Inhibition of cyclooxygenase 2 blocks human cytomegalovirus replication

Hua Zhu*, Jian-Ping Cong†, Deborah Yu†‡, Wade A. Bresnahan†§, and Thomas E. Shenk†¶

*Department of Microbiology and Molecular Genetics, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, Newark, NJ 07103; and †Department of Molecular Biology, Princeton University, Princeton, NJ 08544-1014

Contributed by Thomas E. Shenk, December 31, 2001

Cyclooxygenase 2 (COX-2) mRNA, protein, and activity are transiently induced after infection of human fibroblasts with human cytomegalovirus. Prostaglandin E_2, the product of COX-2 activity, is transiently increased by a factor of >50 in cultures of virus-infected fibroblasts. Both specific (BMS-279652, 279654, and 279655) and nonspecific (indomethacin) COX-2 inhibitors can abrogate the virus-mediated induction of prostaglandin E_2 accumulation. Levels of COX-2 inhibitors that completely block the induction of COX-2 activity, but do not compromise cell viability, reduce the yield of human cytomegalovirus in human fibroblasts by a factor of >100. Importantly, the yield of infectious virus can be substantially restored by the addition of prostaglandin E_2 together with the inhibitory drug. This finding argues that elevated levels of prostaglandin E_2 are required for efficient replication of human cytomegalovirus in fibroblasts. COX-2 inhibitors block the accumulation of immediate-early 2 mRNA and protein, but have little effect on the levels of immediate-early 1 mRNA and protein. Viral DNA replication and the accumulation of some, but not all, early and late mRNAs are substantially blocked by COX-2 inhibitors. Elevated levels of prostaglandin E_2 apparently facilitate the production of immediate-early 2 protein. The failure to produce normal levels of this critical viral regulatory protein in the presence of COX-2 inhibitors might block normal progression beyond the immediate-early phase of human cytomegalovirus infection.

Human cytomegalovirus (HCMV) is a ubiquitous member of the herpesvirus family. HCMV infection of healthy adults is generally subclinical. However, the virus is a major infectious cause of birth defects, and it is an adventitious pathogen that causes disease and mortality in immunocompromised individuals (1).

HCMV infection dramatically alters the steady state levels of many cellular mRNAs (2–4). The quantity of 1,425 cellular mRNAs, of ≈12,600 assayed, changed by a factor of ≥3 during the first 48 h after infection of human fibroblasts (3).

The mRNAs encoding several constituents of the pathway that produces prostaglandin H_2 (PGH_2) from arachidonic acid are among those modulated by HCMV infection (2, 3). The mRNAs encoding cytosolic phospholipase A2 (cPLA2) and cyclooxygenase 2 (COX-2) increase, and the mRNA encoding lipocortin 1, also known as annexin 1, decreases after infection. When activated by phosphorylation, cPLA2 cleaves and releases arachidonic acid from membranes (5, 6), which can then be converted by COX-2 to PGH_2. Lipocortin 1 binds to cPLA2 and inhibits its activation (7–9), blocking the release of arachidonic acid that can serve as a substrate for COX-2. If mRNA levels reflect enzymatic activity, then one would expect that the production of PGH_2 and its metabolites is strongly induced after HCMV infection. Consistent with this prediction, arachidonic acid release (10–12) and prostaglandin E_2 synthesis (PGE_2 is made from PGH_2 by PGE_2 synthase) are induced after infection of monocytes with HCMV (10, 13). Prostaglandins serve as second messages that elicit a wide range of physiological responses in cells and tissues. They have the potential to exert profound effects on HCMV replication and pathogenesis, given their ability to modulate gene expression and immune function.

A specific inhibitor of COX-2 has been shown to block the accumulation of PGE_2 after HCMV infection (13), and nonspecific inhibitors of the enzyme, i.e., compounds that affect COX-2, as well as other targets, reduce virus yields in cultured cells (13, 14). The mechanism for this inhibition is not clear, although PGE_2 has been found to stimulate the activity of the HCMV major immediate-early promoter in transfected THP-1 cells (15) and within HCMV-infected smooth muscle cells (13). The major immediate-early promoter controls the synthesis of viral regulatory proteins that are essential for HCMV replication (16).

Here, we demonstrate that the level of COX-2 protein and activity is dramatically induced after infection of human diploid fibroblasts with HCMV. The production of infectious progeny virus was reduced by a factor of >100 when cells were treated with a specific COX-2 inhibitor, and, importantly, virus replication was substantially restored when drug-blocked cultures were supplemented with PGE_2. This finding argues that the induction of COX-2 and synthesis of PGE_2 are essential for efficient HCMV replication in human fibroblasts. When COX-2 activity was blocked, many viral mRNAs and proteins, including the immediate-early 2 (IE2) transcriptional activator, failed to accumulate to normal levels, and viral DNA synthesis was substantially blocked.

Materials and Methods

Cells, Viruses, and Reagents. All cell culture experiments used primary human foreskin fibroblasts (HFFs), which were propagated in medium containing 10% FCS. Cell viability was tested by using the Cell Titer Aqueous One Solution cell Proliferation Assay (Promega) following the vendor's protocol. Infections were performed with human cytomegalovirus strain AD169 (17) at a multiplicity of 3 plaque-forming units (pfu) per cell, except for the analysis of virus growth kinetics where a multiplicity of 0.1 pfu per cell was used. Virus titers were determined by plaque assay on HFFs.

Three experimental small molecule inhibitors of COX-2 (BMS-279652, -279654, and -279655) were a generous gift from W. Koster (Bristol-Myers Squibb). These related, specific inhibitors and the nonspecific inhibitor, indomethacin (Sigma), were added to human foreskin fibroblast cultures immediately after mock-infection or infection. Cultures were refed with fresh medium plus COX-2 inhibitor every 24 h.

Assay for PGE_2. Arachidonic acid (30 nM) was added to the medium of cells to be assayed for PGE_2, and the culture was

Abbreviations: HCMV, human cytomegalovirus; PG, prostaglandin; COX, cyclooxygenase; IE, immediate-early; pfu, plaque-forming unit.

See commentary on page 3362.

‡Present Address: Georgetown University School of Medicine, Washington, DC 20007.

§Present Address: Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, TX 75390.

¶To whom reprint requests should be addressed. E-mail: tshenk@princeton.edu.

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incubated at 37°C for 15 min. PGE_2 levels were then assayed by using the Biotrak Prostaglandin E_2 Enzyme Immunoassay System (Amersham Pharmacia Biotech) according to the vendor's protocol.

Assays for viral DNA, mRNA, and Protein Accumulation. To monitor viral DNA accumulation, total DNA was prepared from infected cells, after denaturation portions were applied to a membrane by using a slot blot apparatus, and then the membrane-bound DNA was probed with ^{32}P-labeled DNA derived by random priming of the entire HCMV genome with a hexanucleotide primer mix and Exo$^-$ Klenow DNA polymerase (New England Biolabs). The intensities of bands were quantified by using a PhosphorImager (Molecular Dynamics).

To measure viral mRNA accumulation by Northern blot assay, total RNA was isolated from infected cells by using the TRIZOL reagent (GIBCO/Invitrogen). RNA was subjected to electrophoresis in a 1% agarose denaturing gel and blotted to a membrane. The membrane-bound RNAs were then probed with ^{32}P-labeled DNAs specific for individual ORFs that were prepared by random priming as described above. The intensities of bands were quantified by using a PhosphorImager. RNAs corresponding to all known HCMV ORFs were simultaneously assayed by using an HCMV gene array (18). Total RNA was prepared from infected cells, subjected to reverse transcription in the presence of [^{32}P]dCTP, and used to probe the gene array.

To measure protein accumulation by Western blot, cell lysates were subjected to electrophoresis in SDS-containing 8% polyacrylamide gels; proteins were transferred to a membrane and probed with specific antibodies that were then visualized by using the ECL system (Amersham Pharmacia Biotech). Antibodies to the following proteins were used: COX-1 (H3) and COX-2 (C-20) (Santa Cruz Biotechnology); IE1 + IE2 (MAB810) (Chemicon); pUL99 (M. Silva, P. Robinson, and T.S., unpublished results).

Results

HCMV Induces the Production of PGE_2 in Human Fibroblasts. A Northern blot experiment was performed after infection at a multiplicity of 3 pfu per cell to determine the kinetics of COX-2 mRNA induction by HCMV (Fig. 1*A*). Relatively little COX-2 mRNA was detected in mock-infected cells. After infection, an increased amount of the mRNA was evident at 1 h, and the induction continued until 8 h. Less COX-2 mRNA was present at the next time tested, 36 h, and mRNA levels had returned to the basal level at 48 h after infection. COX-2 mRNA was induced by one or more virion constituents because UV-irradiated virus, which is unable to express products from the viral genome (19), was nevertheless able to induce the cellular mRNA (Fig. 1*B*). Adenovirus infection did not induce the cellular mRNA (Fig. 1*B*), demonstrating that the induction is not a generic response to virus infection. A Western blot experiment (Fig. 1*C*) demonstrated that COX-2 protein was induced, whereas the level of the constitutively expressed COX-1 protein was not affected. Although COX-2 mRNA levels were transiently induced, COX-2 protein levels remained substantially induced at 48 h after infection. Apparently, the protein has a longer half-life than the mRNA. COX-2 activity was monitored by measuring the accumulation of PGE_2 (Fig. 1*D*). The product of COX-2 activity, PGH_2, is rapidly converted to PGE_2, which was quantified by enzyme immuno-assay. PGE_2 was strongly but transiently induced. A maximal induction of 50-fold was observed at 24 h after infection, but too few time points were assayed to know whether this was the peak of induction. In other experiments (Fig. 2*A*), a 250-fold induction was observed at 24 h after infection. By 72 h after infection, PGE_2 had returned to basal levels.

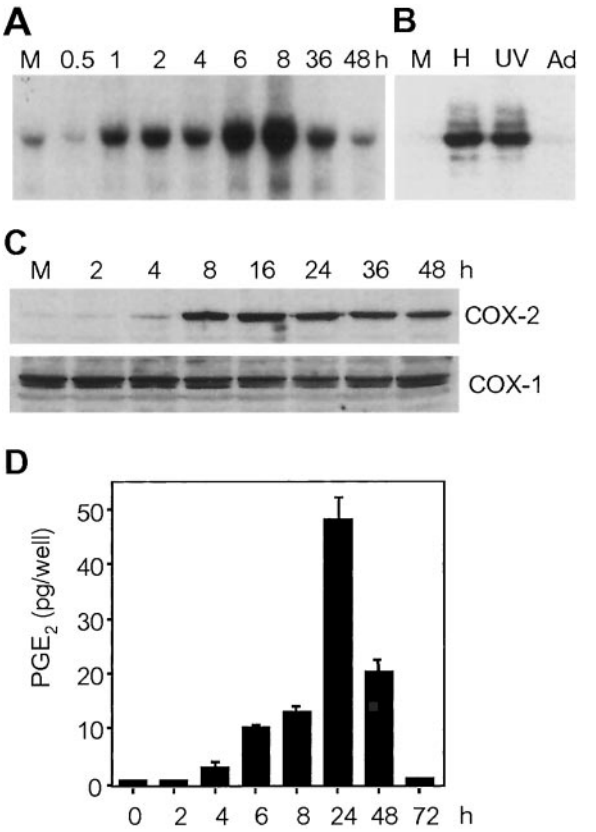

Fig. 1. Induction of COX-2 in HCMV-infected human fibroblasts. Infected cells were harvested at the times after infection (h, hours) indicated. (*A*) Northern blot assay of COX-2 mRNA accumulation after infection with HCMV. Total cellular mRNA was analyzed by using a COX-2-specific ^{32}P-labeled probe. (*B*) Northern blot assays of COX-2 mRNA in mock-infected (M), HCMV-infected (H), UV-inactivated HCMV-infected (UV), or adenovirus-infected (Ad) cells. (*C*) Western blot assay of COX-1 and 2 protein accumulation after infection with HCMV. Extracts were prepared and proteins assayed by using specific antibodies. (*D*) Enzyme immuno-assay of PGE_2 accumulation in cultures of infected cells.

COX-2 Inhibitors Block the Production of PGE_2 in HCMV-Infected Fibroblasts. Three structurally related, specific COX-2 inhibitors, BMS-279652, -279654, and -279655 (Fig. 2*A*), and a nonspecific inhibitor, indomethacin (Fig. 2*B*), were tested for their ability to block the accumulation of PGE_2 in response to infection. All four inhibitors were able to completely block the induction of PGE_2. To rule out the possibility that nonspecific toxicity caused by the drugs was responsible for the block to PGE_2 accumulation, cell viability was tested after drug treatment for 7 days by using a colorimetric assay (Fig. 2*C*). Little toxicity was observed after treatment with as much as 25 μg/ml BMS-279655 or 500 μM indomethacin. Cell viability was significantly compromised at higher concentrations of the drugs.

COX-2 Inhibitors Substantially Reduce the Production of Infectious HCMV Progeny. PGE_2 accumulation could be a host cell anti-viral response, a virus-induced alteration that is required for successful virus replication, or a neutral change that benefits neither the virus nor its infected host cell. To test the possibility that HCMV replication requires the induction of PGE_2, cells were treated immediately after infection with quantities of BMS-279654 (25 μg/ml) or -279655 (25 μg/ml) or indomethacin (500 μM) that completely blocked the virus-mediated induction of PGE_2 accumulation but had little effect on cell viability. Every 24 h, the culture medium was replaced with medium containing fresh

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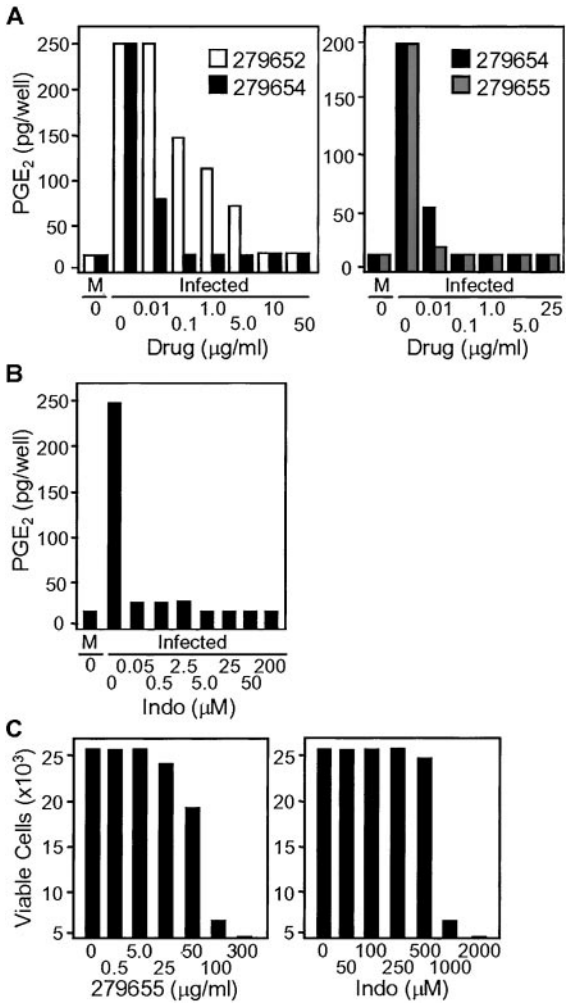

Fig. 2. COX-2 inhibitors block the induction of COX-2 activity in HCMV-infected human fibroblasts. Cells were harvested at 24 h after mock-infection (M) or infection. Each sample was analyzed in duplicate, and the average is presented. (*A*) Enzyme immuno-assay of PGE_2 accumulation in cultures of mock-infected or infected cells that were treated with the indicated concentrations of a specific COX-2 inhibitor (BMS-279652, -279654, or -279655). (*B*) Enzyme immuno-assay of PGE_2 accumulation in cultures of mock-infected or infected cells that were treated with the indicated concentrations of indomethacin (Indo). (*C*) Determination by colorimetric assay of the number of viable cells after culturing for 7 days in medium with no drug or in the presence of a COX-2 inhibitor (BMS-279655 or Indo).

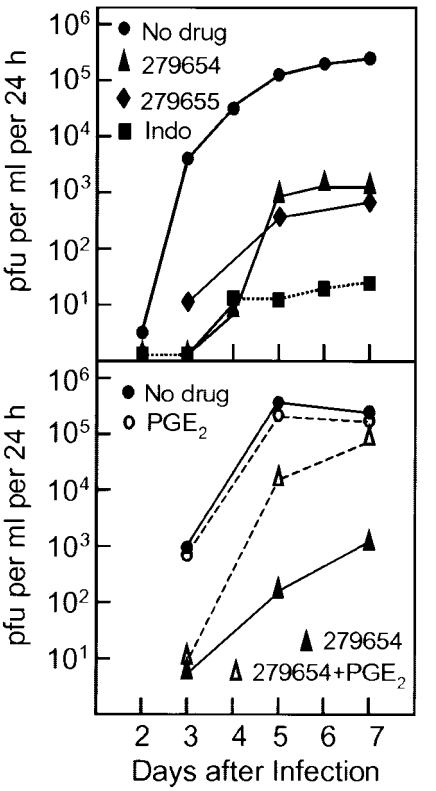

Fig. 3. COX-2 inhibitors reduce the production of infectious HCMV in human fibroblasts, and PGE_2 overcomes the inhibition. Infected cells were fed with fresh medium plus drug every 24 h. Infectious virus released into the medium was quantified by plaque assay on human fibroblasts. (*Upper*) COX-2 inhibitors block the growth of HCMV. Immediately after infection, cells were treated with no drug (●), 25 μg/ml BMS-279654 (♦), 25 μg/ml BMS-279655 (▲), or 500 μM indomethacin (■). (*Lower*) PGE2 reverses the block to HCMV growth mediated by COX-2 inhibitors. Immediately before infection, cells were treated with no drug (●), 0.1 μM PGE_2 (○), 25 μg/ml BMS-279654 (▲), or a mixture of the COX-2 inhibitor plus PGE_2 (△).

drug, and virus released into the medium was quantified by plaque assay (Fig. 3 *Upper*). BMS-279654 or -279655 reduced the virus yield by a factor of ≈120 on day 7 after infection, and indomethacin reduced the yield by a factor of ≈3,000.

The observation that multiple COX-2 inhibitors block virus growth suggests that COX-2 inhibition, rather than some other effect of the drug, was likely responsible for the anti-viral effect. To confirm that this is the case, PGE_2 was added to the culture medium of BMS-278654-treated cells (Fig. 3 *Lower*). Because PGE_2 is the product of COX-2 action, it should reverse the drug-induced block to virus infection. Although it did not increase the virus yield in the absence of the COX-2 inhibitor, exogenously added PGE_2 substantially relieved the inhibitory effect of the drug. On day 7 after infection, BMS-279654-treated cultures produced ≈150-fold less infectious virus than cultures that did not receive the drug. In contrast, the inclusion of PGE_2 with the drug reduced the inhibition to ≈2-fold. PGE_2 production is essential for the efficient production of HCMV progeny in human fibroblasts.

PGE_2 Induction Is Required for the Efficient Accumulation of HCMV DNA, mRNAs, and Proteins. To identify the site of the drug-induced block to viral replication in human fibroblasts, we initially monitored the accumulation of viral DNA after infection of human fibroblasts at a multiplicity of 3 pfu/ml (Fig. 4). Samples of total infected cell DNA were applied to a membrane by using

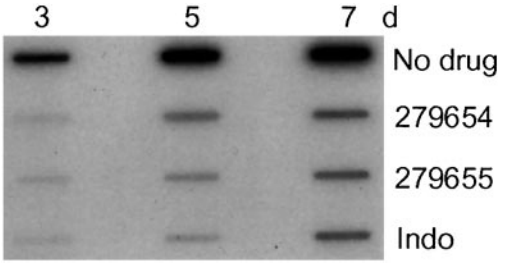

Fig. 4. COX-2 inhibitors reduce the accumulation of HCMV DNA in human fibroblasts. Infected cells were maintained in medium containing no drug, 25 μg/ml BMS-279654, 25 μg/ml BMS-278655, or 500 μM indomethacin (Indo). Cultures were fed every day with fresh medium containing drug. Total DNA was prepared from cells at the indicated times (d, day) and analyzed by slot blot by using ^{32}P-labeled full-length viral DNA as a probe.

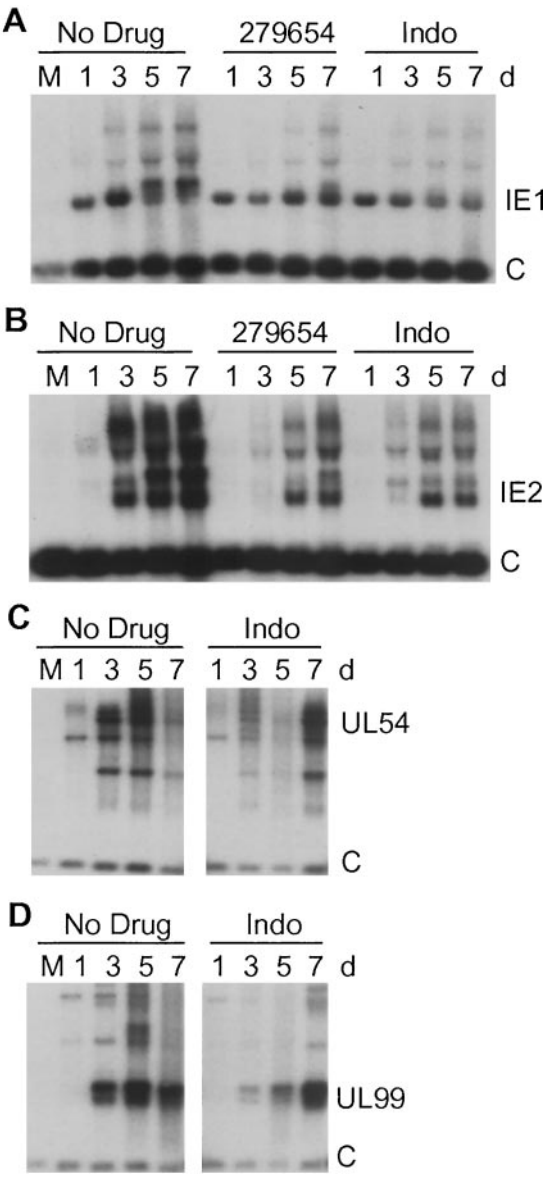

Fig. 5. COX-2 inhibitors reduce the accumulation of some viral mRNAs. Mock-infected (M) and infected cells were maintained in medium containing no drug, 25 μg/ml BMS-279654, or 500 μM indomethacin (Indo). Cultures were fed every day with fresh medium containing drug. Total cell RNA was prepared at the indicated times (d, day) and analyzed by Northern blot by using ^{32}P-labeled probes specific for IE1 (*A*), IE2 (*B*), UL54 (*C*), or UL99 (*D*) mRNA. A probe for the cellular 7SK RNA (*C*) was included in each reaction as a loading control.

a slot-blot apparatus and were assayed for the presence of viral DNA by using ^{32}P-labeled viral DNA as a probe. BMS-279654 (25 μg/ml) or -279655 (25 μg/ml) or indomethacin (500 μM) substantially blocked the accumulation of viral DNA, each reducing its level by a factor of $\approx$15.

Because viral DNA synthesis was compromised by the COX-2 inhibitors, we next examined their effect on the accumulation of immediate-early mRNAs, the first class of viral mRNAs to be produced after infection. The IE1 and IE2 mRNAs are produced from the major immediate-early transcription unit, a unit that gives rise to a set of mRNAs that are synthesized from different start sites and are differentially processed (20–22). They were assayed by Northern blot by using probes corresponding to either the IE1-specific exon 4 (Fig. 5*A*) or IE2-specific exon 5 (Fig. 5*B*). One major species of IE1 mRNA was evident at 3 days after infection of human fibroblasts at a multiplicity of 3 pfu per cell, and treatment with COX-2 inhibitors reduced its level to a limited extent ($\approx$2-fold; Fig. 5*A*). The inhibitors substantially prevented the accumulation of a larger IE1-specific mRNA, which might result from transcription initiation at an upstream site (22), on days 5 and 7 after infection. IE2-specific mRNAs were influenced to a much greater extent (Fig. 5*B*). There was a delay in their appearance and they failed to accumulate to normal levels in the presence of COX-2 inhibitors. If the intensities of all IE2-specific bands observed in the Northern blot are summed, then, on day 3, IE2 mRNAs were reduced by a factor of >100 in the presence of either BMS-279654 or indomethacin.

We also examined the effect of indomethacin on two viral mRNAs that are produced later in the replication cycle: UL54, an early mRNA (Fig. 5*C*), and UL99 (Fig. 5*D*), a late product. The accumulation of both mRNAs was substantially inhibited on days 1–5, although near normal levels were produced by day 7 after infection. UL54 encodes a subunit of the viral DNA polymerase (16). Consequently, a reduction in this product would directly influence viral DNA replication, consistent with the reduced accumulation of viral DNA in the presence of COX-2 inhibitors (Fig. 4). The synthesis of the late UL99 mRNA requires ongoing viral DNA replication (16), and its reduction is also consistent with the drug-induced block to DNA accumulation.

Western blot assays were performed on extracts of infected human fibroblasts to monitor the accumulation of viral proteins. The level IE1 protein was not measurably altered by treatment with BMS-279654 or indomethacin (Fig. 6 *A* and *B*), consistent with the relatively minor effect of COX-2 inhibitors on IE1 mRNA. In contrast, IE2 protein levels were substantially reduced (Fig. 6*A*), as predicted by the large reduction in IE2-specific mRNAs (Fig. 5*B*). Whereas IE2 protein was easily detected on day 3 after infection in the absence of drugs, both BMS-279654 and indomethacin delayed its appearance until day 5, and the protein never reached wild-type levels. We tested the accumulation of two additional immediate-early proteins, pTRS1 and pUL69 (data not shown). There was little effect on pUL69, but pTRS1 accumulation was delayed by about a day in the presence of either COX-2 inhibitor. It is difficult to be certain whether the effect on pTRS1 is direct, because IE2, whose expression is inhibited by the drugs, is a potent transcriptional activator (23, 24), and it could contribute to the expression of TRS1 mRNA. Finally, we examined the accumulation of pUL99, a late protein (Fig. 6*B*), and found that its accumulation was almost completely blocked by the drugs. This observation is consistent with the RNA accumulation data (Fig. 5*D*).

The effect of BMS-279654 on the full set of viral mRNAs was determined at 3 days after infection. We prepared a ^{32}P-labeled cDNA from infected cell RNA and used it to probe an array (18) containing cDNAs corresponding to all known HCMV ORFs. Although some viral mRNAs were present at normal levels, e.g., IRL/TRL2–7 (Fig. 7, compare B and C boxes in the two panels), the COX-2 inhibitor reduced the level of most viral mRNAs, e.g., UL80–84 (Fig. 7, compare A boxes in the two panels). BMS-279654 had a profound effect on the accumulation of mRNAs encoded by the viral genome.

Discussion

COX-2 mRNA (Fig. 1*A*), protein (Fig. 1*C*), and activity (Fig. 1*D*) are dramatically induced after infection of human fibroblasts by HCMV. A constituent of the virus particle is responsible for the induction, because UV-irradiated virus that cannot express its genome is able to efficiently elevate COX-2 mRNAs (Fig. 1*B*). The external domain of the virion glycoprotein, gB, has been shown to induce the accumulation of a wide variety of

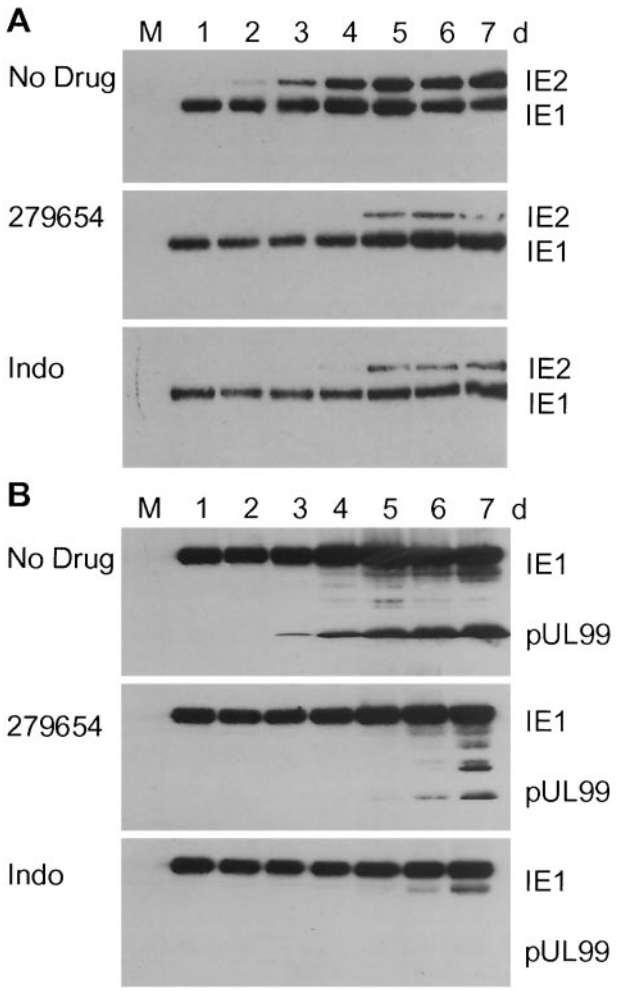

Fig. 6. COX-2 inhibitors reduce the accumulation of some viral proteins. Mock-infected (M) and infected cells were maintained in medium containing no drug, 25 μg/ml BMS-279654, or 500 μM indomethacin (Indo). Cultures were fed every day with fresh medium containing drug. Cell extracts were prepared at the indicated times (d, day) and analyzed by Western blot assay by using monoclonal antibodies specific for the domain shared by IE1 and IE2 or for pUL99.

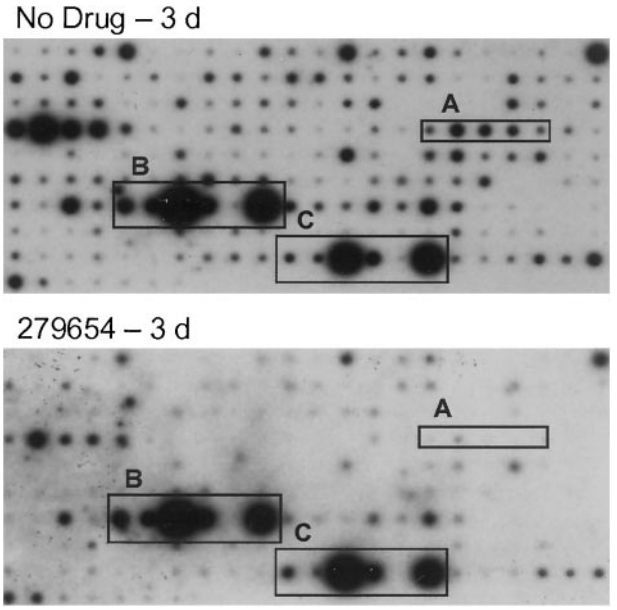

Fig. 7. A COX-2 inhibitor reduces the accumulation of many viral mRNAs. Infected cells maintained in medium containing no drug or 25 μg/ml BMS-279654. Cultures were fed every day with fresh medium containing drug. After 3 days, total RNA was isolated and subjected to reverse transcription. The resulting ^{32}P-labeled cDNA was used to probe an HCMV gene array on a membrane containing all predicted viral ORFs. The same sets of genes are identified in *Upper* and *Lower*. Box A, UL80–84; box B, IRL2–7; box C, TRL2–7. Key to array: line 1, UL1–22; line 2, UL23–42; line 3, UL43–64; line 4, UL65–86; line 5, UL87–108; line 6, UL109-UL129; line 7, UL130-US5; line 8, US6-US27; line 9, US28-TRL13; and line 10, TRL14-actin.

cellular mRNAs when added to fibroblasts (4). Apparently, it binds to a cellular receptor and initiates a signal that propagates to the nucleus and modulates cellular gene expression. NF-κB is activated by gB (25), and the COX-2 promoter contains multiple NF-κB binding sites that are important for its activity (26). Consequently, it is possible that gB present in the virion is responsible for the induction of COX-2.

Both specific (BMS-279652, -279654, and -279655) and nonspecific (indomethacin) COX-2 inhibitors block the virus-mediated induction of COX-2 activity in human fibroblasts (Fig. 2 *A* and *B*) at concentrations that do not compromise cell proliferation and viability (Fig. 2*C*). When the induction of COX-2 activity is completely blocked, the yield of infectious virus is reduced by more than a factor of 100 (Fig. 3 *Upper*). Earlier work demonstrated that indomethacin substantially blocked the growth of HCMV in TPC-1 cells (14), where the virus replicates poorly. Also, Spier *et al.* (13) have shown that the nonspecific COX-2 inhibitor, aspirin, at doses that can be achieved in the plasma of patients, can reduce the yield of HCMV in cultures of smooth muscle cells by a factor of 2–3. We have been able to extend these earlier results to experiments in fibroblasts where the virus grows efficiently and by showing that the COX-2 inhibitors are acting by blocking the production of PGE_2. Addition of exogenous PGE_2 can substantially overcome the block to HCMV growth mediated by BMS-279654 (Fig. 3 *Lower*).

COX-2 inhibitors interfere with the accumulation of many different HCMV mRNAs (Fig. 7). It is not clear how many viral transcription units are directly affected by the drugs. BMS-27954 and indomethacin markedly block the accumulation of IE2 mRNA (Fig. 5*B*). Because IE2 is a transcriptional regulatory protein (23, 24) that is produced at the very start of infection, it is possible that the drugs act primarily by blocking IE2 accumulation. The failure to produce IE2 protein could then lead to reduced activity of many other viral promoters. It is possible that additional immediate-early genes are also directly affected by the COX-2 inhibitors and that the loss of their products might also affect downstream events.

Exogenously added PGE_2 has been shown to activate the HCMV major immediate-early promoter in transfected cells (15). Consequently, COX-2 inhibitors, which block the production of PGE_2, might be expected to interfere with the activation of the major immediate-early promoter. However, an effect at the level of transcription should block the production of both IE1 and IE2 mRNAs because they are differentially processed products of the same primary transcripts. This is not what we observed. IE1 mRNA (Fig. 5*A*) and protein (Fig. 6 *A* and *B*) were affected very little, whereas the levels of IE2 mRNA (Fig. 5*B*) and protein (Fig. 6*A*) were substantially reduced. It is conceivable that, by blocking PGE_2 accumulation, the drugs differentially influence the expression of IE1 and IE2 mRNAs at the level of transcription. The mechanism that might underlie such an activity is not clear. Alternatively, the COX-2 inhibitors might influence mRNA

processing or stability. Experiments are in progress to further probe the role of PGE_2 in HCMV gene expression.

As has been mentioned previously (13), COX-2 inhibitors might prove to be useful anti-viral therapeutics. We have used high concentrations of the inhibitors, together with relatively high multiplicity infections, in the experiments reported here. The lower concentrations of COX-2 inhibitors that can be administered to patients, who are at risk for HCMV disease or are suffering from active disease, might nevertheless prove to be therapeutically beneficial. Further, it is possible that COX-2 inhibitors will synergize with other anti-HCMV compounds, such as ganciclovir, that block viral replication through different mechanisms.

We thank W. Koster (Bristol-Myers Squibb) for the generous gift of specific COX-2 inhibitors, and we thank L. Enquist (Princeton University) for thoughtful comments on the manuscript. This work was supported by a grant form the National Cancer Institute (CA87661). W.A.B. was supported by a postdoctoral fellowship from the National Institute of Allergy and Infectious Diseases (AI10448).

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