Graefe's Arch Clin Exp Ophthalmol
(2005) 243: 1080–1090

CLINICAL INVESTIGATION

DOI 10.1007/s00417-005-1169-y

Murat V. Kalayoglu
Deisy Bula
Jorge Arroyo
Evangelos S. Gragoudas
Donald D'Amico
Joan W. Miller

Identification of *Chlamydia pneumoniae* within human choroidal neovascular membranes secondary to age-related macular degeneration

Received: 23 October 2004
Revised: 25 January 2005
Accepted: 3 March 2005
Published online: 21 May 2005

This study was presented in part at the 2003 and 2004 meetings of the Association for Research in Vision and Ophthalmology, Fort Lauderdale, FL.

M. V. Kalayoglu · D. Bula ·
E. S. Gragoudas · J. W. Miller
Angiogenesis Laboratory,
Department of Ophthalmology,
Massachusetts Eye and Ear Infirmary,
Harvard Medical School,
Boston, MA, USA

D. Bula · J. Arroyo · E. S. Gragoudas ·
D. D'Amico · J. W. Miller (✉)
Retina Service,
Massachusetts Eye and Ear Infirmary,
Harvard Medical School,
8th Floor, 243 Charles Street,
Boston, MA 02114, USA
e-mail: Joan_Miller@meei.harvard.edu
Tel.: +1-617-5733259
Fax: +1-617-5733364

Abstract Age-related macular degeneration (AMD) is a leading cause of blindness in the United States, and increasing evidence suggests that it is an inflammatory disease. The prokaryotic obligate intracellular pathogen *Chlamydia pneumoniae* is emerging as a novel risk factor in cardiovascular disease, and recent sero-epidemiological data suggest that *C. pneumoniae* infection is also associated with AMD. In this study, we examined choroidal neovascular membrane (CNV) tissue from patients with neovascular AMD for the presence of *C. pneumoniae* and determined whether the pathogen can dysregulate the function of key cell types in ways that can cause neovascular AMD. Nine CNV removed from patients with neovascular AMD were examined for the presence of *C. pneumoniae* by immunohistochemistry (IHC) and polymerase chain reaction (PCR); in addition, we performed PCR on nine non-AMD eyes, and IHC on five non-AMD CNV, seven non-AMD eyes, and one internal limiting membrane specimen. Finally, human monocyte-derived macrophages and retinal pigment epithelial (RPE) cells were exposed to *C. pneumoniae* and assayed in vitro for the production of pro-angiogenic immunomodulators (VEGF, IL-8, and MCP-1). *C. pneumoniae* was detected in four of nine AMD CNV by IHC and two of nine AMD CNV by PCR, induced VEGF production by human macrophages, and increased production of IL-8 and MCP-1 by RPE cells. In contrast, none of the 22 non-AMD specimens showed evidence for *C. pneumoniae*. These data indicate that a pathogen capable of inducing chronic inflammation and pro-angiogenic cytokines can be detected in some AMD CNV, and suggest that infection may contribute to the pathogenesis of AMD.

Keywords Infection · Inflammation · Age-related macular degeneration

Introduction

Age-related macular degeneration (AMD) is a chronic, progressive disease that remains the leading cause of blindness in Americans over the age of 55 years [14]. The majority of vision loss is due to neovascular AMD, the advanced form of the disease where immature vessels grow from the choroid into the subretinal space [12]. Neovascular AMD is characterized by the formation of a choroidal neovascular membrane (CNV) in the macula and, subsequently, a severe decrease in vision. Neovascular AMD is mediated by specific pro-inflammatory and angiogenic cytokines secreted by relevant cell types [i.e., retinal pigment epithelial (RPE) cells and choroidal macrophages]. Vascular endothelial growth factor (VEGF), a key regulator of physiological angiogenesis [11], is one such cytokine central to the development of neovascular AMD. Data in support of a central role for VEGF in AMD come from several lines of evidence which indicate that VEGF is sufficient to induce CNV [2, 11, 13, 15, 31–33, 38, 40, 43] . VEGF has been

localized to CNV from AMD patients [31], and anti-VEGF therapy leads to decreased angiographic leakage in experimental models of CNV [27]. In addition, early data from clinical trials suggest a beneficial effect of anti-VEGF therapy on CNV leakage and vision [10] . Interleukin-8 (IL-8) and monocyte chemotactic protein 1 (MCP-1), inflammatory chemokines implicated in the pathogenesis of AMD [16], also have the potential to lead to angiogenesis [4, 37]. However, although CNV formation appears to be mediated by secretion of VEGF and other pro-angiogenic factors, the inflammatory stimuli that induce these factors remain poorly defined.

The obligate intracellular prokaryotic human pathogen *Chlamydia pneumoniae* is emerging as a novel risk factor in cardiovascular diseases [23]. Evidence to implicate this pathogen in atherosclerosis, now appreciated to be an inflammatory disease [18], comes from a variety of epidemiological, pathology-based, animal model, cell biology, and human antibiotic treatment studies [23]. The hallmark of chlamydial infections is their capacity to cause chronic inflammation [18], and persistent *C. pneumoniae* infections may promote inflammatory age-related diseases other than atherosclerosis. Indeed, AMD, which shares several risk factors with cardiovascular disease, recently was associated with *C. pneumoniae* by an epidemiological study [20]; a subsequent report supported this association [17]. To further explore the association between *C. pneumoniae* infection and AMD, we examined archival CNV specimens surgically extracted from AMD patients for the presence of *C. pneumoniae* and studied pro-angiogenic cytokine secretion profiles in human RPE cells and macrophages in vitro following infection with *C. pneumoniae*.

Methods

Reagents and chlamydiae

Unless otherwise noted, all reagents were purchased from Sigma (St. Louis, MO). Density-gradient purified *C. pneumoniae* (strain AR-39) and *C. trachomatis* (serovar A) were kindly provided by Dr. Gerald Byrne (Memphis, TN).

Tissue preparation

The use of human tissue was in accordance with the guidelines and approved by the Institutional Review Boards of the Massachusetts Eye & Ear Infirmary and Massachusetts General Hospital (Boston, MA). Sterile technique was used to handle all specimens. Since most patients with 'wet' AMD undergo photodynamic therapy, surgical removal of CNV from AMD eyes has become a very rare procedure. We therefore were able to obtain only nine AMD CNV specimens for immunohistochemistry (IHC) and polymerase chain reaction (PCR) analysis. Formalin-fixed archival CNV specimens surgically extracted from patients with AMD were obtained from the Massachusetts Eye and Ear Infirmary Eye pathology laboratory. CNV specimens were obtained from patients who had severe vision loss due to subfoveal CNV; each patient chose between surgery and PDT after the risks, benefits and alternatives of each procedure had been explained in full. The nine patients with AMD CNV were between 76 and 90 years old and consisted of five males and four females. The five patients with non-AMD CNV were between 12 and 50 years of age and consisted of one male and four females. Surgical excision of submacular CNV had been performed according to previously described techniques [30]. Immediately after surgical excision, CNV were placed in formalin overnight, washed in phosphate-balanced salt solution (PBS) at 4°C for 4 h, dehydrated with a series of graded alcohol solutions, and embedded in paraffin. In addition, seven eyes without AMD enucleated for uveal melanoma were formalin-fixed, paraffin-embedded and axially sectioned at the optic nerve for IHC. Furthermore, nine frozen whole eyes from patients without AMD (ages 70–85 years) were obtained from the New England Eye Bank (Boston, MA). Eyes were thawed, sectioned at the coronal equator, and examined under a dissecting microscope to ensure absence of drusen and CNV. Retina, choroid, and iris were meticulously dissected under sterile conditions and placed in separate tubes for DNA extraction. All eye-bank eyes were frozen at −7°C within 24 h of death, and globes underwent DNA extraction within 1 week of freezing. Sera were not available for testing chlamydial titers in corresponding archival specimens or eye-bank donor tissues. Most archival specimens were stored in formalin for less than 2 years.

For IHC, paraffin-embedded specimens were serially sectioned at 4–5 μm thickness, placed on glass slides and deparaffinized by xylene-alcohol treatment. Enough tissue was available to obtain two to six sections per specimen. When available, two to three adjacent sections were placed on a single slide to be stained simultaneously.

For PCR of AMD CNV, 20–30 μm sections were obtained from each specimen and total DNA was extracted with the Qiamp DNA mini kit (Qiagen, Valencia, CA), using a modification of the manufacturer's protocol to remove paraffin [42]. For PCR of control eye-bank retina, choroid, and iris specimens, DNA was extracted from approximately 1–2 mg of each specimen using the Qiamp DNA mini kit.

Immunohistochemistry

Antigen retrieval was performed by treatment with target retrieval solution (Dako S1700; Dako, Carpinteria, CA) in a water bath at 95°C for 20 minutes. Sections were in-

cubated for one hour at room temperature with RR-402 mouse monoclonal anti-*C. pneumoniae* antibody (1:50 dilution; Dakocytomation, Carpinteria, CA). Negative controls either omitted the primary antibody or substituted an isotype-specific monoclonal antibody at a similar concentration. Positive controls consisted of human RPE cells or human monocyte-derived macrophages infected with *C. pneumoniae* and fixed in formalin. A standard IHC protocol was then performed with the HRP-AEC anti-mouse cell and tissue staining kit (Dako AP EnVision System; Dakocytomation) according to the manufacturer's recommendations. All incubations were carried out in a humidifier chamber. After incubation, slides were washed in PBS or TBS, counterstained with Mayer's hematoxylin (Sigma), mounted with aqueous mounting medium (Farmount; Dakocytomation), and examined by light microscopy.

PCR and automated sequencing

Meticulous care was taken to prevent contamination and amplicon carryover. All PCR was done under sterile conditions. Positive control DNA was dispensed only after all other specimen tubes were capped-on and removed from the culture hood. All PCR tubes, Qiamp DNA extraction kit reagents and spin columns were treated with UV light (Stratagene UV crosslinker, Stratagene, La Jolla, CA) to ensure absence of amplifiable DNA; PCR did not amplify any product from UV-treated tubes and reagents when targeting a universal bacterial 16S ribosomal RNA sequence [34].

DNA was amplified with touchdown-nested PCR with primers targeting the *C. pneumoniae* major outer membrane protein sequence (CP1-CP2/CPC-CPD) [39]. Bovine serum albumin 0.1–1% was used in each reaction to allow amplification in the presence of melanin, a PCR inhibitor present in uveal tissue [9]. Samples were electrophoresed on 1.5% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

Ethidium bromide-stained gel bands were cut with a sterile razor blade, and the band DNA was extracted and purified with a Qiagen gel extraction kit (Valencia, CA). Purified DNA was analyzed using an automated sequencer at the DNA Sequencing Center for Vision Research (DSCVR; Massachusetts Eye and Ear Infirmary, Boston, MA). Sequencing was performed first with the CPD primer, and sequence identity was confirmed by re-sequencing with the CPC primer. Sequences were analyzed with Chromas software (Technelysium, Helensvale, Australia) and compared with sequences in the National Center for Biotechnology Information database using Basic Local Alignment Search Tool (BLAST). Data are representative of at least two separate experiments.

Cell culture

Human peripheral blood mononuclear cells were isolated by Ficoll-Hypaque centrifugation from healthy donor blood. Monocytes were separated from lymphocytes by incubating cells at 37°C for 1 h in RPMI-1640 medium (Sigma), followed by washing 5 times with HBSS (Sigma). In separate experiments, isolated cells were >90% monocytes by anti-CD14 staining [19] and viability was >95% as assessed by trypan-blue dye exclusion. Monocytes were cultured at 37°C for 5–7 days in RPMI-1640 medium supplemented with 10% FBS, 10 μg/ml gentamycin and 25 mM HEPES buffer to allow maturation into macrophages. Human RPE cells (ARPE-19 cell line; ATCC, Manassas, VA) were propagated in 1:1 DMEM/Ham's F12 medium (ATCC) supplemented with 10% FBS and 10 μg/ml gentamycin. All cells were plated in 96-well microtiter wells or LabTek slides for the experiments (Nalge Nunc International, Rochester, NY). Cells were mock-infected or infected with varying doses of *C. pneumoniae* by incubating for 2 h at 37°C in 50 μl medium, then cultured for 10–64 h in 200 μl medium. Media were assayed by commercially available ELISA kits for the presence of VEGF (R&D Systems), IL-8, and MCP-1 (Anogen, Mississauga, Ontario). Separate cell protein assays indicated that infection did not globally increase protein secretion by the tested cell types (not shown). Cells were fixed in methanol and stained with the Pathfinder FITC-conjugated anti-chlamydial monoclonal antibody (Bio-Rad Laboratories, Hercules, CA). Assays were conducted in triplicate and data are representative of at least two similar experiments.

Results

Detection of *C. pneumoniae* in CNV by IHC

Serial sections of the CNV specimens (Tables 1, 2, 3, 4) were stained with anti-*C. pneumoniae* monoclonal antibody (RR-402) for IHC. Four of nine specimens showed evidence of *C. pneumoniae* [specimens 1.3 (Fig. 1a–d), 1.5 (Fig. 1e, f), 1.6 (Fig. 1g–j), and 1.9 (Fig. 1k)]. Most specimens had an adjacent serial section available on the same slide, and were considered positive only if intracellular staining was observed in corresponding locations of the two adjacent sections (compare Fig. 1a to b, e to f, and h to i). Staining was not noted in CNV specimens incubated with an isotype-specific control antibody or without primary antibody. In addition, an internal limiting membrane (ILM) peel specimen that was incubated with anti-*C. pneumoniae* antibody as a negative control did not show evidence of staining. Furthermore, no staining was observed in serial sections of five CNV surgically extracted from patients without evidence of AMD (Table 3), or from axial

Table 1 Demographics of patients with AMD CNV

Patient	*Age*	*Gender*	*Eye*	*PDT*	*DM*	*CAD*	*HTN*	*Smoking*	*Other history*
1.1	82	M	OD	Yes	No	No	Yes	Never	Cataract
1.2	78	F	OS	No	No	No	No	Never	No
1.3	87	F	OS	No	No	No	Yes	Never	No
1.4	90	F	OD	No	No	No	Yes	Never	Breast CA
1.5	87	F	OD	No	No	No	Yes	Never	Cataract
1.6	76	M	OS	Yes	No	Yes	Yes	Never	s/p CABG
1.7	83	M	OD	Yes	No	No	Yes	Never	Cataract
1.8	77	M	OS	Yes	No	No	Yes	Never	No
1.9	83	M	OS	Yes	No	Yes	Yes	(Occasional)	s/p Hernia repair

Table 2 Demographics of non-AMD eye-bank donors

Patient	*Age*	*Gender*	*Eye*	*Cause of death*	*Death-preservation time*
2.1	72	M	OD	MI	13 h 31 min
2.2	75	M	OD	CVA	10 h 55 min
2.3	81	M	OS	Throat Cancer	4 h 50 min
2.4	75	M	OD	MI	17 h 20 min
2.5	73	M	OS	MI	23 h 15 min
2.6	74	F	OD	Respiratory Failure	11 h 30 min
2.7	73	M	OD	MI	20 h 20 min
2.8	71	M	OD	MI	8 h 38 min
2.9	73	M	OS	MI	not known

Table 3 Demographics of patients with non-AMD CNV

Patient	*Age*	*Gender*	*Eye*	*CNV Cause*	*PDT/Laser*	*DM*	*CAD*	*HTN*	*Smoking*	*Other history*
3.1	43	F	OD	Idiopathic	No	No	No	Yes	Never	None
3.2	50	F	OD	Idiopathic	No	No	No	No	Never	Cataract
3.3	42	F	OD	Idiopathic	No	No	Yes	Yes	Never	Mitral valve prolapse
3.4	41	F	OS	Multifocal choroiditis	No	No	Yes	Yes	Never	None
3.5	12	M	OS	Post-trauma	No	No	No	Yes	Never	Choroidal ruptures

sections of seven non-AMD eyes enucleated for uveal melanoma (Table 4). Some staining in AMD CNV specimens localized within lipofuscin-laden cells (Fig. 1e, f) characteristic of RPE. Two of the four positive specimens (1.6 and 1.9) were extracted from patients previously treated with photodynamic therapy. Since CNV resulting from non-AMD disease tend to occur in younger patients than AMD CNV, IHC control specimens were not age-matched to AMD CNV (compare Table 3 to Table 1). Surgically extracted CNV specimens are very small, and therefore no extra tissue was available to perform double staining to determine which cell types are preferentially infected with *C. pneumoniae*. We did not notice a correlation between *C. pneumoniae* infection and the amount of inflammatory activity within CNV specimens.

Table 4 Demographics of patients without AMD enucleated for uveal melanoma

Patient	*Age*	*Gender*	*Eye*
4.1	68	F	OS
4.2	83	F	OS
4.3	56	F	OD
4.4	64	M	OD
4.5	51	M	OD
4.6	71	F	OD
4.7	83	M	OS

Detection of *C. pneumoniae* in CNV by PCR

To further substantiate the presence of *C. pneumoniae* in AMD CNV, several sections of each archival AMD CNV specimen (Table 1) were examined by touchdown-nested

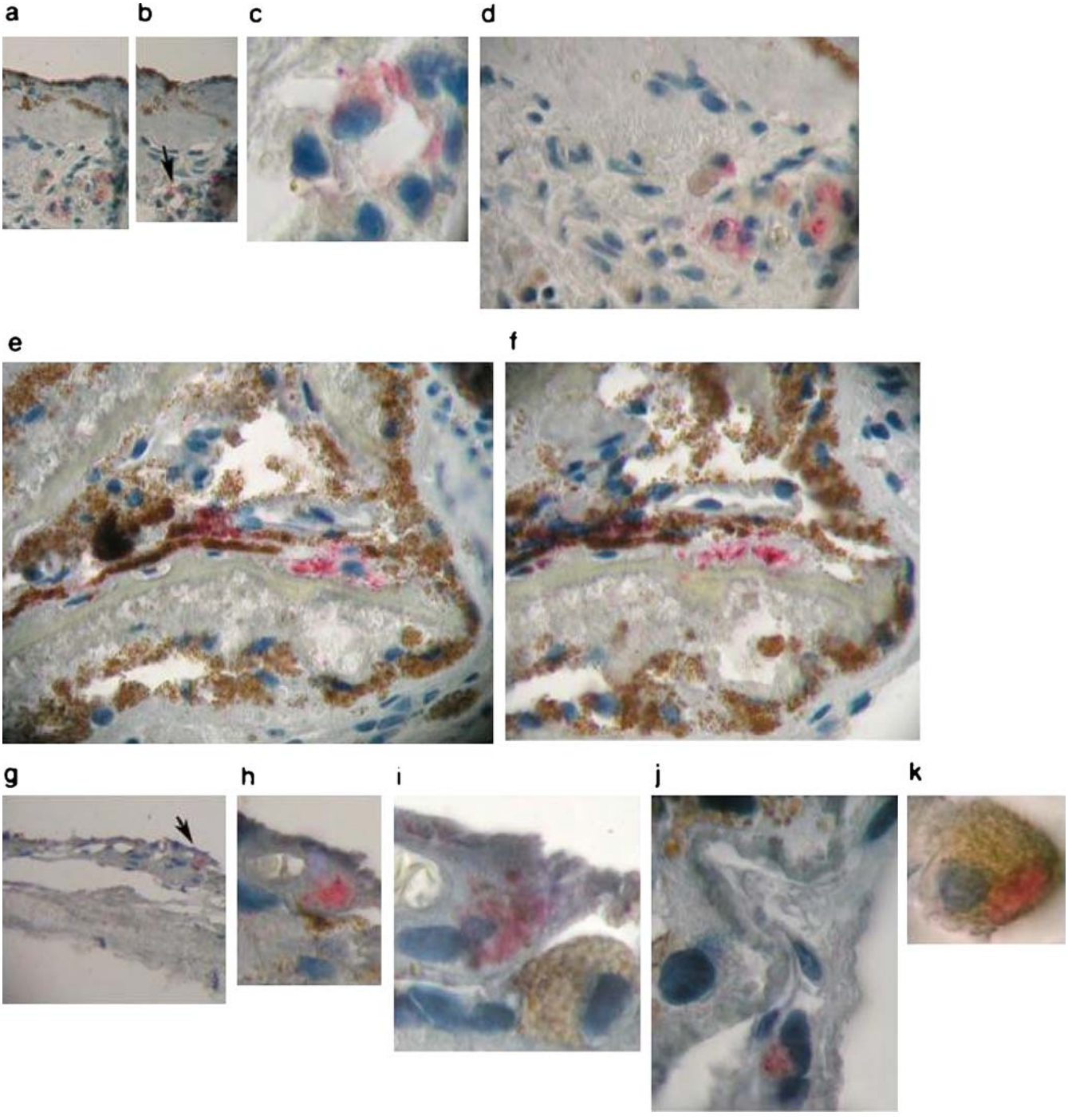

Fig. 1 Detection of *C. pneumoniae* in AMD CNV by IHC. A horseradish peroxidase method using monoclonal antibody (RR-402) to the *C. pneumoniae* major outer membrane protein and AEC (*red*) as substrate was used to detect the pathogen in CNV sections from four separate specimens [1.3 (**a**–**d**), 1.5 (**e**, **f**), 1.6 (**g**–**j**), and 1.9 (**k**)]. Adjacent serial sections (**a** and **b**, **e** and **f**, and **h** and **i**) were used to ensure specific staining of positive samples with RR-402. Negative controls incubated with an isotype-specific monoclonal antibody or by omitting the primary antibody did not show staining. **a** 10× magnification of specimen 1.3 showed staining with RR-402. **b** 10× magnification of the adjacent serial section shown in (**a**) with staining at the specimen's corresponding location (*arrow*). **c** 100× magnification of the area indicated by the arrow in (**b**) showing intracellular staining. **d** 40× magnification of (**a**) showing intracellular staining. **e** 40× magnification of specimen 1.5, and **f** its adjacent serial section showing staining at corresponding locations. **g** 10× magnification of CNV 1.6 with positive staining (*arrow*). **h** 63× magnified view from (**f**) showing staining with RR-402, and **i** a 100× view of the adjacent serial section showing staining at the corresponding location. **j** RR-402 staining of a separate area in CNV 1.6 showing intracellular staining reminiscent of an inclusion. **k** 100× magnification of a section from specimen 1.9 stained with RR-402 shows a pigmented cell with distinct intracellular staining. No adjacent serial section was available for this specimen

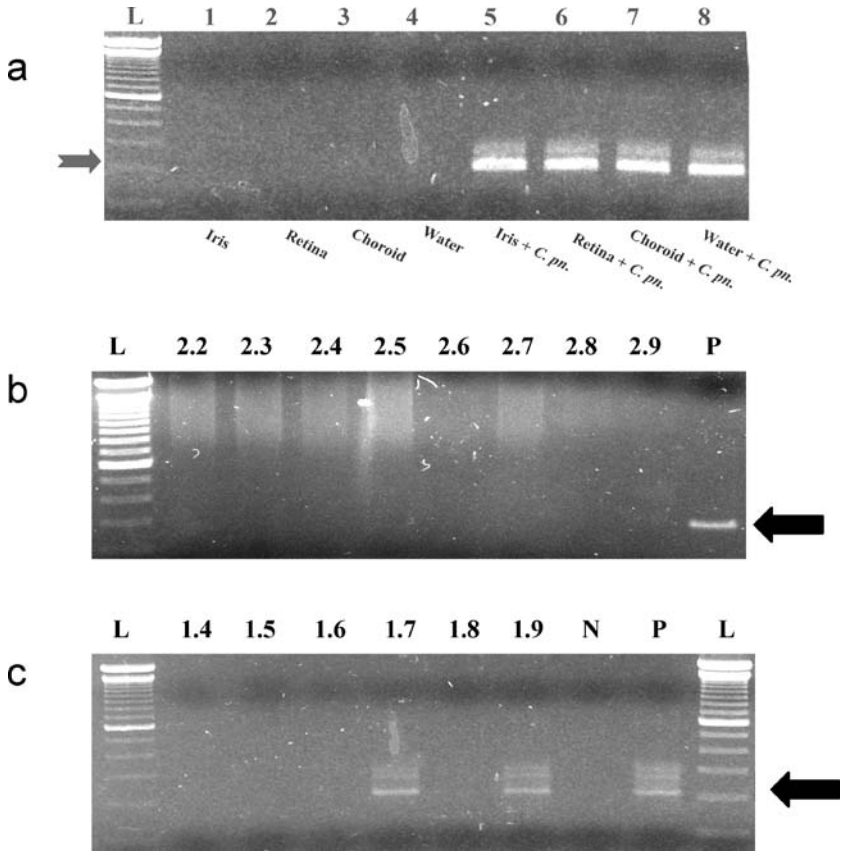

Fig. 2 Detection of *C. pneumoniae* in AMD CNV by PCR. **a** The iris, retina and choroid of an eye bank globe without AMD (Donor 2.1) were separated by dissection and amplified by touchdown-nested PCR [39] with primers specific for the *C. pneumoniae* major outer membrane protein. Position of expected product (207 bp) is shown to the left of the 100 bp ladder (*arrow*). No signal was detected from the iris, retina, choroid, or control sample (water) (*lanes 1–4*). Each sample was then spiked with *C. pneumoniae* DNA (equivalent of 10 IFU, *lanes 5–8*) to demonstrate absence of inhibitors of PCR in the tissue. **b** DNA extracted from the iris, retina, and choroid of each globe was mixed in equivalent volumes (2 ul each) and amplified for the presence of *C. pneumoniae* DNA. No detectable DNA was present from donors 2.2–2.9. *Lane P*, DNA from sample 2.9+*C. pneumoniae* DNA (positive control DNA from 10 IFU, shown by arrow). **c** DNA extracted from CNV isolated from AMD samples 1.4–1.9 was amplified for the presence of *C. pneumoniae* DNA. Sample 1.7 and 1.9 showed amplified product of the expected size, which was cut from the gel for sequencing. Samples 1.1–1.3 (not shown) did not amplify a product in separate experiments. *Lane N*, negative control (*C. trachomatis* serovar A, 10 IFU); *Lane P*, positive control (*C. pneumoniae* AR-39 DNA, 10 IFU). The position of the expected product (207 bp) is shown by an *arrow. Lane L*, 100 bp ladder

PCR [39] for the presence of *C. pneumoniae* DNA. Meticulous care was taken to prevent contamination and amplicon carry-over, including use of sterile technique, UV-irradiation of all DNA extraction reagents and tubes, and use of low *C. pneumoniae* DNA levels for positive controls (see Methods). Since formalin fixation degrades DNA, we chose as control tissues nine fresh, frozen eye-bank eyes without evidence of AMD (Table 2); retina, iris, and choroid tissue were dissected from each of these eyes and the extracted DNA from each tissue was amplified individually (Fig. 2a) or in combination (Fig. 2b). *C. pneumoniae* DNA was not detected from any tissue isolated from the nine control eyes (Fig. 2a, b), even though the tissue was fresh and frozen (e.g. not fixed in formalin). In contrast, DNA extracted from two of the nine archival AMD CNV specimens (25 μm sections, specimens 1.7 and 1.9) amplified the expected *C. pneumoniae* major outer membrane gene segment (Fig. 2c). A negative control (*Chlamydia trachomatis* serovar A DNA) and positive control (*C. pneumoniae* AR-39 DNA) gave expected results in each reaction. In addition, the original primer pair produced products of the expected size for each positive reaction (not shown), indicating that the final products did not result from amplicon carryover during nested PCR. For additional confirmation of the specificity of the PCR, the amplified product bands were extracted from the gel, purified and subjected to automated sequencing, revealing 100% identity to the expected *C. pneumoniae* major outer membrane gene segment (not shown).

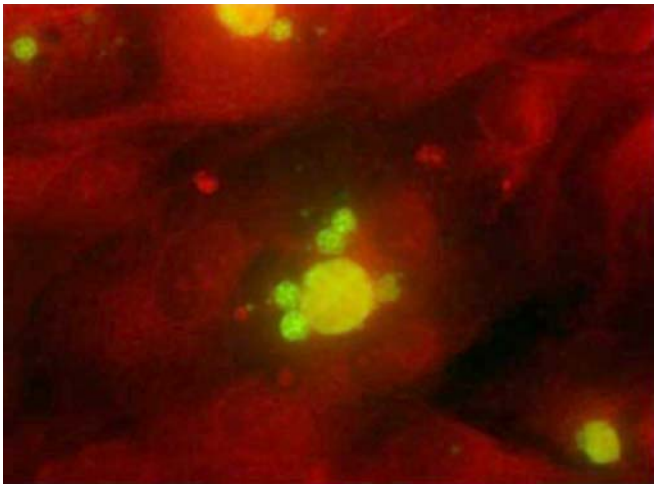

Fig. 3 Infection of ARPE-19 cells by *C. pneumoniae*. Human RPE cells (ARPE-19 cell line) were infected with *C. pneumoniae* (1 IFU/cell), incubated for 68 h, and fixed for immunofluorescent staining. Note the well-defined, multiple reticulated chlamydial inclusions in ARPE-19 cells. Approximately 70% of RPE cells were infected at this dose (1 IFU/cell)

C. pneumoniae infects human macrophages and RPE cells, and exposure to *C. pneumoniae* induces production of pro-angiogenic cytokines by these cell types

To determine whether the pathogen can also alter cell function in ways that may cause neovascular AMD, human monocyte-derived macrophages and ARPE-19 cells were infected with varying doses of *C. pneumoniae* and assayed for the production of pro-angiogenic cytokines. *C. pneumoniae* was found to infect both human macrophages and ARPE-19 cells, establishing large, multiple inclusions within ARPE-19 cells at relatively low multiplicities of infection [1 infection forming unit (IFU)/cell] (Fig. 3). Cells also were assayed for increased production of angiogenic cytokines. Exposure to *C. pneumoniae* induced VEGF by macrophages in a dose-dependent (Fig. 4a) and time-dependent (Fig. 4b) manner. Higher doses of *C. pneumoniae* led to an approximately 50-fold increase in VEGF secretion, and stimulation was evident within 24 h of infection. As expected, ARPE-19 cells secreted high basal levels of VEGF, similar to native RPE [26], and *C. pneumoniae* did not appreciably increase VEGF production by this cell type (not shown). However, *C. pneumoniae*-mediated dysregulation of RPE function was evident by a dose- and time-dependent increase in IL-8 (Fig. 5a, b) and MCP-1 (Fig. 5c, d) by infected ARPE-19 cells. Higher doses of *C. pneumoniae* led to a 10- to 20-fold increase ($P<0.05$) in IL-8 levels and a twofold increase ($P<0.05$) in MCP-1 production by ARPE-19 cells. Specific pathogenic mechanisms (i.e., a role for virulence determinants such as chlamydial heat shock protein 60 and lipopolysaccharide, or requirement for live vs. heat- or UV-killed organisms) were not investigated in this initial set of experiments.

Discussion

Data presented in these experiments show that an infectious agent capable of inducing pro-angiogenic cytokines is present in some AMD neovascular membranes. *C. pneumoniae* was detected in four of nine CNV by IHC (specimens 1.3, 1.5, 1.6, and 1.9), induced VEGF by macrophages, and increased production of IL-8 and MCP-1 by ARPE-19 cells. In contrast, five CNV surgically extracted from patients without AMD (Table 3), and sections from seven non-AMD eyes enucleated for uveal melanoma (Table 4), did not show evidence for *C. pneumoniae* by IHC. These data indicate that an infectious agent may con-

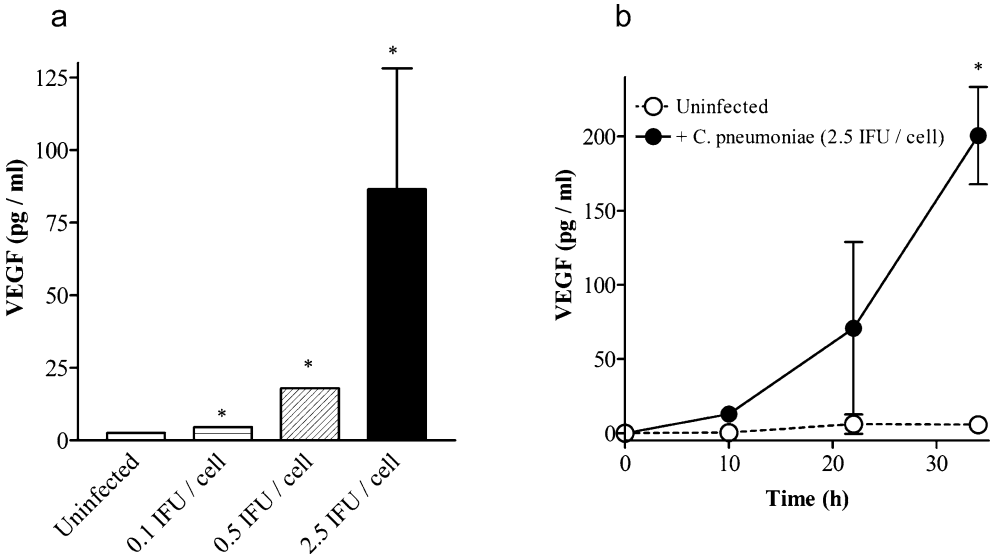

Fig. 4 *C. pneumoniae* infects human monocyte-derived macrophages and induces VEGF production. Human peripheral blood monocyte-derived macrophages were infected with varying doses of *C. pneumoniae* and assayed for VEGF production within 2 days. *C. pneumoniae* induces VEGF production by macrophages in dose-dependent (**a**) and time-dependent (**b**) manner. *, Statistically significant difference ($P<0.05$) compared with uninfected cells. Results are representative of three separate experiments

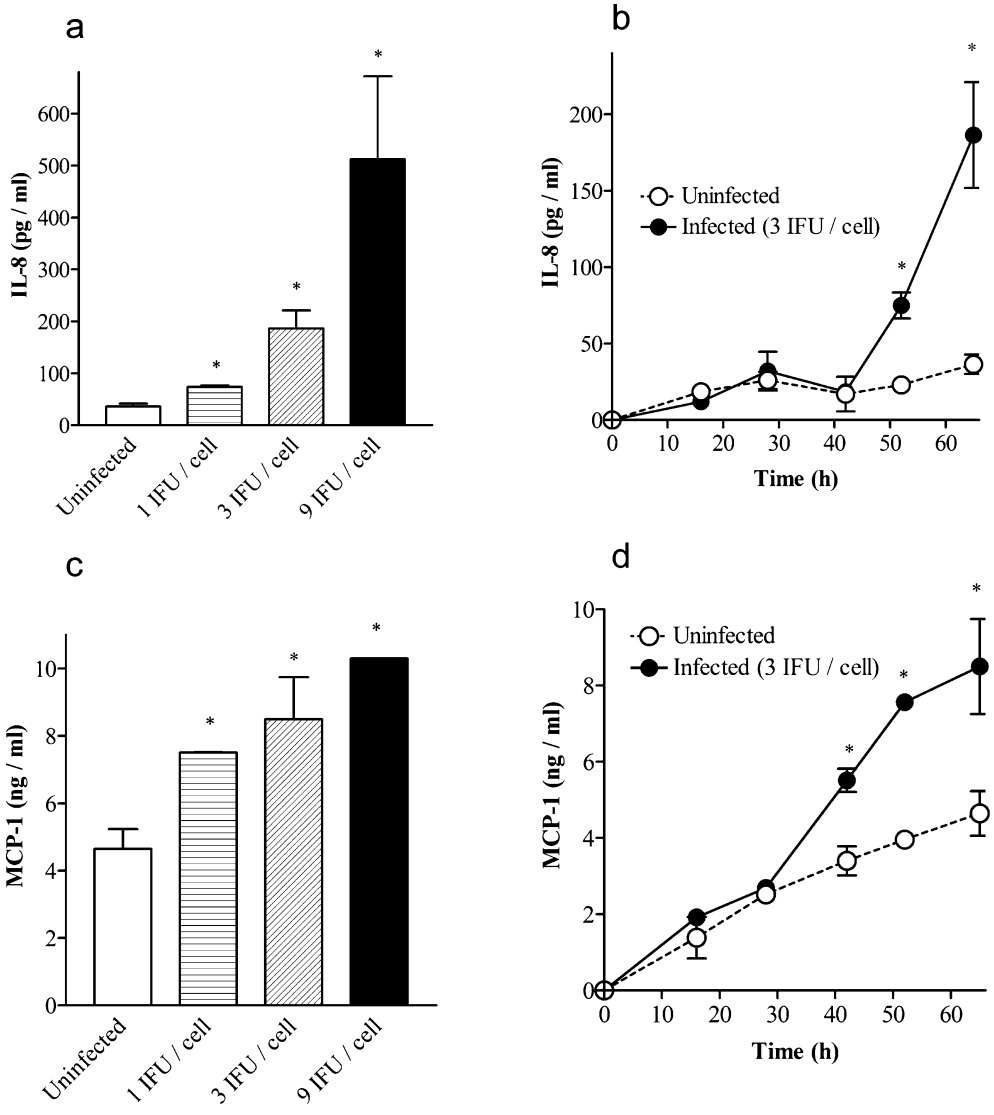

Fig. 5 *C. pneumoniae* infects human RPE cells and induces IL-8 and MCP-1 production. Human RPE cells (ARPE-19 cell line) were infected with *C. pneumoniae* at varying doses, incubated for 68 h, and assayed for the production of IL-8 and MCP-1. **a**, **b***C. pneumoniae* induces production of IL8 in a dose-dependent (**a**) and time-dependent (**b**) manner. **c**, **d** *C. pneumoniae* also induces production of MCP-1 in a dose-dependent (**c**) and time-dependent (**d**) manner. *, Statistically significant difference ($P<0.05$) compared with uninfected cells

tribute to the pathogenesis of AMD and strengthen the hypothesis that chronic inflammation promotes progression to neovascular AMD. However, data provided in these experiments do not negate the "innocent bystander hypothesis", which argues that inactive *C. pneumoniae* arrives at inflamed tissues within macrophages and that its presence is a marker of macrophage recruitment but not a trigger for inflammation. Furthermore, since *C. pneumoniae* was not detected in all CNV, these data suggest that *C. pneumoniae* infection is insufficient to cause CNV. However, the presence of a pathogen—one capable of establishing chronic infection and inducing chronic inflammation—at the site of the disease process may accelerate AMD in some individuals.

Even though specimens were preserved in formalin for extended periods, PCR was also attempted on the archival CNV. PCR showed that two of nine AMD CNV were positive by PCR (specimens 1.7 and 1.9). However, PCR data may be less reliable than IHC given that these archival specimens were preserved in formalin, which degrades DNA. Indeed, overlap between PCR and IHC was observed only in one positive specimen (1.9). Extraction of DNA from formalin-fixed samples significantly degrades amplifiable DNA [41], especially if the amplified segment is larger than 300 bp [5]. We picked a small DNA fragment to amplify and used a modified DNA extraction technique [42] to increase yield; even so, it is unlikely that PCR would amplify DNA from all formalin-fixed specimens containing *C. pneumoniae*. Difficulty in extracting and amplifying chlamydial DNA from formalin-fixed atherosclerotic plaques may also have resulted in lower detection rates with PCR than IHC [28], similar to our study (2/9 positive by PCR versus 4/9 positive by IHC). In contrast, high levels of amplifiable DNA can be extracted from unfixed, frozen specimens such as eye-bank eyes without AMD (negative controls, Table 2), and lack of PCR prod-

ucts from these specimens likely indicates absence of *C. pneumoniae* in these tissues. In addition, PCR and IHC should both be positive for specimens containing an abundant number of chlamydiae; however, if the specimen contained few organisms with patchy distribution, a specimen would be unlikely to be positive by both PCR and IHC, since sections assayed by PCR cannot be stained with IHC (and vice versa). Indeed, PCR and IHC have correlated poorly in detecting *C. pneumoniae* from atheromatous lesions [28], in part because the distribution of the pathogen in these lesions is patchy [29]. Our IHC studies indicate that the distribution of organisms in CNV also is patchy (Fig. 1), since only some sections from positive CNV stain positive for the pathogen. Future studies with freshly isolated AMD tissues will probably help detect *C. pneumoniae* and other pathogens by PCR.

We detected *C. pneumoniae* in specimens 1.3, 1.5, 1.6, 1.7 and 1.9. Table 1 shows that none of the five donors had diabetes, only one (1.9) was an occasional smoker, only two (1.6 and 1.9) had evidence of coronary artery disease, all had a history of hypertension, and three (1.6, 1.7, 1.9) had been previously treated with PDT. Unfortunately, too few specimens were available to study further the possibility that hypertension and PDT may promote infection and inflammation in neovascular AMD.

Serological analysis of these patients may have shown a correlation between *C. pneumoniae* seropositivity and CNV positivity and strengthened our observations. However, the CNV specimens were formalin-preserved archival samples collected and stored for several years, and sera from these patients had not been collected at the time of surgery. We did not attempt to re-contact these individuals for serological analysis, for two reasons. Antibody titers to *C. pneumoniae* fluctuate over time, so that prior infection may subside by the time of the sampling [7]. In addition, serology correlates poorly with detection of *C. pneumoniae* within atheromatous tissues, casting doubt on the reliability of IgG titers for predicting the presence of the pathogen within CNV [8].

The hallmark of chlamydial infections is their capacity to cause chronic inflammation, and *C. pneumoniae* is equipped with several virulence determinants that allow the pathogen to cause such inflammation [22]. *C. pneumoniae* can establish persistent infection [3], induce pro-inflammatory cytokines [25], dysregulate host-cell lipid metabolism [19, 21, 24], and modulate apoptotic pathways [6] in several cell types. Many of these mechanisms may promote AMD and atherosclerosis, two chronic, age-related diseases mediated in part by inflammation [1, 35]. The observation that *C. pneumoniae* can infect RPE cells (Fig. 3) and macrophages [23], cell types central to the pathogenesis of AMD, itself indicates that *C. pneumoniae* may dysregulate their function to promote AMD. Indeed, *C. pneumoniae* was found to induce key angiogenic cytokines by ARPE-19 cells and macrophages in a dose- and time-dependent manner (Figs. 4 and 5). One of these cytokines, VEGF, has been localized to surgically extracted CNV from AMD patients [31]. Furthermore, adenovirus-mediated overexpression of VEGF in the retina is sufficient to induce CNV in animal models [2], and treatment with anti-VEGF drugs dramatically reduces angiogenesis and vascular leakage in animal models of AMD [2]. Bacterial lipopolysaccharide (LPS) can induce VEGF by human macrophages through CD14/NFκβ-mediated activation; however, chlamydial LPS contains a modified lipid A moiety [36], which predicts that it should have low biologic activity. The observed dramatic increase in macrophage VEGF post-infection suggests that virulence determinants other than cLPS may mediate VEGF secretion. Future experiments will determine which chlamydial virulence determinants mediate the induction of VEGF by human macrophages.

It is not yet known whether *C. pneumoniae* can induce VEGF in vivo to levels sufficient to cause angiogenesis; however, the finding that a pathogen capable of inducing pro-angiogenic factors can be detected within AMD CNV does implicate *C. pneumoniae* in the pathogenesis of AMD. Importantly, it is also not clear whether the pathogen initiates inflammation within choroidal tissues to cause CNV de novo; an alternative explanation is that *C. pneumoniae* is delivered to CNV by infected monocytes that are recruited to the lesion by inflammatory stimuli.

Such questions must be addressed to strengthen the association between *C. pneumoniae* infection and AMD, and require additional pathology-based, animal model, and cell culture studies. For example, future IHC studies will use multiple monoclonal antibodies to localize chlamydial antigens to specific CNV cell types and to determine whether pathogen-laden CNV has increased expression of VEGF compared with uninfected CNV. Unfortunately, such studies are challenging in light of the difficulty in obtaining primary AMD CNV tissue, the scarcity of donor eyes available for AMD research from eye banks, and the unavailability of suitable AMD animal models capable of supporting persistent chlamydial infection. Nevertheless, such studies are needed to establish a definitive role for infection in AMD. If additional work supports the association, then human antibiotic treatment trials may determine a role for anti-infective therapy in AMD patients.

Acknowledgements We thank the New England Eye Bank for its generous gift of whole eye tissue, Gerry Byrne and Ted Dryja for reviewing the manuscript, and Ed Connolly, Holly Goolsby, and Cheryl Greene for their technical expertise and help. This work was supported by an institutional grant from Research to Prevent Blindness (Massachusetts Eye and Ear Infirmary).

References

1. Anderson DH, Mullins RF, Hageman GS, Johnson LV (2002) A role for local inflammation in the formation of drusen in the aging eye. Am J Ophthalmol 134:411–431
2. Baffi J, Byrnes G, Chan CC, Csaky KG (2000) Choroidal neovascularization in the rat induced by adenovirus mediated expression of vascular endothelial growth factor. Invest Ophthalmol Vis Sci 41:3582–3589
3. Beatty WL, Byrne GI, Morrison RP (1993) Morphologic and antigenic characterization of interferon gamma-mediated persistent *Chlamydia trachomatis* infection in vitro. Proc Natl Acad Sci U S A 90:3998–4002
4. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, Strieter RM (2000) CXC chemokines in angiogenesis. J Leukoc Biol 68:1–8
5. Bonin S, Petrera F, Niccolini B, Stanta G (2003) PCR analysis in archival postmortem tissues. Mol Pathol 56:184–186
6. Carratelli CR, Rizzo A, Catania MR, Galle F, Losi E, Hasty DL, Rossano F (2002) *Chlamydia pneumoniae* infections prevent the programmed cell death on THP-1 cell line. FEMS Microbiol Lett 215:69–74
7. Danesh J,Collins R, Peto R (1997) Chronic infections and coronary heart disease: is there a link? Lancet 350:430–436
8. Danesh J, Whincup P, Walker M, Lennon L, Thomson A, Appleby P, Wong Y, Bernardes-Silva M, Ward M (2000) *Chlamydia pneumoniae* IgG titres and coronary heart disease: prospective study and meta-analysis. BMJ 321:208–213
9. Eckhart L, Bach J, Ban J, Tschachler E (2000) Melanin binds reversibly to thermostable DNA polymerase and inhibits its activity. Biochem Biophys Res Commun 271:726–730
10. Eyetech Study Group (2003) Anti-vascular endothelial growth factor therapy for subfoveal choroidal neovascularization secondary to age-related macular degeneration: phase II study results. Ophthalmology 110:979–986
11. Ferrara N, Gerber HP, LeCouter J (2003) The biology of VEGF and its receptors. Nat Med 9:669–676
12. Fine SL, Berger JW, Maguire MG, Ho AC (2000) Age-related macular degeneration. N Engl J Med 342:483–492
13. Frank RN, Amin RH, Eliott D, Puklin JE, Abrams GW (1996) Basic fibroblast growth factor and vascular endothelial growth factor are present in epiretinal and choroidal neovascular membranes. Am J Ophthalmol 122:393–403
14. Gottlieb JL (2002) Age-related macular degeneration. JAMA 288:2233–2236
15. Hattenbach LO, Falk B, Nurnberger F, Koch FH, Ohrloff C (2002) Detection of inducible nitric oxide synthase and vascular endothelial growth factor in choroidal neovascular membranes. Ophthalmologica 216:209–214
16. Higgins GT, Wang JH, Dockery P, Cleary PE, Redmond HP (2003) Induction of angiogenic cytokine expression in cultured RPE by ingestion of oxidized photoreceptor outer segments. Invest Ophthalmol Vis Sci 44:1775–1782
17. Ishida O, Oku H, Ikeda T, Nishimura M, Kawagoe K, Nakamura K (2003) Is *Chlamydia pneumoniae* infection a risk factor for age related macular degeneration? Br J Ophthalmol 87:523–524
18. Kalayoglu MV, Byrne GI (2001) *Chlamydia*. In: Dworkin et al (eds) Prokaryotes. Springer, Berlin Heidelberg New York, http://www.link.springer-ny.com.ezp2.harvard.edu/link/service/books/10125/
19. Kalayoglu MV, Byrne GI (1998) Induction of macrophage foam cell formation by *Chlamydia pneumoniae*. J Infect Dis 177:725–729
20. Kalayoglu MV, Galvan C, Mahdi OS, Byrne GI, Mansour S (2003) Serological association between *Chlamydia pneumoniae* infection and age-related macular degeneration. Arch Ophthalmol 121:478–482
21. Kalayoglu MV, Hoerneman B, LaVerda D, Morrison SG, Morrison RP, Byrne GI (1999) Cellular oxidation of low-density lipoprotein by *Chlamydia pneumoniae*. J Infect Dis 180:780–790
22. Kalayoglu MV, Indrawati, Morrison RP, Morrison SG, Yuan Y, Byrne GI (2000) Chlamydial virulence determinants in atherogenesis: the role of chlamydial lipopolysaccharide and heat shock protein 60 in macrophage-lipoprotein interactions. J Infect Dis 181 (Suppl 3):S483–S489
23. Kalayoglu MV, Libby P, Byrne GI (2002) *Chlamydia pneumoniae* as an emerging risk factor in cardiovascular disease. JAMA 288:2724–2731
24. Kalayoglu MV, Miranpuri GS, Golenbock DT, Byrne GI (1999) Characterization of low-density lipoprotein uptake by murine macrophages exposed to *Chlamydia pneumoniae*. Microbes Infect 1:409–418
25. Kaukoranta-Tolvanen SS, Teppo AM, Laitinen K, Saikku P, Linnavuori K, Leinonen M (1996) Growth of *Chlamydia pneumoniae* in cultured human peripheral blood mononuclear cells and induction of a cytokine response. Microb Pathog 21:215–221
26. Kociok N, Heppekausen H, Schraermeyer U, Esser P, Thumann G, Grisanti S, Heimann K (1998) The mRNA expression of cytokines and their receptors in cultured iris pigment epithelial cells: a comparison with retinal pigment epithelial cells. Exp Eye Res 67:237–250
27. Krzystolik MG, Afshari MA, Adamis AP, Gaudreault J, Gragoudas ES, Michaud NA, Li W, Connolly E, O'Neill CA, Miller JW (2002) Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 120:338–346
28. Kuo C, Campbell LA (2000) Detection of *Chlamydia pneumoniae* in arterial tissues. J Infect Dis 181(Suppl 3): S432–S436
29. Kuo CC (1999) Pathologic manifestation of chlamydial infection. Am Heart J 138:S496–S499
30. Lambert HM, Lopez PF (1993) Surgical excision of subfoveal choroidal neovascular membranes. Curr Opin Ophthalmol 4:19–24
31. Lopez PF, Sippy BD, Lambert HM, Thach AB, Hinton DR (1996) Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related choroidal neovascular membranes. Invest Ophthalmol Vis Sci 37:855–868
32. Lutty GA, McLeod DS, Merges C, Diggs A, Plouet J (1996) Localization of vascular endothelial growth factor in human retina and choroid. Arch Ophthalmol 114:971–977
33. Miller JW, Adamis AP, Shima DT, D'Amore PA, Moulton RS, O'Reilly MS, Folkman J, Dvorak HF, Brown LF, Berse B et al (1994) Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. Am J Pathol 145:574–584

34. Relman DA, Schmidt TM, MacDermott RP, Falkow S (1992) Identification of the uncultured bacillus of Whipple's disease. N Engl J Med 327:293–301
35. Ross R (1999) Atherosclerosis—an inflammatory disease. N Engl J Med 340:115–126
36. Rund S, Lindner B, Brade H, Holst O (1999) Structural analysis of the lipopolysaccharide from *Chlamydia trachomatis* serotype L2. J Biol Chem 274:16819–16824
37. Salcedo R, Ponce ML, Young HA, Wasserman K, Ward JM, Kleinman HK, Oppenheim JJ, Murphy WJ (2000) Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. Blood 96:34–40
38. Spilsbury K, Garrett KL, Shen WY, Constable IJ, Rakoczy PE (2000) Overexpression of vascular endothelial growth factor (VEGF) in the retinal pigment epithelium leads to the development of choroidal neovascularization. Am J Pathol 157:135–144
39. Tong CY, Sillis M (1993) Detection of *Chlamydia pneumoniae* and *Chlamydia psittaci* in sputum samples by PCR. J Clin Pathol 46:313–317
40. Wada M, Ogata N, Otsuji T, Uyama M (1999) Expression of vascular endothelial growth factor and its receptor (KDR/flk-1) mRNA in experimental choroidal neovascularization. Curr Eye Res 18:203–213
41. Wiegand P, Domhover J, Brinkmann B (1996) DNA degradation in formalin fixed tissues. Pathologe 17:451–454
42. Wu L, Patten N, Yamashiro CT, Chui B (2002) Extraction and amplification of DNA from formalin-fixed, paraffin-embedded tissues. Appl Immunohistochem Mol Morphol 10:269–274
43. Yu MJ, Shen WY, Lai MC, Constable IJ, Papadimitriou JM, Rakoczy PE (2000) The role of vascular endothelial growth factor (VEGF) in abnormal vascular changes in the adult rat eye. Growth Factors 17:301–312