Effect of 3-Methylcholanthrene on the Development of Aortic Lesions in Mice

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ABSTRACT

The effect of a carcinogen, 3-methylcholanthrene (3-MC), on the formation and growth of atherosclerotic lesions in mice was examined. Increasing doses of 3-MC from 15 to 1500 μg/kg increased the number and size of lipid-staining lesions in the aorta of AKXL-38a mice that were fed an atherogenic diet for 8 weeks. The number of lesions per mouse was 0.85 ± 0.19 (SE) for animals treated with 3-MC (150 μg/kg) compared to 0.10 ± 0.10 lesions/mouse for animals given solvent rather than 3-MC. The progression of lesions over time from 5 to 18 weeks showed that 3-MC-treated mice also differed from controls in the size of lesions. The total score per mouse at 18 weeks of atherogenic diet, based on the number of lesions and the size of each lesion, indicated by a score of 1 to 4, was 4.31 ± 0.71 for 3-MC-treated animals and 2.67 ± 0.74 for animals given solvent. The effect of 3-MC treatment could be observed at 18 weeks even though the entire dose of 3-MC was given during the first week on the atherogenic diet. These experiments do not distinguish whether 3-MC affects atherosclerotic lesions by acting as a mutagen or by some other mechanism.

The composition of an atherogenic diet that produces lesions in mice without high mortality is given as well as a comparison of different methods of evaluating lesion formation.

INTRODUCTION

Benditt and Benditt (1) were the first to observe that an atherosclerotic plaque, like a tumor, appeared to be derived from a single cell. The experimental system utilized the somatic mosaicism arising from X-chromosome inactivation in females heterozygous for the A and B alleles of X-linked enzyme, G6PD.3 The finding that normal sections of arterial wall contained both cells expressing the A allele and cells expressing the B allele, whereas most plaques were monotypic containing cells expressing only the A or B allele, suggested that an individual plaque is a clone from a single cell. The experiments and results paralleled earlier findings on the monoclonal origin of human tumors (2) and led Benditt and Benditt (1) to suggest that a plaque might resemble a benign tumor arising from a mutagenic event in a single cell.

If atherosclerotic plaques are analogous to benign tumors of the arterial wall, then one might expect other mutagens such as chemical carcinogens, radiation, and oncogenic viruses to be involved in the etiology of atherosclerosis. Very early experiments suggested that 3-MC increased the incidence of leukemia in mice fed a low-cystine diet, but these data are difficult to interpret because of a lack of control mice fed the same diet but not given any 3-MC (3, 4). The first clear evidence on the ability of carcinogens to induce atherosclerosis was obtained when Albert et al. (5) in chickens. Weekly i.m. injections of dimethylbenzanthracene or benzopyrene increased the size of atherosclerotic plaques in carcinogen-treated birds without affecting cholesterol levels (5). The increase in size was dose dependent (6). These experiments have been confirmed and extended to show that a tumor promoter did not enhance lesion formation (7). Additional evidence that carcinogenic factors can play a role in atherosclerosis was obtained when Fabricant et al. (8, 9) demonstrated that an oncogenic virus, Marek's disease herpesvirus, increased atherosclerosis in chickens. The percentage of chickens with lesions in the coronary arteries was 4% of control birds, 13% of birds on a diet containing 2% cholesterol, 50% of birds on the normal diet and infected with virus, and 89% of birds on the 2% cholesterol diet and infected with virus. Other investigators have reported that radiation increased atherosclerosis, but this effect was ascribed to arterial wall injury (10).

We have attempted to extend these observations to mammals, using the mouse as an experimental animal, by testing whether a carcinogen can affect the development of atherosclerotic lesions in a dose-dependent manner.

The mouse has not been used extensively in studies of atherosclerosis since early attempts to produce atherosclerotic lesions lead to variable results (11–15). However, Roberts and Thompson (16, 17) overcame these difficulties by using inbred strains as their test animals and a diet containing cholesterol, cocoa butter, and sodium cholate. They found that atherosclerotic lesions were formed reproducibly in mice and that inbred strains of mice differed in their susceptibility to atherosclerosis. Since their work, others have used these techniques to induce atherosclerotic lesions in mice (18, 19). Our present experimental protocols are modified from the procedures of Roberts and Thompson.

MATERIALS AND METHODS

Chemicals. Chemicals were obtained as follows: 3 MC, Sigma Chemical Co., St. Louis, MO; oil red O, Aldrich Chemical Co., Milwaukee, WI; hematoxylin and O.C.T. compound embedding medium, Fisher Chemical Co., Santa Clara, CA. The atherogenic diet was purchased from Teklad Test Diets, Madison, WI, and contained 30% cocoa butter, 5% cholesterol, 2% sodium cholate, 30% casein, 5% Alphacel, 4% vitamin mixture, 4% salt mixture, 6.5% sucrose, 6.5% dextrose, 6.5% dextrin, and 0.5% choline chloride. This diet was mixed with varying concentrations of Purina breeder chow. The fatty acid composition is given in a previous publication (19).

Animals. The mice used in these experiments, strain AKXL-38a, are a recombinant inbred line made from a cross between AKR/J and C57L/J mice. This particular subline carries the A-responsive allele and is characterized by induction of microsomal monoxygenases in re-
sponse to certain carcinogens and drugs (20). The colony is maintained at the NIH, and a breeding colony was set up in our laboratory. Strain AKXL-38a is also maintained at The Jackson Laboratory, Bar Harbor, ME. For experiments using only females, mice were housed 5/cage. For experiments involving males, all animals were caged individually because males of this strain fight too much to be placed together. This particular strain was chosen because it allows comparison with a genetically very similar strain, AKXL-38, which is Ah-nonresponsive.

**Histology.** After sacrifice, the heart and the upper section of the aorta were removed, placed in 10% buffered formalin for 24 h, and imbedded in O.C.T. compound embedding medium for 48 h. The bottom of the heart was removed so that the plane of sectioning was parallel to the tips of the atra. The heart was frozen on a cryostat and 10-μm sections were made. These were examined under a microscope until the 3 valve cusps at the junction of the aorta to the heart could be seen and the cross-section of the aorta was round. Slides with the 3 valve cusps but a highly irregular aorta are actually taken through the aortic sinus. These were not used because it is important to evaluate at a consistent location. This particular location was chosen based on an evaluation of the entire aorta. Because lesions form first in areas of turbulence such as the aortic sinus and the orifice of each artery from the aorta, it was important in choosing a consistent area to either always obtain cross-sections with a particular artery or never obtain such sections. Since always obtaining a particular artery is difficult, we chose an area with no arteries. The ascending aorta, just after the aortic sinus and the exit of the coronary arteries, is such an area. When the appropriate area was reached, as determined by examining unstained slides under the microscope, 15 consecutive sections 10 μm thick were placed on slides, fixed, stained with oil red O, and counterstained with hematoxylin light green.

Sections were examined for oil red O-staining lesions. For most purposes, the number and size of lesions in each animal were recorded. The number of lesions was determined by counting the discrete lipid-staining foci. All 15 cross-sections were used, but each lesion was counted as 1 whether it appeared in one or all 15 cross-sections. We recognize that this count is only an approximation of lesion number since 2 discrete foci in the section of aorta examined might be irregular extensions of a single lesion beyond the area tested. Likewise, a large lesion might have been 2 smaller lesions that grew together. The lesion size, taken from the cross-section with the largest lesion, was scored as 1 to 4 with a score of 1 meaning that the lesion occupied less than one-eighth of the distance from one value cusp to the next, a score of 2 being one-eighth to one-fourth of the distance, a score of 3 being one-fourth to one-half of the distance, or a score of 4 being greater than one-half. When greater precision was required, the lesion was drawn using a drawing lucida attachment on the microscope and using the graphics tablet of an Apple computer to determine the cross-sectional area and the percentage of aortic wall involved.

**RESULTS**

**Diet.** The original Roberts and Thompson diet contained 30% cocoa butter, 5% cholesterol, and 2% sodium cholate (see "Materials and Methods" for exact composition). In order to induce mice to eat this diet, Roberts and Thompson gave weanling animals a mixture of 70% atherogenic diet and 30% normal chow for 2 days, changed to a 80%:20% mixture for 4 days, and then finally changed to a 90%:10% mixture for the remainder of the experiment. In our experience, this diet caused high mortality as also reported by Morrisett et al. (18). Moreover, the very high fat and cholesterol content was not comparable to atherogenic diets used for other species. We therefore tested whether lesions could be produced in mice using diets with lower concentrations of cholesterol and fat. Mice were fed 5 different diets: normal mouse chow which contains 4% fat; breeder chow which contains 10% fat; and 3 different mixtures of the atherogenic diet and breeder chow in the proportions of 25:75, 40:60, and 60:40. These latter 3 diets contained a total of 15% fat, 18% fat, and 22% fat, respectively. The high-fat diets were accepted readily by the animals and did not cause any mortality or reduced weight gain. Six males and 6 females were fed each diet for 8 weeks. Lesions were evaluated in 4 ways: number of lesions per animal; total lesion score per animal based on grading lesions 1 to 4 (see "Materials and Methods"); the fraction of aortic perimeter involved in lesions; and the fraction of cross-sectional area of the aorta occupied by lesions. None of the mice on normal chow containing 4% fat or on breeder chow had any lesions. The number and size of lesions in animals fed the 3 diets containing 15, 18, and 22% fat are shown in Table 1. For females, but not males, lesion number and size were somewhat greater in animals fed 18% fat compared to those fed 15% fat. No further increase in lesion number or size was observed in animals on the 22% fat diet compared to 18% fat. Thus, the 15% fat diet was adequate for lesion formation in these mice; and because it produced lesions without causing mortality, we used it throughout the remainder of the experiments.

The 4 methods of scoring lesions were compared for reproducibility, for ability to distinguish among groups, and for feasibility. All of the methods have fairly high standard deviations and standard errors which may have more to do with variability of lesion formation than scoring method. However, drawing lesions and using the graphics tablet to calculate size is so tedious compared to counting lesions or scores using a 1-to-4 system that we have used these latter techniques for most experiments.

Females gave slightly greater values than males for all methods of evaluation but only the number of lesions per mouse was significantly greater in females than males. Another larger experiment of 288 mice sacrificed after only 6 weeks on the atherogenic diet showed no difference in males or females in the number of lesions per animal.4

The lesions were always scored in those sections of the aorta in which the 3 valve cusps could be seen (Fig. 1A). A typical lesion contained many foam cells and had raised endothelium and medial involvement (Fig. 1B). These are characteristic of early lesions or fatty streaks. The fibrous cap, which is the hallmark of an atherosclerotic plaque, was not seen.

**Dose Response.** In order to determine whether animals treated with carcinogen showed a dose-dependent increase in lesion number or size, 14 female mice/group were placed on the atherogenic diet for 8 weeks, and 3 injections of solvent or 3-MC at concentrations of 5, 15, 50, 150, and 500 μg/kg/injection were given in Days 1, 3, and 8 of the diet. Chart 1 shows that increasing concentrations of 3-MC do increase the atherosclerotic response in mice.

**Time Course.** In order to determine whether the effect of 3-MC was progressive over time, female mice 6 to 10 weeks of age were placed in 4 treatment groups; normal mouse chow (4% fat); atherogenic diet (15% fat), normal chow plus 3-MC; and atherogenic diet and 3-MC. The carcinogen was given 3 injections, each of 2.5 mg/kg, on Days 1, 4, and 8. Mice on normal chow did not have any lesions regardless of carcinogen treatment. The number of lesions per mouse and the lesion size for mice on the atherogenic diet are depicted in Chart 2. Mice

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4 Unpublished data.
CARCINOGENS AND ATHEROSCLEROSIS

Table 1
Effect of various diets on lesion formation in mouse aortas

<table>
<thead>
<tr>
<th>Parameter measured</th>
<th>15% fat</th>
<th>18% fat</th>
<th>22% fat</th>
<th>Sum of 3 diets</th>
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<tbody>
<tr>
<td>No. of mice*</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Diet composition</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>% Teklad atherogenic diet</td>
<td>25</td>
<td>40</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>% cholesterol</td>
<td>12.5</td>
<td>2.0</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>% cholic acid</td>
<td>0.5</td>
<td>0.8</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>% cocoa butter</td>
<td>7.5</td>
<td>12.0</td>
<td>18.0</td>
<td></td>
</tr>
<tr>
<td>No. of lesions/mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>1.5 ± 0.6</td>
<td>2.4 ± 0.9</td>
<td>2.4 ± 0.2</td>
<td>2.1 ± 0.4</td>
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<tr>
<td>Male</td>
<td>1.5 ± 0.6</td>
<td>1.3 ± 0.4</td>
<td>0.8 ± 0.4</td>
<td>1.2 ± 0.3</td>
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<tr>
<td>Total score/mouse</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>3.7 ± 1.4</td>
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<td>6.0 ± 1.6</td>
<td>5.4 ± 1.0</td>
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<td>Male</td>
<td>4.5 ± 1.9</td>
<td>3.3 ± 1.8</td>
<td>1.6 ± 0.8</td>
<td>3.2 ± 0.9</td>
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<tr>
<td>Lesion size as fraction of aortic perimeter/mouse</td>
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<td></td>
</tr>
<tr>
<td>Female</td>
<td>0.23 ± 0.10</td>
<td>0.37 ± 0.10</td>
<td>0.31 ± 0.15</td>
<td>0.30 ± 0.06</td>
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<td>Male</td>
<td>0.28 ± 0.16</td>
<td>0.20 ± 0.14</td>
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<tr>
<td>Lesion size as fraction of aortic cross-sectional area</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>Female</td>
<td>0.07 ± 0.03</td>
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<td>0.09 ± 0.05</td>
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<td>0.05 ± 0.04</td>
<td>0.01 ± 0.004</td>
<td>0.04 ± 0.02</td>
</tr>
</tbody>
</table>

* No mice died while on the diet, but the sections from 3 hearts could not be evaluated due to improper functioning of the cryostat.

DISCUSSION

This report shows that the carcinogen 3-MC increases the number and size of atherosclerotic lesions in a mammalian species. This observation confirms the earlier reports that the carcinogens dimethylbenzanthracene and benzo(a)pyrene increase the incidence and size of atherosclerotic lesions in chickens (5–7). In both the chicken and the mouse experiments, the effect of the carcinogen on plaque size was dose dependent. In the experiments using chickens, the carcinogen was given at weekly intervals throughout the experiment; in this experiment, the carcinogen was given only during the first week. However, the effect of the carcinogen on plaque size was clearly observable at 18 weeks, indicating that carcinogen does not have to be continuously present. Whatever the mechanism by which a carcinogen increases lesion size, the fact that it occurs has public health implications.

Four different laboratories have now shown that 3 polycyclic aromatic hydrocarbons and an oncogenic virus increase the incidence and size of atherosclerotic plaques in 2 animal species, chicken and mouse (5, 7, 8). However, the investigators do not agree on the interpretation of these data. Albert et al. (5, 6) report that the carcinogen increased the size but not the frequency of lesions. If plaque formation were a mutational event, then a chemical carcinogen should increase the number of plaques, but an increase in size only argues for a mitogenic type of action. One could rescue the mutational hypothesis by saying that the small lesions observed by Albert et al. are really fatty streaks, which are usually ditypic in the human, and that the carcinogen causes a mutation in one cell in the fatty streak which has a growth advantage. This overgrows the rest, causing a large lesion or plaque. One way to test this hypothesis is to examine lesions for an X-linked variant with the prediction that the small lesions would be ditypic and the larger lesions would be monotypic. The other 2 laboratories (7, 8) and our data all indicate that the carcinogenic stimulus caused an increase in both the number and size of the plaques; thus, those laboratories lean toward a mutational explanation. However, an increased number may not be a strong argument since, for technical reasons, the lesion count is only an approximation. Small lesions can grow together into one, and one large lesion can have irregular extensions that appear as discrete lesions in an aortal cross-section.

Other types of action other than mutagenic could be evoked for the 3 polycyclic hydrocarbons that have been shown to increase atherosclerosis. These chemicals induce the microsomal monooxygenases, they are cytotoxic, they bind to the lipoproteins, and they might have a promotor or mitogenic effect. Further animal experimentation is needed to determine whether the mechanism by which 3-MC increases atherosclerosis is a mutagenic event or some other mechanism. Some important questions are: (a) do noncarcinogenic chemicals that are closely related to carcinogens fail to increase atherosclerosis; (b) do P-450 inducers that are not carcinogens increase atherosclerosis; and (c) do carcinogens alter the lipid transport system?

Since these experiments were suggested by the experiments indicating that plaques were monoclonal, it might be appropriate to review the data critically. At least 3 laboratories have examined atherosclerotic plaques in females heterozygous for G6PD (1,

* No mice died while on the diet, but the sections from 3 hearts could not be evaluated due to improper functioning of the cryostat.

* Different from males at $P = 0.05$. 

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Chart 1. Effect of increasing carcinogen dose on number and size of lesions in aortic wall. Mean number or size of lesions per mouse after 8 weeks on the atherogenic diet; bars, SE. The number of mice evaluated was 10 with no carcinogen, 12 at 0.3 μg 3-MC, 14 at 0.9 μg 3-MC, 9 at 3.0 μg 3-MC, and 13 at 9.0 and 30 μg 3-MC. The μg 3-MC represents the total dose given. Based on a 20-g mouse, these are equivalent to 15, 45, 150, 450, and 1500 μg/kg. The missing mice were due to poor histological sections rather than death of animals. Scoring was done without the technician’s knowing the treatment.

Chart 2. Effect of carcinogen on number and size of aortic lesions in mice on an atherogenic diet for 5 to 18 weeks. •, mice treated with 3-MC (2.5 mg/kg); ○, mice with no carcinogen treatment. Each point represents the mean value per mouse; bars, SE. The number of animals evaluated at each time point for control and 3-MC-treated animals, respectively, were 12 and 14 at 5 weeks, 14 and 13 at 9 weeks, 15 and 11 at 12 weeks, 13 and 0 at 16 weeks, and 12 and 27 at 18 weeks.

21–26). All laboratories agree that the normal arterial wall is ditypic but that most plaques are monotypic. The percentage of plaque samples that are monotypic vary from 60 to 90%, depending on the laboratory. However, the laboratories differ considerably in their interpretation of these facts. Benditt and Benditt (1) interpret the monotypic characteristic of most plaques to mean a monoclonal origin; i.e., each plaque arises from a single cell. They interpret the ditypic nature found in some plaques as resulting from the technical difficulty of obtaining a sample free of surrounding tissue or 2 different monoclonal plaques growing together or the presence of migratory cells in the plaque such as macrophages. Thomas et al. (26), on the other hand, interpret the data quite differently. They place most of their emphasis on the fact that many plaques are ditypic, and they show data indicating that the more individual samples taken from a particular plaque, the more likely it is that a ditypic sample will be found. If the ditypic nature were due to plaques growing together, then some sections from a large plaque should be monotypic for the A-variant, some ditypic, and some monotypic for the B-variant. Thomas et al. (26) claim that this was found rarely, being seen in only 2 of 64 plaques examined in their laboratory and only 5 of 26 plaques examined by Pearson et al. (23, 24). Thomas et al. use their data to reject the single-cell origin of the plaque; the alternative explanation which they offer for monotypism is clonal selection in proliferating cells. Thomas argues that, following intimal injury, many cells respond by proliferating and that these differ in growth potential; thus, some, or a very few, cells contribute most to the final plaque. Yet a third laboratory, that of Pearson et al. (21–25), appears to lean toward a mutational explanation for monotypism although it fully recognizes the ditypic nature of many plaques and states that more than one mutational event may occur in large plaques. Pearson et al. (25) did an interesting experiment to determine whether proliferation of cells following injury leads to monotypic areas. They examined scar tissue in G6PD heterozygotes and found no evidence for monotypism.

Finally, we find that the mouse can be used effectively as an experimental system in atherosclerosis research, particularly with an improved diet. Recent studies by LeBoeuf et al. (27) and Lusis et al. (28) have characterized lipoprotein chemistry of the mouse system. The advantages of the well-developed genetic system of the mouse in an analysis of the various risk factors affecting atherosclerosis has been demonstrated by several lab-
oratories (16–19, 27, 28). It is likely that the inbred mouse will be a useful addition to the experimental repertoire available for atherosclerosis research.

ACKNOWLEDGMENTS

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Note Added in Proof

Recently, it has been reported that the carcinogen nickel subsulfide causes widespread arteriosclerotic lesions in the rat 7 weeks after one intrarenal injection (Hopfer, S. M., Sunderman, F. W., Jr., McCully, K. S., Reid, M. C., Liber, C., Spears, J., and Serur, J. Ann. Clin. Lab. Sciences, 14: 355–365, 1984).

REFERENCES

Fig. 1. Arteries from mice on normal chow and ethionine diet. A, cross-section of the aorta in a mouse on normal chow. × 40. B, lesion from mouse on ethionine diet for 14 weeks. × 400. C and D, intramycotic light green. × 400.
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