

BASIC SCIENCE

Effects of MF-Tricyclic, a Selective Cyclooxygenase-2 Inhibitor, on Atherosclerosis Progression and Susceptibility to Cytomegalovirus Replication in Apolipoprotein-E Knockout Mice

David Rott, MD, Jianhui Zhu, MD, PhD, Mary Susan Burnett, PhD, Yi Fu Zhou, MD, Alexandra Zalles-Ganley, BS, Jibike Ogunmakina, BS, Stephen E. Epstein, MD
Washington, DC

OBJECTIVES	We examined whether selective cyclooxygenase-2 (COX-2) inhibition in apolipoprotein-E (apoE) deficient mice reduces cytomegalovirus (CMV) replication, and determined whether COX-2 anti-inflammatory activity leads to decreased atherosclerosis.
BACKGROUND	Evidence suggests that CMV infection contributes to atherosclerosis and that this occurs in part through inflammatory mechanisms. Cyclooxygenase-2 inhibitors are potent anti-inflammatory agents. They also inhibit CMV replication in vitro.
METHODS	The apoE deficient mice were either treated or not treated with a selective COX-2 inhibitor, and either infected or not infected with CMV. Viral deoxyribonucleic acid load in salivary glands was determined by quantitative polymerase chain reaction. Atherosclerotic lesion analysis was performed by standard methods.
RESULTS	In vivo COX-2 inhibition, unexpectedly increased viral load: in the CMV-infected animals viral load was 2.58 ± 1.0 in the nontreated group, 4.74 ± 1.38 in the group treated with 12 mg/kg/day MF-tricyclic, and 6.51 ± 1.64 in the group treated with 24 mg/kg/day MF-tricyclic (p trend = 0.050). This increased viral load was paralleled by increased anti-CMV antibody titers. Most surprisingly, COX-2 inhibition significantly increased early atherosclerotic lesion area, independent of viral infection.
CONCLUSIONS	Our study demonstrates that selective inhibition of COX-2 in vivo increases viral load. The finding that inhibition of COX-2 increases atherosclerosis development in apoE deficient mice suggests, unexpectedly, that this enzyme exerts antiatherosclerosis activity, at least in this model. (J Am Coll Cardiol 2003;41:1812-9) © 2003 by the American College of Cardiology Foundation

Evidence suggests that infection with any of several pathogens, including cytomegalovirus (CMV), contributes to atherosclerosis (1-3), and that this occurs in part through inflammatory mechanisms (1,2). Strong evidence suggesting a causal role of CMV in atherogenesis is provided by the demonstration that CMV infection causes atherosclerosis progression in a murine model of atherosclerosis (4-6).

See page 1820

We previously demonstrated that CMV infection of human coronary artery smooth muscle cells (SMCs) generates intracellular reactive oxygen species (ROS) (7). This is undoubtedly part of the host's defense response, as ROS are essential for nuclear translocation of nuclear factor kappa-B (NFκB), where it transactivates many genes involved in inflammatory and immune responses (8). However, CMV also requires NFκB for transactivating its own genes. Its

major immediate early promoter has four NFκB binding sites that, when activated by NFκB, initiate expression of the downstream viral genes essential for viral replication (9).

We further explored the pathway by which CMV generates ROS. We found that CMV activates a pertussis toxin-sensitive G-protein-coupled cascade, leading to a cyclooxygenase-2 (COX-2)-dependent increase in ROS through arachidonic acid release and metabolism via mitogen-activated protein kinase-dependent stimulation of phospholipase A₂ (10). These studies also confirmed the importance of the cyclooxygenase system in the viral pathway of cell activation. Thus, COX-2 inhibition, either with the nonselective COX inhibitors aspirin and indomethacin, or with the selective COX-2 inhibitor NS-398, diminished CMV-induced ROS generation, thereby preventing NFκB activation (11). Moreover, aspirin prevented in vitro CMV replication in human coronary artery SMCs (11). Therefore, we proposed that COX-2 might be a therapeutic target: that its inhibition would diminish viral replication in vivo, thereby inhibiting any contribution of the virus to atherogenesis.

The COX-2 inhibitory drugs are also potent anti-inflammatory agents (12). Because inflammation plays a

From the Cardiovascular Research Institute, Medstar Research Institute, Washington Hospital Center, Washington, DC. This work was supported in part by a grant from Merck Pharmaceuticals.

Manuscript received July 3, 2002; revised manuscript received January 8, 2003, accepted January 16, 2003.

Abbreviations and Acronyms

- apoE = apolipoprotein-E
- CMV = cytomegalovirus
- COX = cyclooxygenase
- DNA = deoxyribonucleic acid
- IFN γ = interferon-gamma
- IgG = immunoglobulin-G
- IL = interleukin
- NF κ B = nuclear factor kappa-B
- PCR = polymerase chain reaction
- PG = prostaglandin
- ROS = reactive oxygen species
- SMCs = smooth muscle cells
- TNF α = tumor necrosis factor-alpha

central role in atherogenesis (13), a compelling case can be made for such drugs inhibiting atherosclerosis progression independent of any antiviral effect. The purpose of the present investigation was to determine in a mouse model of atherosclerosis the validity of two hypotheses: 1) selective COX-2 inhibition, through the mechanisms responsible for its *in vitro* antiviral effects, reduces CMV replication *in vivo*; and 2) selective COX-2 inhibition, through its anti-inflammatory activity, decreases atherosclerosis development independent of infection.

METHODS

Animals and CMV infection. The study protocol was approved by the Medstar Research Institute Animal Care and Use Committee, in accordance with the guide for the care and use of laboratory animals (NIH publication No. 85-23, revised 1996).

The apolipoprotein-E (apoE) deficient mice were purchased from Jackson Laboratory (Bar Harbor, Maine). All mice were housed in microisolator cages in a pathogen-free facility and allocated into six groups (Table 1). Mouse CMV (Smith strain, VR-1399, American Type Culture Collection [ATCC], Manassas, Virginia) propagated in mouse fibroblast SC-1 cells (ATCC). Eight week-old mice (groups 3 to 6) were injected intraperitoneally either with 5×10^4 PFU mouse CMV or with the supernatant of uninfected SC-1 cells (ATCC). Injections were given 48 h after initiation of the feeding protocol. Mice allocated to groups 1 and 2 did not receive the virus. At 11 weeks of age salivary

glands, spleens, and blood were collected and serum was obtained. Samples were stored at -80°C .

Drug. We used the selective COX-2 inhibitor MF-tricyclic [3-(3, 4-difluorophenyl)-4-(4-(methylsulfonyl)phenyl)-2-(5H)-furanone]. This drug has no significant anti-COX-1 activity at clinically effective concentrations (14). Drug-free chow and sterile mixture of drug in mouse chow were obtained from Merck Pharmaceuticals (Merck Frosst Canada and Co., Kirkland, Canada) in two drug concentrations: 0.01334% (w/w) and 0.0067% (w/w); fresh chow was administered daily.

Mice were fed *ad lib* with drug-containing chow or control chow. Mice in all six groups were started on this feeding protocol simultaneously. The feeding protocol was continued for three weeks, when mice were sacrificed. Food intake and body weight were monitored weekly and the drug doses calculated accordingly. Serum samples were shipped to Merck for drug levels testing.

Polymerase chain reaction (PCR) analysis. Samples were coded so that the analysis was done blindly as to sample source.

GENOMIC DEOXYRIBONUCLEIC ACID (DNA) EXTRACTION. DNA was isolated from mouse salivary glands (Genra Systems, Minneapolis, Minnesota).

TAQMAN ANALYSIS. Target gene—mouse CMV IE1 exon 4, accession number M11788, TaqMan probe and primers were selected using Primer Express software (PerkinElmer Life Sciences Inc. Beltsville, Maryland) and were synthesized at BioServe Biotechnologies Ltd. (Laurel, Maryland). Primer and probe optimizations were performed at 60°C . Mouse CMV (TET-labeled probe) and apoB (6FAM-labeled probe) constitutive gene were analyzed alone. All TaqMan reactions were performed in $15 \mu\text{l}$ volumes using $2 \times$ Universal Master Mix (PerkinElmer), 6.25 pmol of each primer, 6.25 pmol of mouse CMV probe, and $1 \mu\text{l}$ of 100 ng/ μl DNA. The “real time” amplification program consisted of 50°C for 2 min (AmpErase UNG cleavage step), 95°C for 10 min (activation of AmpliTaq Gold step), and then 40 cycles of 95°C for 15 s followed by 60°C for 1 min.

A standard curve was generated using 10-fold dilutions of SC-1 cells (ATCC) infected with mouse CMV. The upper and lower limits of DNA concentrations used were 100 ng/ μl and 100 fg/ μl .

Table 1. Mice Allocation

Group (n)	Inoculum	Drug (mg/kg/day)	PCR (n)	Serology (n)	Lesion Analysis (n)
1 (16)	None	None	None	None	11
2 (16)	None	12	None	None	11
3 (17)	Sham	None	17	17	12
4 (17)	CMV	None	17	17	12
5 (17)	CMV	12	17	17	12
6 (17)	CMV	24	17	17	12

CMV = cytomegalovirus; PCR = polymerase chain reaction.

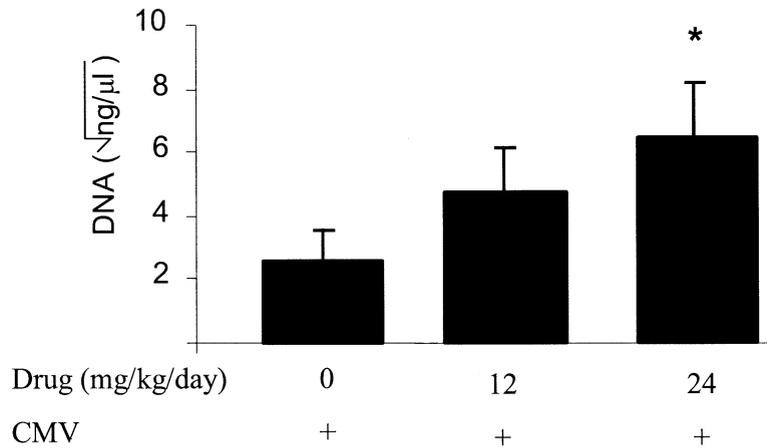


Figure 1. Mouse cytomegalovirus (CMV) deoxyribonucleic acid (DNA) concentration in mouse salivary glands. This value (which underwent square root transformation before statistical analysis) was taken as a polymerase chain reaction (PCR)-derived index of the number of viral particles present in the tissue. The results show a dose-response curve, with highest drug level associated with highest viral load. All sham-infected mice were negative for CMV DNA. The gene targeted for PCR amplification was mouse CMV IE1 exon 4. * $p = 0.05$ vs. no drug treatment group.

Serology. Anti-mouse CMV immunoglobulin G (IgG) antibody titer was determined in mouse serum by using an anti-mouse CMV IgG enzyme-linked immunosorbent assay kit (Charles River Laboratories, Wilmington, Massachusetts). The presence or absence of anti-CMV IgG was determined by the value (optical density sample – optical density issue control) divided by 0.13. A value above 3 was regarded as positive. The positive sample was further diluted to 1:60, 1:500, 1:1,000, 1:2,000, and 1:3,000. The antibody titer was considered to be the highest dilution at which the sample was reading positive.

Cytokine concentrations. Interferon-gamma (IFN γ), tumor necrosis factor-alpha (TNF α), and interleukin-6 (IL-6) concentrations in mouse serum were determined by using mouse ELISA kits (Biosource International, Camarillo, California). All assays were performed in duplicate. Minimal detectable levels of IL-6, IFN γ , and TNF α were <3.0, <1.0, and <3.0 pg/ml, respectively.

Quantification of atherosclerotic lesions. Hearts were coded so analysis was performed blindly. Lesions were analyzed as described (15). Hearts were fixed in 10% buffered formalin and embedded in 5%, 10%, and then 25% gelatin. Frozen sections (10- μ m) of the aortic sinus were cut and then stained using oil red O and hematoxylin and counterstained with light green. Five sections per mouse were analyzed for lesions. Area analysis was completed using Spot ImagePro Driver software (Diagnostic Instruments).

Cholesterol levels. Serum samples, collected at time of killing, were individually evaluated for cholesterol by Cholesterol-SL-Assay, a 4-aminoantipyrine-based enzymatic assay (Diagnostic Chemical Limited, Oxford, Connecticut).

Statistical analysis. Data are given as mean \pm SEM. Comparisons between multiple experimental groups were made by one-way analysis of variance or Kruskal-Wallis test, as appropriate. Comparisons between two experimental groups were made by Student t test (two-tailed). Values of

$p \leq 0.05$ were considered statistically significant. Polymerase chain reaction results underwent square-root transformation prior to statistical analysis.

RESULTS

Drug consumption and serum level measurements.

Average mouse weight was 20 g, and average chow consumed was 3.6 g/day/mouse. Hence, doses consumed were 24 mg/kg/day for group 6 and 12 mg/kg/day for groups 2 and 5 (Table 1). The corresponding mean serum drug levels (μ g/ml) were 2.09 ± 0.09 versus 1.03 ± 0.15 ($p < 0.001$) in these groups, respectively. Sera of mice from untreated groups were negative for drug.

Cholesterol levels. The total serum cholesterol levels did not differ significantly among the six groups. Mean cholesterol levels (mg/dl) were: 365.9 ± 21.0 , 421.8 ± 32.0 , 451.9 ± 35.6 , 390.1 ± 41.2 , 436.9 ± 79.1 , and 480.8 ± 23.1 in groups 1 to 6, respectively.

Detection of CMV DNA by PCR. As shown in Figure 1, the mean mouse CMV DNA in salivary glands, a PCR-derived index of the number of viral particles present in the tissue, was: 2.58 ± 1.0 in the CMV-infected/no drug group, 4.74 ± 1.38 in the CMV-infected/12 mg/kg dose group, and 6.51 ± 1.64 in the CMV-infected/24 mg/kg dose treatment group (p trend = 0.050). A dose-response curve was evident; highest drug level was associated with highest viral load. Sham-infected mice were negative for CMV DNA (group 3) (Table 1). All positive and negative controls worked as expected, and all samples were positive when entered into a PCR with primers for the apoB gene, indicating adequate quality of DNA.

Mouse anti-CMV IgG antibody titers. Figure 2 shows mean anti CMV IgG antibody titers in serum were: 602.4 ± 93.4 in the CMV-infected/no drug group, $1,031.3 \pm 154.6$ in the CMV-infected/12 mg/kg dose group, and $1,264.7 \pm 232.7$ in the CMV-infected/24 mg/kg dose

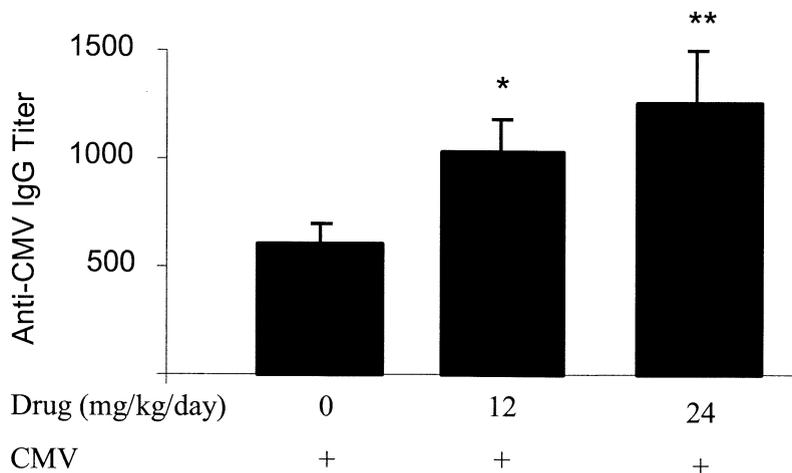


Figure 2. Anti-mouse cytomegalovirus (CMV) immunoglobulin G (IgG) antibody titer in mice sera. All mice were infected with the same concentration of virus. Cytomegalovirus antibody titers increased with increasing dose of the cyclooxygenase-2 inhibitor, compatible with a drug-induced increase in viral load seen in Figure 1. All sham-infected mice were negative for anti-CMV IgG antibodies. **p* = 0.025 vs. no drug treatment group; ***p* = 0.015 vs. no drug treatment group.

group (*p* trend = 0.025). Sham-infected mice were negative for anti-CMV IgG antibodies.

Quantification of atherosclerotic lesions. Analysis included 11 or 12 samples from each of the six groups (Table 1). Figure 3 shows mean lesion area (μm^2) was 6,096.5 \pm 1,837 in the noninfected/no-drug group, 11,215.8 \pm 1,369 in the noninfected/12 mg/kg dose group, 6,036.5 \pm 154 in the sham-infected/no-drug group, 6,947.8 \pm 1,166 in the CMV-infected/no-drug group, 11,785.7 \pm 1,512 in the infected/12 mg/kg dose group, and 12,514.2 \pm 1,252 in the infected/24 mg/kg dose group (*p* trend < 0.001). Drug treatment consistently increased lesion size: by 84% in the noninfected group (Fig. 4) and by 70% and by 80% in the CMV-infected low-dose and high-dose groups, respectively. Thus, significant differences were detected between each individual drug-treated group (groups 2, 5, and 6) (Table 1) when compared with each individual nontreated

group (groups 1, 3, and 4) (Table 1). In this study infection with CMV did not increase lesion size.

Cytokine concentrations. Interferon-gamma, TNF α , and IL-6 were undetectable in the serum of all six groups (data not shown). However, all controls worked as expected, indicating adequate test quality.

DISCUSSION

Previous investigations from our laboratory, described in the introduction, demonstrated that aspirin, a nonselective COX inhibitor, inhibits in vitro CMV replication in coronary artery SMCs by preventing CMV-induced ROS generation. This latter effect was also shared by indomethacin, another nonselective COX inhibitor, and by the selective COX-2 inhibitor NS-398 (11).

Cyclooxygenase-2, one of two isoforms of COX (16), is

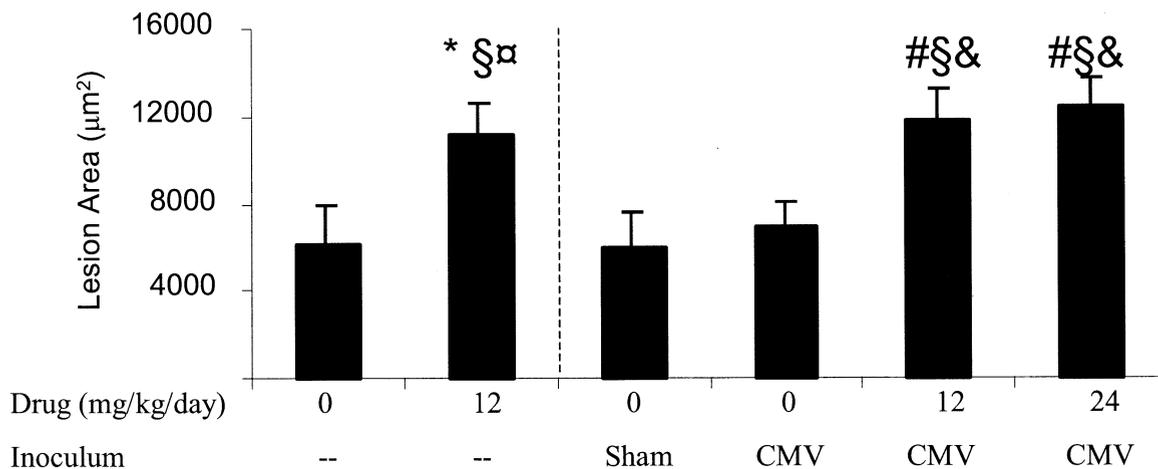
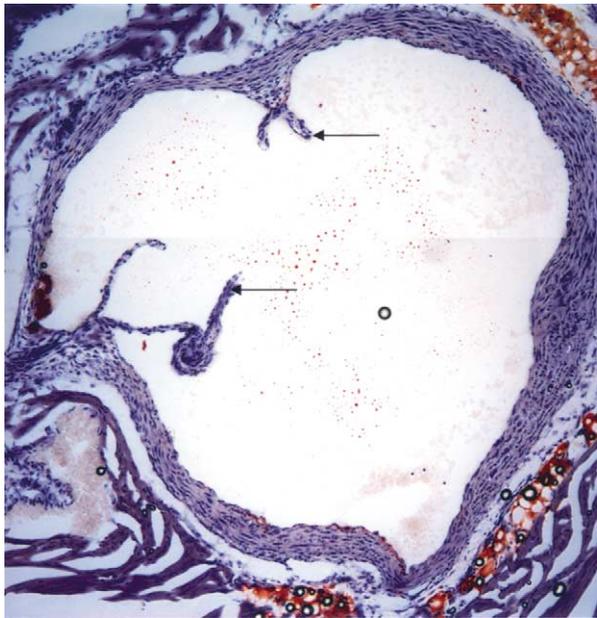
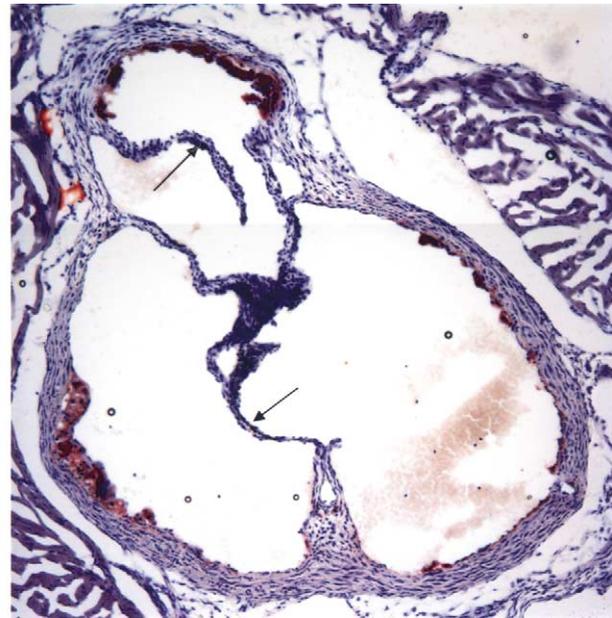


Figure 3. Atherosclerotic lesion area. Selective cyclooxygenase-2 inhibition consistently increased lesion size: by 84% in the noninfected group, and by 70% and by 80% in the cytomegalovirus (CMV)-infected low-dose and high-dose groups respectively. In this study, infection with CMV did not increase lesion size. **p* = 0.013 vs. no drug treatment/no inoculum group; #*p* < 0.01 vs. no drug treatment/no inoculum group; §*p* < 0.01 vs. no drug treatment/sham infection group; &*p* = 0.02 vs. no drug treatment group/CMV infection group; α*p* = 0.05 vs. no drug treatment group/CMV infection group.



A. Untreated



B. Treated with MF tricyclic

Figure 4. Oil red O staining of atherosclerotic lesions in the aorta. Representative photomicrographs ($\times 10$ magnification) of the lesions present in two uninfected mice. (A) Untreated with MF-tricyclic; (B) treated with MF-tricyclic, 12 mg/kg/day. The arrows point to some of the aortic valve leaflets, indicating that the sections employed for measuring lesion size are located, as is traditional, at the root of the aorta.

induced by mitogens, cytokines, certain inflammatory agents (17), and, as we showed, CMV infection (7,10,11). It is the isoform thought to promote inflammation. Cyclooxygenase-1 is constitutively expressed in most tissues and serves as the “housekeeping” isoform; its presence in platelets leads to platelet aggregation via COX-1-mediated thromboxane A_2 (17) production.

Because of the critical role of CMV-induced ROS generation in both viral and cellular gene expression, and because inflammation plays a critical role in atherogenesis, we considered that COX-2 might constitute a therapeutic target whereby its inhibition would decrease CMV replication *in vivo* and inhibit inflammatory changes that might contribute to atherogenesis. Therefore, the present investigation was initiated to determine the validity of two hypotheses: 1) selective COX-2 inhibition, through the mechanisms responsible for its *in vitro* antiviral effects, reduces CMV replication *in vivo*; and 2) selective COX-2 inhibition, through its anti-inflammatory activity, decreases atherosclerosis development independent of infection.

To our surprise, we found just the opposite. Regarding the first hypothesis, selective COX-2 inhibition with MF-tricyclic increased viral load in a dose-dependent fashion, with the highest dose increasing viral load by about 100% (Fig. 1). We also found a parallel dose-response effect of MF-tricyclic on anti-CMV antibody titers (Fig. 2), suggesting that the increase in viral load found in the treated animals represented a biologically relevant change.

In the experimental design we employed for this investigation, CMV infection itself had no effect on lesion size. Therefore, we were unable to determine whether selective

COX-2 inhibition diminishes CMV-induced increase in atherosclerosis development. In two separate studies we found that CMV infection increases lesion development in apoE knockout mice (4,6). The differences between this and the prior publications are undoubtedly due to the fact that in the present study we employed a brief three-week period between infection and sacrifice, whereas in the previous studies sacrifice occurred 14 weeks after infection. Three weeks appears too brief a time for any pro-atherosclerotic effects of CMV to become manifest.

The result of the study exploring the validity of the second hypothesis we examined was also unexpected. Lesion analysis showed that selective COX-2 inhibition with MF-tricyclic increases atherosclerotic lesion area (Figs. 3 and 4). It should be noted that lesions in this particular model, in which apoE knockout mice were sacrificed at only 11 weeks of age and after only 3 weeks on the selective COX-2 inhibitor, were very early lesions. Therefore, our results are probably more relevant to lesion initiation.

Selective COX-2 inhibition and CMV replication.

Although initially surprising, upon further reflection the contrast between *in vitro* and *in vivo* COX-2 inhibition effects should have been anticipated. The *in vitro* experiment consists of a simple system, one cell type and only two variables: 1) virus and 2) drug treatment. In this system, drug-induced selective COX-2 inhibition exerted a significant antiviral effect. The putative mechanisms, relating to inhibition of ROS generation, have been discussed. The *in vivo* situation, however, is considerably more complex, with multiple possible consequences of drug activity. For instance, COX-2 inhibition has, *in vivo*, potent anti-

inflammatory effects. These effects include hindering recruitment of neutrophils to sites of injury (18) and interfering with monocyte recruitment (19). Both neutrophils and monocytes, components of the innate immune response, function as the initial host defense response to the invading pathogen and contain infection until more efficient but slower adaptive immune responses develop. Cyclooxygenase-2 inhibition of the innate immune response probably contributes to increasing in vivo susceptibility to viral infection. In addition, COX-2 participates in the synthesis of cyclopentenone prostaglandins (PG) (20), potent inhibitors of viral replication (21). This COX-2 activity provides another possible mechanism for increased viral replication following COX-2 inhibition.

We are not familiar with any evidence that COX-2 inhibition alters the cellular component of the adaptive immune response. Our results suggest that COX-2 inhibition probably does not interfere with the humoral component, as we observed higher anti-CMV IgG titers of the mice treated with the COX-2 inhibitor, which paralleled their higher viral load.

Interferon-gamma was undetectable in the serum of all six groups in the current study. An earlier report by our group showed CMV induces IFN γ in mouse serum (22). In that study mice were infected at two weeks of age and sacrificed four weeks later. Because it is well known that immune responses of neonates evolve markedly over the first weeks and months of life, we can only speculate that two-week-old mice react differently to infection than do adult mice. Such age-related differences in responses could account for the different IFN γ responses to infection we observed between the two studies.

Selective COX-2 inhibition and atherosclerosis. The second unanticipated finding of our study was that in contrast to our hypothesis, selective COX-2 inhibition actually *increased* early lipid aortic accumulation in the apoE knockout mice. This raises a major conundrum: why should an anti-inflammatory drug *accelerate* the initiation of atherosclerosis, a disease recognized as having a major inflammatory component?

Recent studies have demonstrated a complex role for COX-2 in inflammation, in that the enzyme appears to possess not only its well-known inflammatory activity, but also *anti-inflammatory* properties (23-25). This latter activity resides in a previously unrecognized role of COX-2 in the resolution phase of the inflammatory response.

Some mechanistic insight relevant to these studies is provided by the investigation of Gilroy et al. (26). They showed in a rat model of inflammation that there are two phases of COX-2 expression: an early phase associated with high levels of PGE₂ synthesis and a later phase associated with a second peak that, paradoxically, coincides with inflammatory *resolution*. This second peak is associated with minimal PGE₂ synthesis and high levels of both PGD₂ and PGJ₂.

Compatible with this concept of dual time-delayed peaks

of COX-2 that exert opposite effects on inflammation are the findings that the selective COX-2 inhibitor NS-398 and the nonselective COX inhibitor indomethacin inhibit inflammation when given at the early phase of inflammation, but exacerbate inflammation when given at the late phase (20). Consequently, Colville-Nash and Gilroy (27) suggested that COX-2 is pro-inflammatory early in inflammation, but facilitates inflammatory resolution later.

These findings, suggesting a dual role for COX-2 in inflammation and inflammation resolution, have been demonstrated only in rodent models, and no evidence is yet available that such a dual role is present in humans. Nonetheless, this concept may explain our unexpected results. The ApoE deficient mice develop spontaneous atherosclerosis. Significant inflammatory changes in the aorta are detected at five to six weeks of age (28). At eight weeks (the age of our mice at the beginning of the study) there is well-established inflammation of the arterial wall. We initiated selective COX-2 inhibition at a time of ongoing vessel wall inflammation, a time that COX-2, according to this concept, is anti-inflammatory and, therefore, when its inhibition would exacerbate atherogenesis.

Recently, studies in mice suggested that selective COX-2 inhibition, through inhibition of PGI₂ activity, may exert pro-proliferative vascular effects. Thus, Cheng et al. (29) demonstrated that mice genetically deficient in the PGI₂ receptor exhibit an increased proliferative response to vascular injury, an effect that could contribute to a gradual increase in atherosclerotic lesion size through enhanced proliferation of vascular SMCs.

Potential deleterious effects of selective COX-2 inhibition on the course of atherosclerosis *in humans* were suggested by the demonstration in patients with rheumatoid arthritis that rofecoxib, a selective COX-2 inhibitor, was associated with a higher number of acute coronary events than occurred in patients treated with naproxen, a nonselective COX inhibitor (30). Moreover, a meta-analysis (with all the limitations inherent in such an analysis) of several published studies employing selective COX-2 inhibitors suggested that such treatment increased the incidence of myocardial infarction (31). Another examination of the same issue concluded that whereas there was a significant increase in cardiovascular events when rofecoxib was compared with naproxen, there was no evidence of excess cardiovascular events for rofecoxib relative to either placebo or to non-naproxen nonselective COX inhibitors (32).

The mechanisms responsible for precipitation of acute coronary events derive from plaque instability and plaque rupture. Following rupture, the highly thrombogenic plaque core is exposed to blood within the vessel lumen, resulting in platelet aggregation, thrombus formation, and acute vascular occlusion (13). Thus, any deleterious effects of selective COX-2 inhibitors on the course of atherosclerosis could involve three separate processes: 1) a *proinflammatory effect* that accelerates the initiation and chronic progression of the atherogenesis process, and that may induce plaque instabil-

ity and rupture; 2) a *procoagulant effect* (induced by inhibition of COX-2-mediated expression of the highly anti-thrombotic PGI₂ without concomitant reduction of COX-1-mediated expression of the highly thrombotic thromboxane A₂ (31) that precipitates an acute coronary syndrome; and 3) a *pro-proliferative effect* that could increase lesion size through accumulation of SMCs.

Of additional relevance is the finding that indomethacin, a nonselective COX inhibitor, significantly *reduced* atherosclerosis in mice (33). Indomethacin is more potent in inhibiting COX-1 compared with COX-2 (34). Therefore, the data taken in their entirety, suggest that COX-1 has pro-atherosclerotic activity, whereas COX-2 is not pro-atherosclerotic and may even have a protective effect; conversely, inhibitors of COX-1 appear to protect against atherosclerosis, and selective inhibitors of COX-2 may have deleterious consequences.

These concepts, however, must still be considered hypotheses to be validated, as other data relating to selective COX-2 inhibition suggest possible anti-atherogenesis effects (through inhibition of inflammation) and a plaque stabilization effect (through inhibition of PGE₂-dependent matrix metalloproteinase expression by macrophages within plaques) (35). Moreover, our results may be model-, therapy duration-, and drug-specific. For example, Pratico et al. (33) used an low-density lipoprotein receptor knockout mouse, employed the selective COX-2 inhibitor nimesulide, and sacrificed mice at 26 weeks of age compared with our 11 weeks of age; they found the COX-2 inhibitor exerted no effect on the rate of atherogenesis. Although these caveats relating to the conclusions of our investigation must be acknowledged, the important fact emerging from our study is that a selective COX-2 inhibitor, under the experimental conditions we studied, can increase early atherosclerosis lesion formation.

In conclusion, the results of this investigation raise disturbing questions about possible adverse effects deriving from chronic use of selective COX-2 inhibitors on susceptibility to viral infection and on early atherosclerosis lesion development. However, extrapolation of our results obtained in a mouse model to humans taking COX-2 inhibitors may not necessarily be valid. Moreover, the doses given to the mice resulted in serum levels that were at least four times higher (using the low dose of the COX-2 inhibitor, 12 mg/kg/day), on a molar basis, than those seen in patients receiving the usual clinical dose of rofecoxib (25 mg once daily).

Because of the importance of the implications of the conclusions deriving from our investigation, and because of the intrinsic limitations of an animal model outlined above, the conclusions must be regarded as tentative and hypothesis generating. Our results emphasize the critical need for more information regarding the relative biologic roles of both COX isoforms, and in particular their roles in inflammation, in resistance to infection, and in atherogenesis.

Acknowledgments

The authors would like to thank Ms. Pauline Luk from Merck Frosst Canada, and Mr. Michael Siddon from BioServe Biotechnologies, Ltd., Laurel, Maryland, for outstanding technical help.

Reprint requests and correspondence: Dr. Stephen E. Epstein, Cardiovascular Research Institute, Washington Hospital Center, 110 Irving Street NW, Suite 4B-1, Washington, DC 20010. E-mail: stephen.epstein@medstar.net.

REFERENCES

1. Epstein SE, Zhou YF, Zhu J. Infection and atherosclerosis: emerging mechanistic paradigms. *Circulation* 1999;100:20-8.
2. Zhu J, Quyyumi AA, Norman JE, et al. Cytomegalovirus in the pathogenesis of atherosclerosis: the role of inflammation as reflected by elevated C-reactive protein levels. *J Am Coll Cardiol* 1999;34:1738-43.
3. Thom DM, Grayston JT, Siscovitch DS, et al. Association of prior infection with *Chlamydia pneumoniae* and angiographically demonstrated coronary artery disease. *JAMA* 1992;268:68-72.
4. Burnett MS, Gaydos CA, Madico GE, et al. Atherosclerosis in apoE knockout mice infected with multiple pathogens. *J Infect Dis* 2001;183:226-31.
5. Berenci K, Endresz V, Klurfeld D, et al. Early atherosclerotic plaques in the aorta following cytomegalovirus infection of mice. *Cell Adhes Commun* 1998;5:39-47.
6. Hsich E, Zhou YF, Paigen B, et al. Cytomegalovirus infection increases development of atherosclerosis in Apolipoprotein-E knockout mice. *Atherosclerosis* 2001;156:23-8.
7. Speir E, Shibutani T, Yu ZX, et al. Role of reactive oxygen intermediates in cytomegalovirus gene expression and in response of human smooth muscle cells to viral infection. *Circ Res* 1996;79:1143-52.
8. Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J* 1991;10:2247-58.
9. Sambucetti LC, Cherrington JM, Wilokinson GW, Mocarski ES. NF-kappa B activation of the cytomegalovirus enhancer is mediated by a viral transactivator and by T cell stimulation. *EMBO J* 1998;8:4251-8.
10. Shibutani T, Johnson TM, Yu Z-X, Ferrans VJ, Moss J, Epstein SE. Pertussis toxin-sensitive G proteins as mediators of the signal transduction pathways activated by cytomegalovirus infection of smooth muscle cells. *J Clin Invest* 1997;100:2054-61.
11. Speir E, Yu ZX, Ferrans VJ, et al. Aspirin attenuates cytomegalovirus infectivity and gene expression mediated by cyclooxygenase-2 in coronary artery smooth muscle cells. *Circ Res* 1998;83:210-6.
12. Lefkowitz JB. Cyclooxygenase-2 specificity and its clinical implications. *Am J Med* 1999;106:43S-50S.
13. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999;340:115-26.
14. Black SC, Brideau C, Cirino M, et al. Differential effect of a selective cyclooxygenase-2 inhibitor versus indomethacin on renal blood flow in conscious volume-depleted dogs. *J Cardiovasc Pharmacol* 1998;32:686-94.
15. Paigen B, Morrow A, Holmes PA, et al. Quantitative assessment of atherosclerotic lesions in mice. *Atherosclerosis* 1987;68:231-40.
16. Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J Biol Chem* 1996;271:33157-60.
17. Smith WL, Dewitt DL. Prostaglandin endoperoxide H synthases-1 and -2. *Adv Immunol* 1996;62:167-215.
18. Wilgus TA, Ross MS, Parrett ML, et al. Topical application of a selective cyclooxygenase inhibitor suppresses UVB mediated cutaneous inflammation. *Prostaglandins Other Lipid Mediat* 2000;62:367-84.
19. Langenbach R, Loftin C, Lee C, Tian H. Cyclooxygenase knockout mice: models for elucidating isoform-specific functions. *Biochem Pharmacol* 1999;58:1237-46.

20. Gilroy DW, Colville-Nash PR, Willis D, et al. Inducible cyclooxygenase may have anti-inflammatory properties. *Nat Med* 1999;5:698-701.
21. Santoro MG. Antiviral activity of cyclopentenone prostanoids. *Trends Microbiol* 1997;5:276-81.
22. Rott D, Zhu J, Brunett MS, et al. Serum of cytomegalovirus infected mice induces monocyte chemoattractant protein-1 expression by endothelial cells. *J Infect Dis* 2001;184:1109-13.
23. Langenbach R, Loftin C, Lee C, et al. Cyclooxygenase knockout mice: models for elucidating isoform-specific functions. *Biochem Pharmacol* 1999;58:1237-46.
24. Morteau O. Prostaglandins and inflammation: the cyclooxygenase controversy. *Arch Immunol Ther Exp* 2000;48:473-80.
25. Smith WL, Langenbach R. Why there are two cyclooxygenase isoenzymes. *J Clin Inv* 2001;107:1491-5.
26. Gilroy DW, Tomlinson A, Willoughby DA. Differential effects of inhibitors of cyclooxygenase (cyclooxygenase 1 and cyclooxygenase 2) in acute inflammation. *Eur J Pharmacol* 1998;355:211-7.
27. Colville-Nash PR, Gilroy DW. COX-2 and the cyclopentenone prostaglandins—a new chapter in the book of inflammation? *Prostaglandins Other Lipid Mediat* 2000;62:33-43.
28. Breslow JL. Mouse models of atherosclerosis. *Science* 1996;272:685-8.
29. Cheng Y, Austin SC, Rocca B, et al. Role of prostacyclin in the cardiovascular response to thromboxane A₂. *Science* 2002;296:539-41.
30. Bombardier C, Laine L, Reicin A, et al. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. *N Engl J Med* 2000;343:1520-8.
31. Mukherjee D, Nissen SE, Topol EJ. Risk of cardiovascular events associated with selective COX-2 inhibitors. *JAMA* 2001;286:954-9.
32. Konstam MA, Weir MR, Reicin A, et al. Cardiovascular thrombotic events in controlled, clinical trials of rofecoxib. *Circulation* 2001;104:2280-8.
33. Pratico D, Tillmann C, Zhang ZB, et al. Acceleration of atherogenesis by COX-1-dependent prostanoid formation in low density lipoprotein receptor knockout mice. *Proc Natl Acad Sci USA* 2001;98:3358-63.
34. Mitchell JA, Akarasereenont P, Thiemermann C, et al. Selectivity of nonsteroidal anti-inflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. *Proc Natl Acad Sci USA* 1994;90:11693-7.
35. Cipollone F, Prontera C, Pini B, et al. Over expression of functionally coupled cyclooxygenase-2 and prostaglandin in symptomatic atherosclerotic plaques as a basis of prostaglandin E₂-dependent plaque instability. *Circulation* 2001;104:921-7.