Brief Definitive Report

VIRUS-INDUCED ATHEROSCLEROSIS*

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It has been suggested that atherosclerosis may result from alterations in lipid metabolism, arterial injury, or the effects of chemical or viral mutagens on vascular smooth muscle cells (1–3). Viruses may be inciters of arterial injury, mutagens, or they may alter lipid metabolism of cells (4). Despite these considerations, the role of viruses in the pathogenesis of atherosclerosis has received little attention. Results of experiments reported here indicate that infection with a virus, Marek's disease herpesvirus (MDV), will lead to occlusive atherosclerosis of large muscular arteries in hypercholesterolemic and normocholesterolemic chickens. The atherosclerosis in these chickens closely resembles chronic atherosclerosis in human arteries.

Materials and Methods

Chickens. The 130 chickens used were from a strain of specific-pathogen-free (SPF) White Leghorn chickens maintained by the Department of Avian and Aquatic Animal Medicine at the New York State College of Veterinary Medicine. The genetic strain used, P-line, is moderately susceptible to infection with MDV (5).

Virus Stock. The virus used in this experiment was CU-2, a cell-free, clone-purified strain of MDV. This strain is of relatively low virulence, and it produced primarily neural and gonadal tumors in susceptible birds (6).

Collection of Serum and Estimation of Cholesterol Concentrations. Concentrations of total serum cholesterol were determined by a modification of the Liebermann-Burchard method (7) using a Coulter Automated Analyzer (Coulter Electronics Inc., Hialeah, Fla.). Chickens of all groups were bled before initiation of cholesterol feeding, at wk 15 of the experiment, and thereafter at approximately 5-wk intervals.

Agar Gel Precipitin Tests. Agar gel precipitin (AGP) tests for antibody to MDV were performed on serum collected from all birds at 30 wk of age by methods described previously (8). Positive AGP tests have been demonstrated to indicate persistent MDV infection (9).

Experimental Groups. Four comparably sized groups, a total of 130 chickens, were randomly selected and each group was housed in an isolation unit and initially fed a commercial diet low in cholesterol (not more than 0.02% cholesterol by weight). Groups I and II were inoculated intratracheally at 2 days of age with the MDV. At 15 wk, one group of virus-inoculated chickens (group II) and one group of uninoculated controls (group IV) were fed the same diet supplemented with 2% (wt/vol) cholesterol for an additional 15 wk (cholesterol, U.S.P.; ICN Pharmaceuticals Inc., Cleveland, Ohio). Groups I and III were continued on the diet low in cholesterol. All surviving birds were sacrificed at 30 wk of age. Only male birds surviving for the 7-mo experimental period were included in this report.

Autopsy Procedures. Chickens were sacrificed by cervical dislocation and examined for

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grossly visible atherosclerosis and for gross lesions of Marek's disease. Hearts, aortas, celiac, gastric, and mesenteric arteries were dissected and fixed in 10% neutral buffered formalin. A small sample of one brachiocephalic artery was prepared for immunofluorescence microscopy and examined as described previously (10). After fixation, the brachiocephalic arteries and the thoracic and lumbar portions of the descending aorta were opened, and their luminal surfaces were examined for grossly visible atherosclerosis. Comparable numbers of tissue blocks were taken in each group from the brachiocephalic artery, proximal and distal segments of the thoracic aorta, distal lumbar aorta, celiac, gastric, and mesenteric arteries. In addition, comparable numbers of tissue blocks, usually 12–14, were taken from the hearts of chickens of all groups in a manner similar to that used by Patterson (11). Sections from tissue blocks were routinely stained with hematoxylin and eosin, and selected specimens were stained with Weigert-Van Gieson for elastic tissue and Oil red 0 for fat.

Sections of these aortas and their major branches were examined for microscopic lesions. One section from each of the tissue blocks of heart was examined, and arterial lesions were enumerated and classified according to the size of the artery involved and the qualitative character of the lesion, fatty, proliferative, or fatty-proliferative (12).

Fluorescent Antibody Tests. Frozen sections of branchiocephalic arteries were examined by staining with specific MDV antibody conjugated with fluorescein isothiocyanate as described previously (13).

Statistical Analysis. All chi-squares were calculated using a 2 × k contingency table. Where appropriate, *t* tests assumed nonhomogeneity of variance.

Results

MDV infection in groups I and II was confirmed by positive AGP tests for antibody to MDV, and the death of ≅30% of the inoculated chickens with characteristic gross lesions of Marek's disease. No evidence of mortality due to MDV or positive AGP tests was found in control groups III and IV.

Before feeding the cholesterol-supplemented diet, the concentration of total serum cholesterol was normal for chickens of all groups and the overall means ranged between 134 and 147 mg/100 ml. Serum cholesterol concentrations for chickens of groups I and III remained within a normal range for the experimental period, with overall means of 147 ± 15 and 137 ± 5 mg/100 ml, respectively (mean ± SEM). During feeding of cholesterol-supplemented diet, the average total serum cholesterol concentrations for chickens of groups II and IV increased to between 198 and 902 mg/100 ml, and the overall means for each group, 425 ± 30 and 461 ± 40 mg/100 ml, respectively, were not significantly different (*t* tests = 0.72, NS).

Grossly visible, often occlusive atherosclerosis was seen in coronary arteries, aortas, and major branches of 4 of 18 chickens of group II infected with MDV and fed cholesterol supplement. Grossly similar, but less fatty lesions were seen in arteries of 3 of 22 chickens of group I, infected with the virus but not fed cholesterol supplement. In contrast, grossly visible atherosclerosis was not seen in 24 normocholesterolemic chickens of group III and 23 hypercholesterolemic chickens of group IV not infected with MDV. Thus, grossly visible atherosclerosis was seen in 7 of 42 chickens infected with MDV and in none of 47 uninfected controls ($X_1^2 = 8.50$ $P < 0.005$).

Microscopically, proliferative and fatty-proliferative arterial lesions were often seen in aortas, coronary, celiac, gastric, and mesenteric arteries in chickens of groups I and II. Fatty-proliferative lesions contained intimal and medial foam cells, extracellular lipid, cholesterol clefts, and calcium deposits (Figs. 1–3). Many fatty proliferative lesions bore close resemblance to chronic

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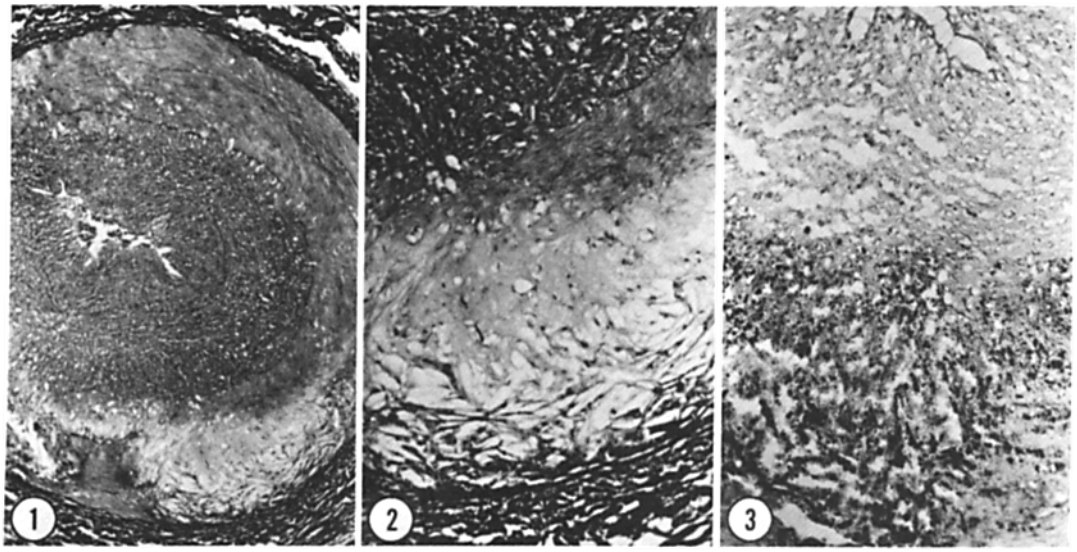

FIG. 1. Gastric artery of normocholesterolemic group I chicken infected with MDV and fed diet low in lipid. Lumen of artery is occluded by thickened intima with atheromatous change. This degree of atherosclerotic change was present in celiac, gastric, mesenteric, and coronary arteries of chickens of groups I and II. These changes were not seen in uninfected chickens of groups III and IV. Weigert-Van Gieson, × 36.

FIG. 2. Higher magnification of atheromatous change illustrated in intima and media of mesenteric arteries of chicken in Fig. 3. Note foam cells, extracellular lipid, and cholesterol clefts in media. Weigert-Van Gieson, × 190.

FIG. 3. Frozen section of gastric artery adjacent to that illustrated in Fig. 4. Note large quantities of lipid, appearing black, deep in the intima and underlying media. Oil red 0, × 95.

human atherosclerosis. Proliferative lesions resembled human arteriosclerosis without fatty change. Microscopic lesions were rarely found in these arteries in groups III and IV with the exception of the distal aorta. Coronary arterial lesions in chickens of groups I and II most frequently involved large and medium sized coronary arteries and were also proliferative and fatty-proliferative in character. In groups I and II there were 43 and 161 lesions, respectively, in large coronary arteries whereas in groups III and IV there were only 2 and 3 lesions, respectively (group I vs. II, t test = 2.71, $P < 0.02$; group II vs. IV, t test = 2.37, $P < 0.05$, t tests calculated assuming nonhomogeneity of variance). The vast majority of arterial lesions in uninfected, cholesterol-fed chickens were found in small intramyocardial arteries, were fatty in character, and bore little resemblance to human atherosclerosis.

The number of chickens with microscopic arterial lesions in mesenteric and gastric arteries, coronary arteries, and proximal aortas was significantly greater in virus-infected chickens than in uninoculated control groups (Table I). The number of chickens with microscopic lesions of the distal descending aorta was significantly different in groups I and III, but not in groups II and IV.

MDV-specific antigens were observed by immunofluorescence microscopy in arterial lesions of four infected birds. No viral antigen was found in the aortas of uninfected control birds.

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TABLE I
Number of Chickens with Microscopic Arterial Lesions

Group	Large coronary arteries	Gastric and mesenteric arteries	Brachiocephalic arteries and proximal decending aorta	Distal descending aorta
I Virus alone N = 23 [41]*	11 (22)‡	8 (22)	10 (23)	18 (23)
II Virus and cholesterol N = 19 [41]	17 (19)	5 (9)	16 (18)	14 (18)
III Untreated N = 24 [25]	1 (24)	0 (16)	1 (23)	6 (23)
IV Cholesterol alone N = 23 [23]	3 (23)	1 (18)	1 (22)	16 (22)

* Number of chickens in each group on day 1 of experiment. 27 chickens in groups I and II died with Marek's disease and 13 females were removed from the experiment.
N, Number of chickens autopsied after 7 mo.
‡ Number in which arterial specimens were available.
Coronary arteries:
I vs. III, $X_1^2 = 9.83$, $P < 0.005$.
II vs. IV, $X_1^2 = 24.37$, $P < 0.001$.
Gastric and mesenteric arteries:
I vs. III, $X_1^2 = 7.37$, $P < 0.01$.
II vs. IV, $X_1^2 = 8.68$, $P < 0.005$.
Brachiocephalic arteries and aorta:
I vs. III, $X_1^2 = 9.68$, $P < 0.005$.
II vs. IV, $X_1^2 = 28.82$, $P < 0.001$.
Distal aorta:
I vs. III, $X_1^2 = 12.55$, $P < 0.001$.
II vs. IV, $X_1^2 = 0.13$, NS.

Discussion

Results of these experiments indicate that atheroarteriosclerosis closely resembling that in man was induced in normocholesterolemic as well as hypercholesterolemic SPF chickens by infection with MDV. The character and distribution of the arterial lesions induced in chickens infected with MDV was significantly different from that induced by cholesterol feeding. Moreover, the number of animals with lesions in large arteries was significantly greater in both of the virus-infected groups than in uninfected control groups with or without supplemental cholesterol. Thus, infection with MDV induced arterial lesions closely resembling human atherosclerosis in portions of the arterial tree not involved in animals fed cholesterol, and also significantly increased the number of birds with arterial lesions. In addition, the increased number of chickens with arterial lesions as well as the increased number of coronary lesions in group II as compared to group I, suggest a synergistic action between the virus infection and cholesterol feeding.

The distal segment of the descending aorta was the only site where a significant number of lesions was found in uninfected birds. These lesions in

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group IV may represent modification of arterial lesions induced by other unknown factors, since an appreciable number of lesions were also found in group III untreated controls. For example, carcinogens have been shown to increase the incidence and severity of this type of lesion (14). Similarly distributed aortic lesions are known to occur in the distal aorta of the chicken (15), and in at least one other avian species, the pigeon (16).

Of particular interest is the occurrence of fatty-proliferative lesions in arteries of the normocholesterolemic, virus-infected birds of group I. This could result from the effect of virus on lipid metabolism of arterial cells (4), or from sustained arterial injury (17).

The pathogenesis of this viral-induced atherosclerosis is unknown. The lesions may result from oncogenic transformation of arterial smooth muscle cells as suggested by Benditt (3), since MDV is an oncogenic virus. The lesions may also result from a response of the arterial wall to viral-induced injury or an immunologic response either to the virus or to tissue altered by the virus. It is possible that all of these may be important. Whatever the mechanism, the finding that herpesviruses can induce atherosclerosis in chickens may be important to our understanding of human atherosclerosis, since five herpesviruses are known to produce persistent infections in man.

Summary

Of four groups of chickens, two (groups I and II) were infected with MDV and two were not (groups III and IV). Groups I and III were fed diets low in lipid, and groups II and IV were fed cholesterol-supplemented diets. Striking grossly visible atherosclerotic lesions were seen in large coronary arteries, aortas, and major aortic branches of infected normocholesterolemic and hypercholesterolemic chickens (groups I and II). In contrast, grossly visible atherosclerotic lesions were not seen in uninfected normocholesterolemic chickens (group III), nor in uninfected hypercholesterolemic chickens (group IV). Microscopically, arterial changes in the infected animals were characterized by occlusive fibromuscular intimal thickening which formed fibrous caps overlying areas of atheromatous change. This change closely resembled chronic atherosclerosis in man. These results may have important bearing on our understanding of the etiology and pathogenesis of human arteriosclerosis since there is widespread and persistent infection of human populations with up to five different herpesviruses.

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