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Retinoic acid prevents *Chlamydia pneumoniae* induced foam cell development in a mouse model of atherosclerosis

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Abstract

Chlamydia pneumoniae, a common respiratory pathogen, has been associated with cardiovascular disease. *C. pneumoniae* infection accelerates atherosclerotic lesion development in hyperlipidemic animals. Retinoic acid, an anti-oxidant, inhibits infection of endothelial cells by *C. pneumoniae*. The present study demonstrated that retinoic acid suppresses the acceleration of foam cell lesion development induced by *C. pneumoniae* in hyperlipidemic C57BL/6J mice. Retinoic acid treatment had no effect on foam cell lesion development in uninfected animals. Lung infection and duration was decreased in treated mice, suggesting one mechanism by which retinoic acid reduces *C. pneumoniae* accelerated foam cell lesion formation in hyperlipidemic mice.

Keywords

Chlamydia pneumoniae; Atherosclerosis; Mouse model Retinoic acid treatment

1. Introduction

An association of *Chlamydia pneumoniae* with atherosclerosis has been well established by seroepidemiology, detection and isolation of the organism from atherosclerotic lesions in humans [1]. Experiments in mouse models of atherosclerosis have shown that *C. pneumoniae* promotes atherosclerotic lesion development in hyperlipidemic mice [1]. The mechanisms by which *C. pneumoniae* promotes atherosclerosis have not been well defined. In addition to the activation of vascular cells to produce proinflammatory cytokines and other proatherogenic factors, *C. pneumoniae* has been shown to induce oxidation of LDL and foam cell formation [2].

In previous studies, we showed that the major outer membrane protein (MOMP) of *Chlamydia trachomatis* is glycosylated and the structure of the glycan is a high mannose oligosaccharide [3]. The mannose oligosaccharide mediates attachment and infectivity of chlamydiae in cell culture. Cleavage of the glycan from the surface of *C. pneumoniae* organisms decreased infectivity of chlamydiae in cell culture [4] and mouse models of lung infection [5]. We further

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demonstrated that *C. pneumoniae*, but not *C. trachomatis*, binds to the mannose 6-phosphate/insulin-like growth factor-2 (M6P/IGF-2) receptor in endothelial cells, based on the finding that mannose 6-phosphate and retinoic acid, which bind to different sites on the receptor, inhibited attachment and infectivity of *C. pneumoniae* [6,7]. These findings suggest that the terminal mannose of the mannose oligosaccharide is phosphorylated [6]. Interestingly, simultaneous intranasal inoculation of mice with M6P-PAA and *C. pneumoniae* significantly inhibits lung burden in comparison to inoculation with *C. pneumoniae* alone [8].

In addition to binding to the M6P/IGF2 receptor [9], retinoic acid has anti-oxidant properties, elicits a variety of cellular responses which affect cell growth, differentiation, and metabolism through binding to its nuclear receptors [10], and interferes with glycosylation of viral proteins [11]. Because retinoic acid is a ligand for the M6P/IGF-2 receptor and an anti-oxidant, the effects of retinoic acid on the acceleration of atherosclerosis by *C. pneumoniae* was evaluated in C57BL/6J mice fed an atherogenic diet.

2. Materials and Methods

2.1 Experimental animals and study design

Eight-week-old pathogen-free male C57BL/6J mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Five mice per cage were kept in a filter-top cage under pathogen free conditions. Mice were fed with a standard chow or an atherogenic diet consisting 15% fat, 1.25% cholesterol, and 0.5% sodium cholate (TD 88051, Harlan Teklad, Madison, WI) starting at 8 weeks of age and water *ad libitum* throughout the study as previously described [12]. The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Washington.

2.2 Inoculation

Mice were mildly sedated by intraperitoneal injections of a mixture of ketamine (Fort Dodge Laboratories, Shenandoah, IA) and xylazine (Lloyd Laboratories, Shenandoah, IA). Mice were inoculated intranasally with 1×10^7 inclusion forming units (IFU) of *C. pneumoniae* (strain AR-39) at 9, 11 and 13 weeks of age. The organism was grown in HL cells and purified by density gradient centrifugation using diatrizoate meglumine (Hypaque-76; Winthrop-Breon Laboratories, New York). The purified organisms were resuspended in sucrose phosphate glutamic acid chlamydial transport medium and frozen at -70°C until its use. Control mice were sham-inoculated with sterile PBS. The inoculated and sham-inoculated animals were further divided into treatment and no-treatment groups.

2.3 Retinoic acid treatment

Retinoic acid (Sigma, St. Louis, MO) was administered at 50 mg/kg body weight in corn oil by gavages into the stomach. Retinoic acid was given three times a week starting at the beginning of the high cholesterol diet and throughout the experimental period. Control mice were gavaged with corn oil. The study design is illustrated in Figure 1.

2.3 Collection of tissue

Necropsies were performed at 20 weeks after the first inoculation. Mice were sedated with a mixture of 15 mg/kg Acepromazine Maleate (Fermenta Animal Health Co., Kansas City, MO) and 150 mg/kg Ketamine HCL (Phoenix Pharmaceutical Inc., St. Joseph, MO). Blood was collected by exsanguination from the femoral arteries at necropsy. The hearts and aortas were perfusion fixed with 10% buffered formalin administered through the left ventricle. Lungs, hearts and thoracic aortas with its main branches attached were dissected *in toto*. The aortas were separated from the hearts. The bases of the hearts were placed in embedding medium for

frozen section (OCT™, Sakura Finetek, Torrance, CA) and sectioned on a cryostat at the level of the aortic sinus.

2.5 Assay of lung infectivity

For determination of the effect of retinoic acid gavage on lung infectivity, mice were treated with retinoic acid or corn oil 2 hr prior to infection and 3 times per week, thereafter. Lungs were dissected intact, immediately placed in sterile glass vials, and placed on ice prior to freezing at -70°C . Subsequently, lung tissues were thawed and homogenized with a sterile pestle and mortar in sucrose-phosphate-glutamic acid medium to make a 10% (wt/vol) solution. Homogenates were centrifuged at $500 \times g$ for 10 min. The supernatant was saved and the tissue sediment discarded. Cell monolayers in shell vials were inoculated with 100 μl of the supernatant in triplicate. Following incubation at 37°C for 3 days coverslips were stained with a *C. pneumoniae* specific monoclonal antibody TT-401 conjugated to fluorescein isothiocyanate. Infectivity titer was quantified by counting inclusions and expressed as IFU per ml. Lung burden was calculated by determining the IFU/gm of lung.

2.6 Quantification of lesion area

Tissue sections were stained with Movat's pentachrome stain. Histological staining with the Movat's pentachrome stain rather than use of the more conventional Oil red O enables identification of foam cell aggregates (fatty streaks) as compared to areas of the lesions that are enriched in collagen and proteoglycans. The foam cell area was measured as a separate parameter because it has a defined histology of atherosclerosis. For quantification of atherosclerosis, both foam cell area and the adjacent and underlying areas enriched in connective tissue were measured separately and expressed as foam cell area and combined as total lesion area (foam cell area plus adjacent area of inflammatory infiltration), respectively. The measurement of the lesions was done by computer assisted morphometry using Image-Pro software (Media Cybernetics, Bethesda, MD). Lesion area was measured at 45 μm intervals along the aortic root (a total of approximately 300 μm) and the average lesion area was calculated. All measurements were done blindly with the investigators unaware of the treatment groups.

2.7 Measurement of antibody and blood cholesterol levels

Plasma was separated from heparinized blood and frozen at -70°C for serology and lipid measurements. *C. pneumoniae*-specific antibody titers were determined by the microimmunofluorescence test using formalin-fixed *C. pneumoniae* elementary bodies as antigen. Antibodies were measured using fluorescein isothiocyanate-conjugated goat anti-mouse IgG and IgM (heavy-chain specific; Sigma, St. Louis, MO). Antibody titers were determined by serial 2-fold dilutions of plasma. Total blood cholesterol was measured using a commercial enzymatic test kit (Sigma, St. Louis, MO). Levels were determined in triplicate and averaged.

2.8 Statistical analysis

Data were expressed as mean $\pm$ SD. Group data were analyzed by unpaired Student's *t* test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1 Clinical response and lipid profiles

Clinical signs of infection included increased respiratory rate and nasal and ocular discharge. The symptoms were most severe within the first few days after the first and second inoculation. These clinical symptoms were resolved within 2 weeks after the third inoculation. No adverse

reactions were observed following retinoic acid treatment. There were no significant differences in body weights or cholesterol levels between the groups at the time of necropsy (Table 1).

3.2 Serology

Antibody titers against *C. pneumoniae* were determined at 29 weeks of age (20 weeks after the first inoculation). All infected mice, except for 1, had IgG antibodies against *C. pneumoniae* at the time of sacrifice. The titers ranged from 1:16 to 1:512. The median antibody titers of infected mice were similar for retinoic acid treated and untreated mice (1:128 vs. 1:64, respectively). All uninfected mice were antibody negative (< 1:8).

3.3 Quantification of lesion area

In untreated groups, infected mice developed significantly larger foam cell areas than uninfected mice ($1859 \pm 1093 \mu\text{m}^2$, $n=9$ versus $939 \pm 686 \mu\text{m}^2$, $n=9$; $P=0.048$) (Table 1). The total lesion areas were also greater in infected than uninfected mice ($5984 \pm 2702 \mu\text{m}^2$, $n=9$, versus $3786 \pm 2411 \mu\text{m}^2$, $n=9$). However, the difference was not statistically significant ($P=0.087$) (Table 1). In retinoic acid treated groups, no difference in foam cell area was observed between infected and uninfected mice ($1209 \pm 722 \mu\text{m}^2$, $n=9$, versus $1185 \pm 1385 \mu\text{m}^2$, $n=8$, $P=0.96$). There was also a trend toward reduced foam cell area ($1209 \pm 722 \mu\text{m}^2$, $n=9$, versus $1859 \pm 1093 \mu\text{m}^2$, $n=9$, $P=0.16$) and total lesion area ($5273 \pm 4633 \mu\text{m}^2$, $n=9$, versus $5984 \pm 2702 \mu\text{m}^2$, $n=9$, $P=0.70$) in infected mice treated with retinoic acid in comparison to untreated infected mice.

3.4 Retinoic acid inhibits lung infection

To investigate whether the suppression of atherosclerotic lesion development by retinoic acid was due to the inhibition of lung infection, the infectivity titer and duration of infection were analyzed. Retinoic acid treatment was shown to decrease the average titers of lung infection, percent of mice infected, and duration of infection (Table 2). These findings indicate that retinoic acid inhibits the lung infection of *C. pneumoniae*.

4. Discussion

It has been thought that antioxidants such as Vitamin E, beta-carotene or Vitamin A, and Vitamin C reduce hypercholesterolemia and coronary heart disease. However, several large scale human therapeutic trials for reducing the risk of coronary artery disease based on the pretext that these antioxidants inhibit oxidation of LDL have not shown beneficial effects in either reducing hypercholesterolemia or coronary heart disease [13-15]. In this current study, treatment of hyperlipidemic mice with retinoic acid, a transcriptionally active product of Vitamin A, had no effect on plasma cholesterol levels and fatty streak development in uninfected hyperlipidemic mice (Table 1). These findings are similar to those observed in the aforementioned human trials. However, treatment of *C. pneumoniae* infected mice with retinoic acid was shown to prevent the acceleration of foam cell lesion development by *C. pneumoniae* (Table 1).

The mechanisms by which retinoic acid treatment prevents accelerated foam cell formation by *C. pneumoniae* infection remains to be defined. One possibility is through the anti-inflammatory effects of retinoic acid [16]. However, this mechanism may not be a major contributing factor, because retinoic acid treatment of uninfected mice had no effect on foam cell formation or total lesion area in this study. Another plausible mechanism is that retinoic acid reduces the severity and duration of *C. pneumoniae* infection as shown in Table 2, thus, ameliorating the atherogenic effects of *C. pneumoniae*. These findings are supported by *in vitro* experiments demonstrating that retinoic acid inhibits attachment of *C. pneumoniae* to

host cells and intracellular growth and these actions are mediated through different receptors. As mentioned previously retinoic acid binds to the M6P/IGF-2 receptor and inhibits attachment and infectivity of *C. pneumoniae* in endothelial cells [6,7]. Similarly, other pathogens including herpes simplex virus [17] and varicella-zoster virus [18] have been reported to bind to M6P/IGF-2 receptors and the infectivity of these pathogens can be inhibited by ligands of the receptor. The other anti-chlamydial activity of retinoic acid is its effect on intracellular growth. This inhibitory effect occurs through its interaction with the nuclear retinoic acid receptor through which retinoic acid is known to elicit a variety of cellular responses that ultimately could affect intracellular growth. For example, retinoic acid has been reported to increase the intracellular ceramide concentration, by increasing the production of acid sphingomyelinase, which results in hydrolysis of sphingomyelin to ceramide [19]. It is known that Chlamydia utilizes host cell derived sphingolipids for synthesis of the inclusion membrane [20]. Therefore, one possible mechanism by which retinoic acid might inhibit chlamydial growth would be by interfering with utilization of sphingolipids by chlamydiae, however; this possibility was not addressed in this study. Therefore retinoic acid may reduce the severity of *C. pneumoniae* infection and ameliorate the atherogenic effects of *C. pneumoniae*.

In summary, retinoic acid treatment of hyperlipidemic mice may ameliorate the acceleration of *C. pneumoniae* induced atherosclerosis by suppressing lung infection.

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Figure 1.
Experimental design.

Table 1

Effect of retinoic acid (RA) treatment on body weight, plasma cholesterol, and development of atherosclerotic lesions in C57BL/6J mice.

Measurements	Sham-inoculated		Infected	
	-RA	+ RA	-RA	+RA
Body weight (gm)	27.7±1.9 ^a (1.0) ^b	26.5±1.1 (1.0)	25.9±1.5 (0.9)	26.7±1.7 (1.0)
Cholesterol (mg/dl)	179±30 (1.0)	175±34 (1.0)	186±24 (1.0)	191±20 (1.1)
Foam cell area (μm ²)	939±686 (1.0)	1185±1385 (1.3)	1859±1093 (2.0) ^c	1209±722 (1.3)
Total lesion area (μm ²)	3786±2411 (1.0)	3025±2373 (0.8)	5984±2702 (1.6)	5273±4633 (1.4)

^a mean ± SD, N=9, except N=8 in uninfected-treated group.

^b () fold difference in comparison to uninfected and untreated.

^c $P < 0.05$, versus uninfected and untreated.

Table 2

Inhibition of lung infection of mice by retinoic acid treatment.

Days post infection	No. of mice positive/no. of mice tested		IFU/gm lung (% decrease) ^a	
	-RA	+ RA	-RA	+ RA
3	5/5	5/5	1.1×10^7	6.8×10^6 (38%)
7	4/4	3/5	5.2×10^6	2.7×10^6 (48%)
21	5/9	0/8 ^b	2.6×10^4	0 (100%)

^a Data are the mean titers in lung. Percent decrease is relative to the IFU/gm of lung in the control.^b $P < 0.05$