5, and 2), the differences in migration stimulated by these two types of preparations were significant ($0.025 < P < 0.05$). In all these homogenates maximal lesion activity was found at a protein concentration of 100 μg protein/ml (log

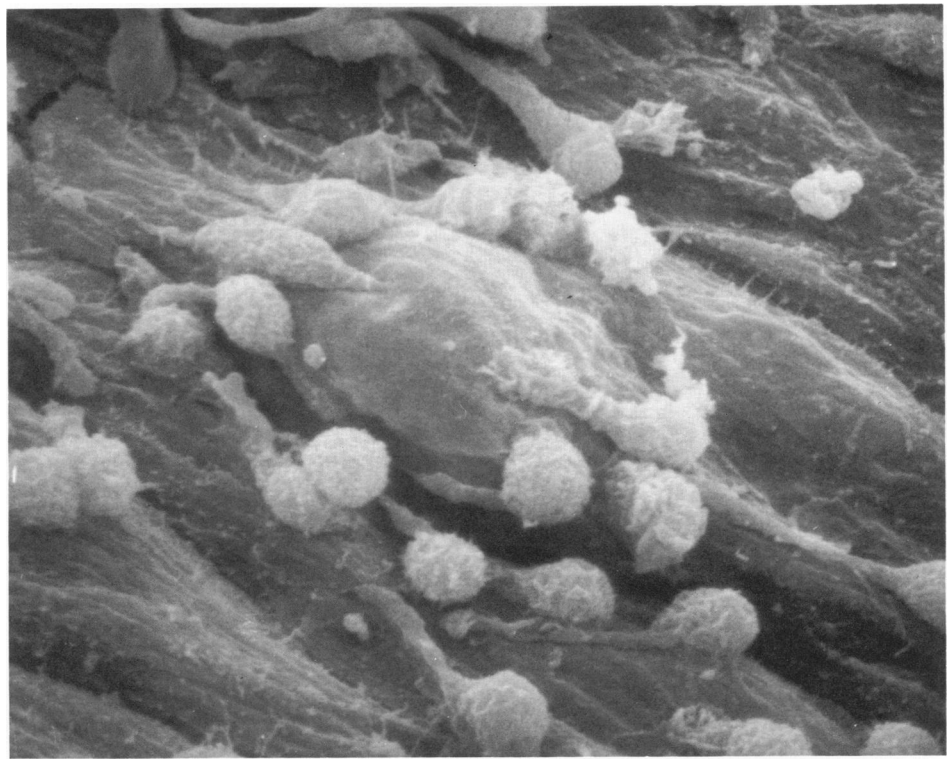

Figure 1—Lesion-prone area of the aorta from a pigeon fed the cholesterol diet for 12 weeks. A small lesion in the center is encircled by leukocytes with lamellåpodia extended toward this lesion; the morphologic features of the adherent leukocytes are suggestive of migration of these cells toward the lesion. (×3750)

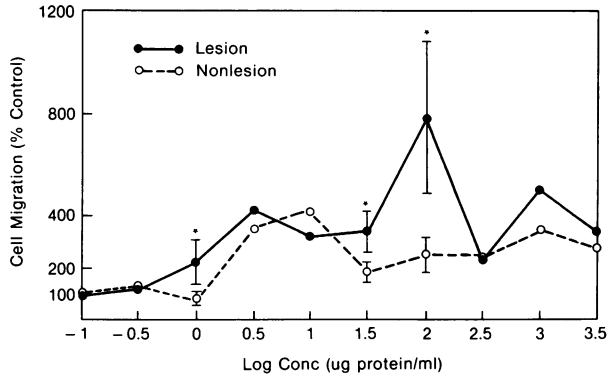

Figure 2—Monocyte response to lesion-prone and nonlesion area homogenates prepared from aortas of pigeons fed cholesterol for 12 weeks. Migration was quantitated as the number of monocytes per ten 1000× fields. Results are expressed as the percentage of the response to PBSA control; 100% represents the control response. Each point represents the mean±SEM of 5–15 experiments done in triplicate. *Concentrations at which there was significant difference ($P < 0.05$) between lesion and nonlesion homogenates. When expressed as cells per ten fields, the response of monocytes to PBSA and 1% ZAS was 17±4 and 165±25, respectively.

2). Since the monocytes are the principal leukocytes that migrate into the artery wall and become foam cells, it was important to determine whether the chemoattractant activity of the lesion homogenates was a general leukocyte attractant or whether it was specific for monocytes. In mammals the most numerous granulocytic leukocyte is the neutrophil, a cell that responds to numerous chemoattractants. The equivalent cell type in avian species is the heterophil[36]; therefore, chemotaxis assays paralleling the monocyte studies were carried out with this cell type. Heterophil migration stimulated by both types of homogenates was significantly less ($P < 0.05$) than monocyte migration (Figure 3) except at the highest protein concentration tested (log 3, 1000 μg/ml). At this high concentration the heterophil response to homogenates from susceptible regions approached, but was still less than, the monocyte response. With this exception, the heterophil response to either of the homogenates was no different from the response to the PBSA control. However, the average heterophil response to ZAS was 5.1 ± 0.8 times that to PBSA, which indicated that the low migration response to the homogenates was not due to inactivation of heterophils during cell preparation. Finally, it was reported for the pig that the chemotactic response to atherosclerotic lesion extracts is greatest with monocytes isolated from hypercholesterolemic animals.[24] To test the potential for a cholesterol effect directly on monocytes, 10 pigeons were maintained for 12 weeks on a diet supplemented with 0.4% cholesterol. Blood samples were obtained at biweekly intervals for lipid and lipoprotein analysis as already described. In addition to plasma lipid analyses, leukocyte differentials and monocyte responsiveness in chemotaxis were monitored. As summarized in Table 1, the total number of leukocytes in circulation decreased dramatically by 42% during the first 6 weeks, then gradually returned to control values over the balance of the experimental period. This variation in total leukocyte number was paralleled by the percentage of monocytes, which decreased from 28% of leukocytes at the beginning of the experiment to 16% at Week 2. The early decrease in monocyte percentage was followed by a period of rebound; and at the end of the experiment, monocytes comprised 35% of the circulating leukocytes. Although both absolute number of monocytes and their percent contribution to the leukocyte pool varied, differences were not found in the chemotactic response.

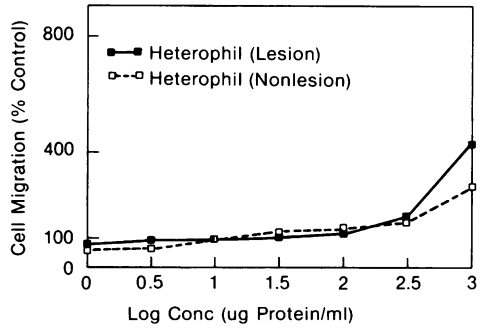

Figure 3—Heterophil response to lesion-prone and nonlesion area homogenates prepared from aortas of pigeons fed cholesterol for 12 weeks. Migration was quantitated as the number of heterophils per ten 1000× fields. Results are expressed as the percentage of the response to the PBSA control; 100% represents the control response. Each point represents the mean of two experiments done in triplicate. When expressed as cells per ten fields, the response of heterophils to PBSA and 1% ZAS was 72±11 and 420±85, respectively.

As a first step in determining the characteristics of the chemoattractant in lesion homogenates, the heat stability of the activity was tested at four temperatures. The monocyte chemoattractant in the homogenates from susceptible regions was found to be heat-stable when homogenates were heated for 30 minutes at temperatures ranging from 22 to 100 C (Figure 4). ZAS was used as a control in these experiments; its chemotactic activity is primarily due to C5a, which is stable at 56 C.[39] The activity of ZAS heated at 80 and 100 C was significantly less ($P<0.01$) than that of ZAS at 22 C (see Figure 4).

In order to further determine the chemical characteristics of the active substance(s), we extracted lesion-prone region homogenates for lipid and assayed the resulting aqueous (proteins and carbohydrates) and organic (lipids) extracts for their ability to induce monocyte migration. As shown in Figure 5A, the bulk of the activity was recovered in the aqueous extract. The magnitude of the monocyte migration in re-

Table 1—Leukocyte Differentials and Plasma Cholesterol Levels in White Carneau Pigeons Maintained on Cholesterol-Supplemented Diets

Weeks on diet	Total plasma cholesterol*	Leukocyte analyses: Total leukocytes†	Differential by percentage‡: Monocytes	Heterophils	Lymphocytes
0	196 ± 15	58,000	28	31	39
2	560 ± 83	55,000	16	37	45
4	758 ± 115	38,000	18	21	56
6	993 ± 126	35,000	32	20	46
8	994 ± 90	49,000	35	26	37
10	841 ± 73	44,000	42	25	32
12	815 ± 121	53,000	34	20	45

*Mean and standard error shown for 10 animals at each sample time.
†Includes lymphocytes, monocytes, heterophils, basophils, and eosinophils. Thrombocytes not shown. Mean value of 10 animals rounded to nearest 1000 cells/μl.
‡Basophils and eosinophils comprised 1–4% of leukocytes throughout the study.

sponse to the aqueous extract (as was also true with the whole homogenate) was dependent upon protein concentration, the maximum response being obtained at 1 μg protein/ml (log 0). Although some activity was detected in the organic extract as well (Figure 5B), it was not significantly different from the control response (100%).

Since most of the activity was found in the aqueous extract, which contains proteins and carbohydrates, we treated homogenates with a protease to determine whether the chemoattractant was protein. Homogenates from lesion-prone regions and ZAS as a control were incubated with three concentrations of pronase for 1 hour, then assayed for chemoattractant activity. ZAS was used as the control because its active substance, C5a, is a protein subject to inactivation by pronase. The activities of both preparations were decreased when incubated with increasing concentrations of pronase (Figure 6). This loss of homogenate activity after pronase treatment, as well as the recovery of activity in the aqueous phase following lipid extraction, suggested that the lesion chemoattractant was protein.

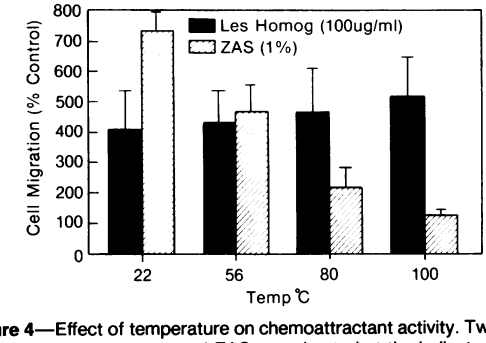

Figure 4—Effect of temperature on chemoattractant activity. Twelve week lesion-area homogenates and ZAS were heated at the indicated temperatures for 30 minutes. Monocyte migration to heated homogenates and ZAS was quantitated as the number of cells per ten 1000× fields and expressed as the percentage of the response to the PBSA control. Each bar in the histogram represents the mean ± SEM of five experiments done in triplicate.

The molecular weight of the active protein(s) in the homogenates from lesion-prone regions was estimated with the use of gel column chromatography. A sample of lesion area homogenate containing approximately 2 mg of protein was applied to a Biogel P-100 column, which has a 5–100-kd fractionation range. The majority of the proteins in the lesion homogenates eluted between 67–100 kd (Figure 7). Maximal monocyte chemoattractant activity was detected in a molecular weight range of approximately 5–10 kd. Activity of 100–200% above PBSA control was found in nearly all fractions tested, with the exception of

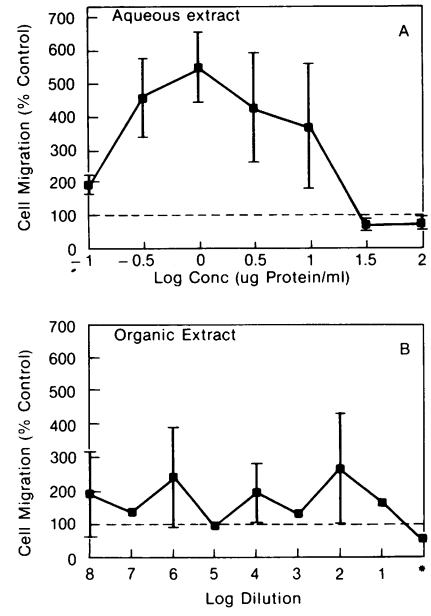

Figure 5—Monocyte migration to the aqueous (**A**) and organic (**B**) phases of lipid extracts of aortic homogenates from lesion-prone area of 12-week cholesterol feeding experiments. In **B**, the asterisk indicates the undiluted organic phase extract. Monocyte migration was quantitated as the number of cells per ten 1000× fields and expressed as the percentage of the response to the PBSA control; 100% (---) represents the control response. Each point represents the mean ± SEM of five experiments done in triplicate.

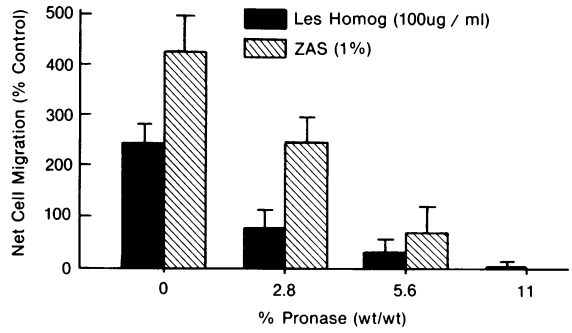

Figure 6—Effect of protease on chemoattractant activity. Homogenates from lesion and nonlesion areas of 12-week cholesterol-fed birds, ZAS, and PS were incubated with the indicated concentrations of pronase for 1 hour at 37 C; percent pronase indicates micrograms pronase/100 μg homogenate or ZAS protein. Monocyte migration was quantitated as the number of cells per ten 1000× fields. Results are net cell migration expressed as a percentage of the response to the PS control incubated with the corresponding amount of pronase.

$$\text{Net migration} = \frac{\text{number of cells migrated} - \text{number of cells migrated control}}{\text{number of cells migrated control}} \times 100\%;$$

so that net migration for control = 0%. Each bar represents the mean ± SEM of three experiments done in triplicate. ZAS + 11% pronase was not done.

pre-void volume fractions, in which no activity was detected. Similar molecular weight ranges of activity were reported by Gerrity et al[24] and have been found by other investigators when collagen[40] and elastin[41,42] fragments were fractionated and assayed for chemotactic activity.

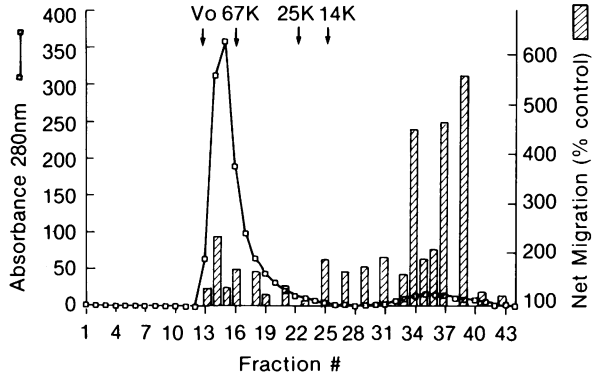

Figure 7—Absorbance and chemoattractant activity of column fractions of 12-week homogenates. A sample of lesion-area homogenate from 12-week cholesterol feeding experiment containing 2 mg protein was applied to a Biogel P-100 column; 44 fractions of 0.5 ml each were collected. The relative protein concentration of each fraction was determined spectrophotometrically by the absorbance at 280 nm. Monocyte response to column fractions was quantitated as the number of cells/ten 1000× fields and expressed as a percentage of the response to the PBSA control; 100% represents the control response. The activity represented here was that found in column fractions diluted 1 : 10 in PBSA. The void volume (*Vo*) and elution of proteins of known molecular weight are indicated by the *vertical arrows*. Standards used were: blue dextran (*Vo*), albumin (67 kd), chymotrypsinogen (25 kd), and ribonuclease (14 kd).

Chemoattractant Activity in Homogenates From Pigeons Fed Cholesterol for 24 Weeks

We fed pigeons 0.4% cholesterol-supplemented diet for 24 weeks to determine the effects of lesion complexity and time of progression on chemoattractant activity. Lesions induced by 24 weeks of cholesterol feeding are in pigeon aorta typically multilayered intimal foam-cell lesions with a moderate amount of extracellular matrix material, extracellular lipid, and a few lipid-containing smooth muscle cells.[35] Grossly, these lesions appeared as yellow fatty streaks or were slightly raised and circular in shape. In general, 30–100% of the lesion prone area (5 × 8 mm) near the celiac bifurcation was involved in the 24-week-diet animals. The mean total plasma cholesterol value in these birds was 1080 ± 440 mg/dl (mean ± SD).

Monocyte migration stimulated by lesion area and nonlesion area homogenates (Figure 8) exceeded that incited by PBSA alone (100%); however, no difference in the relative activity of the two types of homogenates was noted. This was in marked contrast to the 12-week lesions, in which the lesion-prone site was significantly more active. In addition, maximal activity of the 24-week lesion area homogenates was 350% of control at 31.6 μg protein/ml, compared with 750% of control at 100 μg/ml in the 12-week lesion homogenates (Figure 2). This difference suggested that there was much less chemoattractant activity in the older lesions. However, when the 24-week lesion homogenates were lipid-extracted, significant amounts of activity were recovered in both the aqueous and organic phases of the extracts (Figure 9). This indicated that these more advanced lesions contained both lipid and protein/carbohydrate chemoattractants; however, these activities appeared to be masked in the complex homogenate. Because it was conceivable that the organic phase activity was the result of lipid oxidation

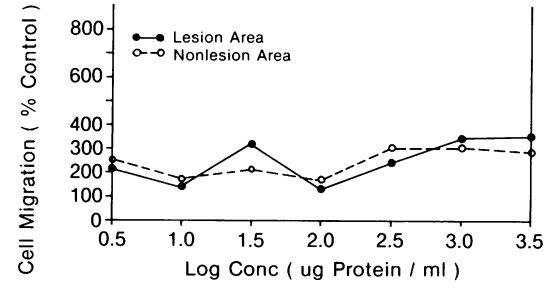

Figure 8—Monocyte response to lesion-prone and nonlesion area homogenates prepared from aortas of pigeons fed cholesterol for 24 weeks. Migration was quantitated as the number of monocytes per ten 1000× fields and expressed as a percentage of the response to the PBSA control; 100% represents the control response. Each point represents the mean of three experiments done in triplicate.

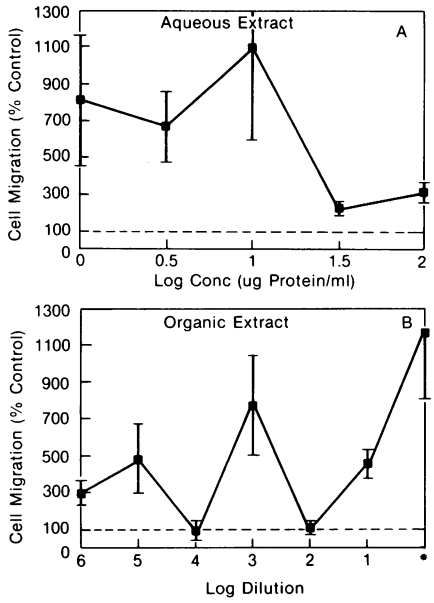

Figure 9—Monocyte migration to the aqueous (**A**) and organic (**B**) phases of lipid extracts of 24-week lesion homogenates. In **B**, the asterisk indicates the undiluted organic extract. Monocyte migration was quantitated as the number of cells per ten 1000× fields and expressed as a percentage of the response to the PBSA control; 100% (---) represents the control response. Each point represents the mean ± SEM of three experiments done in triplicate.

during the extraction process, parallel extractions were done in the presence and absence of the antioxidant, 2,6-di-tert-butyl-P-cresol (BHT). Notably, the inclusion of BHT did not alter the response of monocytes to the organic phase extract from the 24-week-diet animals.

Homogenates Prepared From Pigeons Fed a Cholesterol-Free Diet

Because chemoattractant activity was found in the aorta in very early (12-week) and in later (24-week) lesions, both of which are characterized by the presence of foam cells in the intima, it was of interest to determine whether chemoattractants were also present in the lesion-prone area prior to the appearance of foam cells or adherent leukocytes. It has been reported that the lesion-prone area of the aortas in young birds fed a standard pellet diet is microscopically normal, the intima consisting of an intact endothelium and basement membrane.[35] A small number of adherent leukocytes has been described on the normal aorta, but the adherent cells were scattered without specificity for the lesion-prone area.[13,15] Consistent with these earlier reports, gross lesions were not found in either the lesion-prone or nonlesion areas of the aortas from the group on the normal diet. At necropsy the average total plasma cholesterol level in this group of birds on the standard diet was 202 ± 9 mg/dl, which is in the normal range for pigeons.[43]

The activity of the lesion area homogenates from these pigeons was greater than that in the homogenates from the nonlesion areas (Figure 10); this difference was significant at protein concentrations of 1, 3.16, and 31.6 μg/ml (log 0, 0.5, and 1.5; $P<0.02$). Surprisingly, the chemoattractant activity of the lesion area whole homogenates from these normal diet birds was greater than that of lesion whole homogenates from pigeons fed cholesterol for either 12 or 24 weeks. This relationship cannot be directly interpreted, for the large amount of activity found when the 24-week lesion homogenates were extracted (Figure 9) suggests that the activity detected in the whole homogenates may not be reflective of the total activity present.

Discussion

Monocyte chemoattractants have been demonstrated in the lesion-prone area of the pigeon aorta. The development or generation of these chemoattractants was not dependent on the induction of hypercholesterolemia, because chemoattractants were found in both young pigeons fed a normal diet and age-matched birds fed a cholesterol-containing diet.

The lesion-prone site in the aorta of young normocholesterolemic pigeons is morphologically normal. Scanning electron microscopic studies by Jerome et al[13,15] have shown that the size, shape, and orientation of endothelial cells at this site are similar to cells in other areas of the aorta; and although there are some scattered adherent leukocytes, neither the number nor the orientation of the adherent cells differs from the rest of the aorta. It has been shown that intimal macrophages and foam cells are absent from this re-

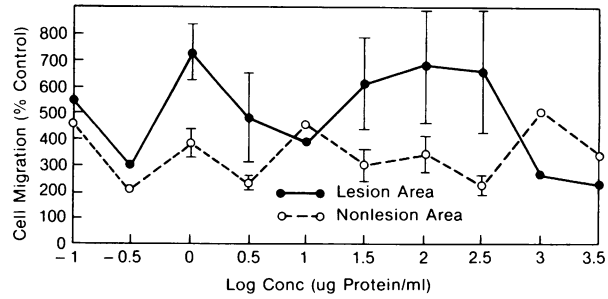

Figure 10—Monocyte response to lesion-prone and nonlesion area homogenates prepared from aortas of pigeons fed a cholesterol-free diet for 12 weeks. Migration was quantitated as the number of monocytes per ten 1000× fields. Results are expressed as the percentage of the response to the PBSA control; 100% represents the control response. Each point represents the mean ± SEM of five experiments done in triplicate. The asterisk indicates concentrations at which there was a significant ($P<0.02$) difference between lesion and nonlesion homogenates.

gion in young animals as included in this study.[35] Therefore, based upon the results reported herein, it appears that monocyte chemoattractants are present in the lesion-prone sites prior to the appearance of the first foam cells. The presence of chemoattractants in the artery wall prior to leukocyte infiltration is consistent with several studies[44-46] which have demonstrated a temporal sequence involving the generation of chemoattractants followed by the focal accumulation of leukocytes during an inflammatory response. This observation suggests that the aortic monocyte chemoattractants may be at least partially responsible for the appearance of the first monocyte-derived foam cells in early lesions and that intimal foam cells are not the initial source of the attractants. As has been shown by several investigators, atherosclerosis with adherent leukocytes does develop naturally in the pigeon; however, the occurrence of monocytes on the endothelium is not consistently observed until the birds are considerably older than in the present study.[15,41] Therefore, it is highly likely that the chemoattractant identified in the prelesion aorta of young birds contributes to the later infiltration of monocytes. Although our studies have demonstrated a monocyte-specific activity, it remains unknown whether the monocyte response is truly chemotactic or whether the primary cellular response is chemokinetic. Checkerboard assays carried out with the use of aortic homogenates from the pigeon have been inclusive, probably because of the biochemical complexity of the homogenates. As indicated in our preliminary fractionation and characterization studies, the arterial extracts contain several active fractions of differing molecular weights. Conceivably, checkerboard assays with both chemotactic and chemokinetic properties are reflecting different cellular responses to each of the extract subfractions. Irrespective of the precise cellular response mechanism, monocyte activation upon exposure to the arterial factor(s) would lead to enhanced margination at lesion sites, as demonstrated in both the aorta and coronary arteries of the pigeon.[13-16,22]

Chemoattractants prior to the development of lesion may be produced locally by the cells of the artery wall or derived from plasma proteins and lipids after transendothelial movement. The hypothesis that these attractants may be produced by cells of the artery wall is supported by the demonstration of chemotactic activity in medium from cultures of smooth muscle cells which were derived from normal aortas of baboons[25,26] or humans.[27] It has also been shown that the endothelium in lesion-prone areas of the aorta[47] is more permeable to plasma proteins than endothelium from other areas of the aorta. This enhanced transport may provide a mechanism by which elements from the plasma enter the artery wall and lead to the local generation of chemotactic substances. Several proteins found in the plasma are chemotactic for leukocytes. These include C5a, thrombin, fibronectin, fibrinopeptide, Angiotensin II, kallikrein, and plasminogen activator.[48-53] Also, fibronectin has been shown to accumulate in tissue during inflammation and does not require activation to be chemotactic.[49]

The chemoattractants found in the early foam-cell lesions produced by feeding cholesterol for 12 weeks have been partially characterized as monocyte-specific, heat-stable protein(s). Indirect evidence from the heat inactivation experiments indicated that the lesion chemoattractant was not C5a. C5a is the major chemotactic factor in ZAS,[54] which was consistently used as the positive migration control in our experiments. Unlike the activity in the lesion area homogenate, ZAS was inactivated when heated at 80 C and 100 C; but the activity in lesion homogenates was unaffected at these temperatures (Figure 4). Another indication that the activity in homogenates from lesion areas was not due to C5a was that these homogenates were relatively monocyte-specific, whereas ZAS (C5a) was chemotactic for monocytes and heterophils at the same concentration (1%). These observations in the pigeon parallel those reported for mammalian leukocytes, because chemotaxis of human neutrophils and monocytes can also be stimulated by equal concentrations of C5a derived from activated serum.[54,55] Heterophils in our experiments migrated to the lesion homogenates, but only at a homogenate protein concentration (1000 μg/ml) that was considerably higher than the concentrations that induced monocyte migration (31.6 and 100 μg/ml). The response of heterophils to homogenates from lesion-prone regions may account for the occasional heterophil seen adherent to and within lesions in pigeons.[13,71] A small number of granulocytes has also been observed in human[2] and African green monkey[56] lesions. Although the proteinaceous chemoattractant in lesion homogenates has not been identified, a few possible candidates are suggested by comparing the lesion chemoattractant characteristics with those attractants described by other investigators. Fragments of both collagen and elastin share many of the same properties as the lesion chemoattractant. They are all heat-stable proteins that are relatively specific for monocytes and are chemotactically active over a wide molecular weight range when fractionated.[38,39] Fibronectin and its fragments may also be considered candidates, because the 60–100-kd activity detected when homogenates were fractionated

overlaps the 90–220-kd range of chemotactically active fragments of fibronectin as described by others.[48,49] In addition, collagen, elastin, and fibronectin are normal components of the artery wall. Collagen and elastin are secreted by smooth muscle cells,[57] and endothelial cells secrete collagen and fibronectin.[58] In addition, monocyte-derived macrophages in the artery wall could contribute to the picture either by directly secreting proteins such as fibronectin[59] or by releasing proteases, which would fragment normal arterial fibers to yield chemotactically active peptides.[60]

The chemoattractants in pigeon aortic lesions appear to change as the lesions progress. Lesions present after birds had been fed a cholesterol-containing diet for 24 weeks contained protein (and/or carbohydrate) as well as lipid chemoattractants. This change in lesion chemoattractants was most probably related to alterations in lesion composition that accompany progression. Aortic lesions produced after 24 weeks are typically multilayered foam-cell lesions containing varying amounts of extracellular matrix material, extracellular lipid, and lipid-laden smooth muscle cells.[33] This increase in morphologic complexity as lesions progress is accompanied by changes in biochemical composition. Increases in the concentrations of all classes of lipids (derived from both local synthesis and the plasma) in the aortic wall during lesion progression are well documented, as are changes in the composition of the extracellular matrix.[61] Although none of these progression-related changes has been specifically associated with monocyte chemotaxis, it seems evident that this biochemical evolution could influence both the amount and the character of the lesion chemoattractants.

The activity in the organic phase extracts of the 24-week lesion area homogenates represents lipid factor(s) not found in earlier lesions (12-week). Although none of the lipids that accumulate in lesions have previously been reported to be chemotactic, the interaction of lipids with reactive metabolites of macrophages in the lesions could produce chemotactic lipids. Monocytes and macrophages in the process of phagocytizing particles,[62] migrating in response to chemotactic factors,[63] or otherwise stimulated by chemoattractants[64,65] secrete the reactive oxygen metabolites superoxide anion (O_2^-), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2). Petrone[66] and Turner[67] have shown that chemotactic lipids can be produced from otherwise inactive lipids when they are oxidized. Furthermore, it has been proposed that oxygen metabolites, secreted by inflammatory leukocytes, may directly oxidize lipids in cell membranes.[68] Interestingly, prostaglandin E_2, which is secreted by both macrophages[61] and smooth muscle cells,[57] is synthesized in greater amounts in the lesion-prone area of pigeon aortas.[69] This lipid is not chemotactic itself, but will enhance monocyte migration to chemotactic factors.[70]

Monocyte chemoattractants have also been described in extracts of foam-cell lesions from pig aortas.[24] These chemoattractants are similar to those of the pigeon in that they are monocyte-specific and consist of active molecules of low and high molecular weights; although similar, differences between the two do exist. The most pronounced difference between the pig and pigeon aortic chemoattractants is the apparent requirement of hypercholesterolemia for the expression of chemoattractant activity in pig aortas. Extracts from aortas of hypercholesterolemic pigs contain monocyte chemoattractants, whereas extracts from normocholesterolemic pigs do not.[24] The reason for the discrepancy between the pigeon and the pig in the appearance of aortic chemoattractants under normocholesterolemic conditions is unclear, although it is apparent that this is not due to a difference in susceptibility to atherosclerosis. Atherosclerosis develops in both the pigeon and the pig whether they are fed a normal or a cholesterol-supplemented diet[45]; however, as noted by numerous authors, the timetable for atherosclerosis to develop naturally in the pig is considerably longer than in the pigeon.

Although there are some differences between the development and activity of chemoattractants found in the pig[24] and those reported here in the pigeon, the occurrence of these factors in the lesions of two disparate species suggests that monocyte chemoattractants may be a common and important feature of atherosclerotic lesions. In addition, the demonstration of chemoattractants in the lesion area of pigeon aortas prior to lesion formation, coupled with the observation that focal leukocyte adhesion is a common feature of pigeon lesions at all stages of development,[13–16,22,71] implies that chemoattractants are being continually produced. Furthermore, the observations suggest that chemoattractants play a role in the recruitment of new monocytes to existing lesions and thereby constitute a driving force in lesion progression.

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