Roles of an IκB Kinase-related Pathway in Human Cytomegalovirus-infected Vascular Smooth Muscle Cells

A MOLECULAR LINK IN PATHOGEN-INDUCED PROATHEROSCLEROTIC CONDITIONS*

Received for publication, September 10, 2004, and in revised form, December 2, 2004
Published, JBC Papers in Press, December 24, 2004, DOI 10.1074/jbc.M410392200

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Viral and bacterial pathogens have long been suspected to affect atherogenesis directly. However, mechanisms linking innate immunity to chronic inflammatory diseases such as atherosclerosis are still poorly defined. Here we show that infection of primary human aortic smooth muscle cells (HAOSMC) with human cytomegalovirus (HCMV) leads to activation of the novel IκB kinase (IKK)-related kinase, Tank-binding kinase-1 (TBK1), a major effector of the cellular innate immune response. We demonstrate that part of the HCMV inflammatory response is most likely mediated via this novel kinase because the canonical IKK complex was only poorly activated upon infection of HAOSMC. An increase in TBK1 phosphotransferase activity led to a strong activation of the interferon regulatory factor (IRF)-3 transcription factor as measured by its C-terminal phosphorylation, dimerization, and DNA binding activity. In addition to TBK1, HAOSMC also express another IKK-related kinase isoform, IKKε, albeit at a lower level. Nevertheless, both isoforms were required for full activation of IRF-3 by HCMV. The transcripts of proatherosclerotic genes Ccl5 (encoding for the chemokine RANTES (regulated upon activation, normal T cell expressed and secreted) and Cxcl10 (encoding for the chemokine IP-10 (interferon-γ-inducible protein 10)) were induced in an IRF-3-dependent manner after HCMV infection of smooth muscle cells. In addition, cytokine arrays analysis showed that RANTES and IP-10 were the predominant chemokines present in the supernatant of HCMV-infected HAOSMC. Activation of the TBK1/IRF-3 pathway was independent of epidermal growth factor receptor and pertussis toxin-sensitive G protein-coupled receptor activation. Our results thus add additional molecular clues to a possible role of HCMV as a modulator of atherogenesis through the induction of a proinflammatory response that is, in part, dependent of an IKK-related kinase pathway.

Inflammation is a key process in the development of atherosclerosis, and one of the rate-limiting steps in the development of atherosclerotic lesions is the production of inflammatory chemokines by endothelial cells and vascular smooth muscle cells (VSMC)1 (for review, see Ref. 1). The two major chemokine groups are the CC chemokines, which include monocyte chemotactic protein-1, RANTES, macrophage inflammatory protein-1α and -1β, and the CXC chemokines interleukin (IL)-8, stromal cell-derived factor-1α, and interferon-γ-inducible protein-10 (IP-10). Chemokines induce chemotaxis of leukocytes in vitro as well as the recruitment of leukocytes to inflammatory sites in vivo. These mediators of inflammation have been detected in atherosclerotic lesions from both human and experimental animals but not in normal arteries, suggesting that they may play a significant role in the pathogenesis of atherosclerosis (2).

Because traditional risk factors such as hyperlipidemia, hypertension, diabetes, age, sex, smoking, and familiar history cannot explain the occurrence of atherosclerosis in about 50% of the cases, other factors could be involved (3). Infectious agents including bacteria such as Chlamydia pneumoniae and Helicobacter pylori or viruses such as human cytomegalovirus (HCMV) have long been suspected to initiate or contribute to the disease (for reviews, see Refs. 4 and 5). After primary infection, the β herpesvirus HCMV establishes lifelong latent infection in the host. In the human aorta, endothelial cells, and VSMC appear to be the primary site of infection, suggesting that the vasculature may serve as a reservoir for HCMV (6). Infection or reactivation from latency is thought to play an essential role in atherogenesis, both in native as well as transplant atherosclerosis. The molecular mechanisms by which HCMV infection contributes to atherosclerosis are still not fully understood. The induction of a repertoire of viral genes responsible for cell cycle control disruption has been suggested (7, 8). In addition, through up-regulation of scavenger receptor-A and platelet-derived growth factor-β receptor, HCMV may play a role in cholesterol uptake (9) as well as proliferation and migration of VSMC, respectively (10, 11). Independent studies also demonstrated the production of the cytokines IL-6 and IL-8 in HCMV-infected VSMC (12, 13). Thus, notably, most of

1 The abbreviations used are: VSMC, vascular smooth muscle cells; EGFR, epidermal growth factor receptor; EMSA, electrophoretic mobility shift assay; ERK1, extracellular signal-regulated kinase-1; gB, glycoprotein B; GST, glutathione S-transferase; HAOSMC, human aortic smooth muscle cells; HCMV, human cytomegalovirus; hpi, hours postinfection; IKK, IκB kinase; Il-β, interleukin; IP-10, interferon-γ-inducible protein 10; IRF, interferon regulatory factor; ISRE, interferon-stimulated response elements; JNK, c-Jun N-terminal kinase; LPA, lysophosphatidic acid; LPS, lipopolysaccharide; NES, nuclear export sequence; NF-κB, nuclear factor-κB; PTX, pertussis toxin; RANTES, regulated upon activation, normal T cell expressed and secreted; RT, reverse transcription; SeV, Sendai virus; SMC, smooth muscle cells; TBK1, Tank-binding kinase-1; TLR, Toll-like receptor; WCE, whole cell extracts; wt, wild-type.
the HCMV infection effects on atherosclerosis progression seem to be mediated by gene regulation through activation of transcription factors.

The transcription factor NF-κB is an important regulator of inflammation, immune response, and cellular survival (14). Its role in atherosclerosis development is strengthened by the observations that it can mediate the induction of more than 150 proatherosclerotic genes (14). The pathways involved in NF-κB have been well characterized. In nonstimulated cells, the NF-κB factors are retained in the cytoplasm in association with inhibitory subunits, IκBs. After viral infection, treatment with proinflammatory stimuli such as tumor necrosis factor-α, IL-1, lipopolysaccharide (LPS), or exposure to double-stranded RNA, a replicative intermediate of RNA viruses, IκBs are phosphorylated at conserved N-terminal residues (serines 32 and 36 for IκBα) by the IκB kinase (IKK) complex, which is composed of two catalytic subunits IKKα and β and one regulatory subunit IκKγ. Phosphorylation triggers a signal that induces ubiquitin-dependent degradation of IκBα and subsequent nuclear translocation of the NF-κB dimers (for review, see Ref. 15). Two related IKK homologs, IKKe (16), also called IKK-i (17), and TBK1 (18), also called NF-κB-activating kinase (19) or tumor necrosis factor receptor-associated factor 2-associated kinase (20, 21), also have been implicated in NF-κB activation (16, 18, 20–23). The targets of TBK1 in the NF-κB activation pathway have not been identified because IκBα does not seem to be the physiological substrate (20). Other possible substrates of TBK1 include the IKK complex as well as the Rela (p65) subunit of NF-κB (19, 21).

Interferon regulatory factor (IRF)-3 is another key transcription factor activated after virus infection. It belongs to the family of IRFs which includes IRF-1 to IRF-9 (24). IRF-3 is essential in the activation of the innate arm of the immune system (25). The pathways regulating IRF-3 phosphorylation and activation are the focus of considerable investigations. Recent studies suggest that TBK1 and IKKe correspond to the kinases that mediate the terminal phosphorylation of IRF-3 (26–30). Upon phosphorylation, IRF-3 homodimerizes and accumulates into the nucleus where it induces gene transcription after recognition of specific DNA response elements called interferon-stimulated response elements (ISRE) or positive regulatory domains I–III located in promoters of chemokines IL-15, IP-10, and RANTES as well as cytokines such as the type I interferon (26, 27; for a recent review, see Ref. 31).

As a mechanism of lifelong latent infection, HCMV executes multiple immune-evasive activities in infected cells (32). Recent reports demonstrated that the tegument protein from HCMV, pp65, was able to prevent NF-κB and IRF-3 activation (33, 34). Earlier studies also suggested that HCMV interferes with the IRF-3 pathway (35, 36). These reports challenge the concept that HCMV infection of VSMC could lead to atherosclerosis development through, in part, the induction of proinflammatory genes. This study was thus undertaken to verify whether one of the major effector pathway of the innate arm of the immune system, the IKK-related kinase pathway, could be involved in HCMV-induced proinflammatory response in VSMC. Our results show for the first time an inducible phosphotransferase activity of TBK1 by a DNA virus such as HCMV. Activation of the kinase was observed in primary human vascular smooth muscle cells (HAOSMC), and led to strong IRF-3 target genes, i.e. the proatherosclerotic chemokines IP-10 (Ccl10) and RANTES (Ccl5). IKKe, albeit expressed at a lower level in primary HAOSMC, was required for full IRF-3 activation by HCMV. Our results thus add additional molecular evidence for a role of HCMV as a modulator of atherogenesis through the induction of an inflammatory response, which in part, results from the activation of an IKK-related kinase pathway.

**EXPERIMENTAL PROCEDURES**

**Reagents, Antibodies, and Plasmids—**Angiotensin II was purchased from Hupakel Scientific (St. Laurent, QC). Lysophosphatidic acid (LPA) was from Sigma. Tyrophostin AG1478 was from Calbiochem. G418 was from Invitrogen. Pertussis toxin (PTX) was from List Biological Laboratories (Campbell, CA). Commercial antibodies were from the following suppliers: anti-IRF-3 (SC-369X), anti-IKKγ (SC-8330), anti-TBK1 (SC-9911), and anti-IκBα (SC-371) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-IKKκ (IMG-270A) (which also recognizes TBK1) was from Imgenex (San Diego). An antibody against the nuclear export sequence of human IRF-3 (anti-NESE) was from Immuno-Biological Laboratories Co. (Irl, Japan). Anti-β-actin clone AC-74 (A5516) was from Sigma. Phospho-IκBα (Ser-32) antibody (9241) was from Cell Signaling Technology (Beverly, MA). A neutralizing antibody against epidermal growth factor receptor (EGRF; clone 05-101), which was recently shown to inhibit HCMV entry (37), was purchased from Upstate Biotechnolog. Anti-HCMV (a mixture of two mouse monoclonal antibodies from Vero, M0854) was from DAKO (Glostrup, Denmark). A rabbit anti-TBK1 antibody (89246) that has been shown to immunoprecipitate endogenous TBK1 (27) was a kind gift from Dr. Tom Maniatis (Harvard University). Polyclonal antibodies to ERK1 (SMI, p38 (HSK 591.1), and JNK (914.1) were all gifts from Dr. Sylvain Moleco (University of Montreal) and have been described elsewhere (38). The anti-phospho-IRF-3 Ser-396 (HPS 5033) was from Dr. John Hiscott (McGill University, Montreal, QC), and its use has been described previously (26, 39). The IRF-3 dominant-negative mutant (ΔNIRF-3-40) was a kind gift from Dr. Rongtuan Lin (McGill University, Montreal, QC). Glutathione S-transferase (GST)-ATF2 recombinant protein was from Santa Cruz Biotechnology. GST-IκBα, GST-IRF-3wt, GST-IRF-3 5A, and GST-IRF-3 J2A were kind gifts from Dr. John Hiscott and have been described previously (26).

**Types and Virus Strains—**Primary human HAOSMC were purchased from Cell Applications, Inc. (San Diego) and cultured in medium purchased from the same manufacturer. Each cell lot was from a single donor. All experiments were repeated for two different donors. Rat aortic SMC and rat aortic SMC overexpressing a dominant-negative version of EGRF (HERC533) (41) were obtained from Dr. Sylvain Moleco (University of Montreal). Growth conditions, starvation media, and stimulation with angiotensin II and LPA have been described elsewhere (41). Human foreskin fibroblasts and MRC-5 fibroblasts were obtained from American Type Culture Collection (ATCC) and cultured in minimal essential medium containing 10% fetal bovine serum. All cells tested negative for mycoplasma contamination. HCMV Towne strain was obtained from ATCC. HCMV Toledo was a kind gift from Dr. Carlos Vieira (Fred Hutchinson Cancer Research Center, Seattle). Sendai virus (SeV) was obtained from Specific Pathogen-Free Avian Supply (North Franklin, CT).

**HCMV Production—**HCMV Towne and Toledo strains were propagated as follows. Subconfluent MRC-5 cells were inoculated with HCMV at a multiplicity of infection of 0.001 plaque-forming unit/cell for 1 h, with rocking every 15 min. After HCMV adsorption, the cells were washed once with phosphate-buffered saline and reincubated with complete growth medium. Medium was changed each 3 days until 100% plaque formation was observed. After plaque formation, the cells were washed once with phosphate-buffered saline and reincubated with complete growth medium for 1 h, with rocking every 15 min. After HCMV infection, the cells were washed once with phosphate-buffered saline and reincubated with complete growth medium. Medium was changed each 3 days until 100% cytopathic effects were observed. 3–4 days later, medium was collected and centrifuged at 1,500 × g for 15 min to remove cellular debris. The precleared supernatant was then ultracentrifuged at 154,000 × g for 30 min at 4 °C. The pellet containing concentrated virus particles was washed once with serum-free medium and resuspended in serum-free medium, and centrifuged at 80 °C. Virus titers were measured by plaque assay on human foreskin fibroblasts (passages 4–6) as follows. Cell monolayers were infected with 10-fold serial dilutions of virus stock. After a 1-h adsorption period, the cells were overlaid with medium containing 1% methyleneululose (Sigma). When plaques were seen, cell monolayers were postfixed with ethanol/methanol, washed twice with phosphate-buffered saline, and then incubated with blocking buffer (6% bovine serum albumin in phosphate-buffered saline) for 30 min at room temperature. Plaques were detected by immunocytochemical staining with mouse monoclonal anti-HCMV (1/200) as the primary antibody and alkaline phosphatase-conjugated anti-mouse immunoglobulin G (1/200) for the secondary antibody (KPL, Gaithersburg, MD) followed by enzymatic deposition of 5-bromo-4-chloro-3-in-
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doly/nitro blue tetrazolium (Sigma). Infectivity was recorded as plaque-forming units, and habitual titers were ~10^3 plaque-forming units/ml.

Infection—Cells were infected with HCMV Towne strain at a multiplicity of infection of 1.0 plaque-forming unit/cell or with SeV at 40 hemagglutinating units/10^6 cells for 2 h in serum-free medium. Then the serum-free medium was replaced with complete medium for the rest of the kinetics. Where indicated, cells were infected with Toledo strain at a multiplicity of infection of 1.0. In some experiments, before infection, a portion of HCMV stock was heat-treated by boiling for 20 min.

Small Interfering RNA (siRNA)—The 21-nucleotide siRNA duplexes with 2-nucleotide (2-deoxy)-thymidine 3' overhangs were obtained from Dr. Haab (Lonza). Cells were transfected with siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. siRNA duplexes were used at a final concentration of 120 nM in transfections. Cells were harvested for analysis 72 h after transfection.

Retrovector Construction, Transduction, and Generation of Rat VSMC-overexpressing ΔNIRF-3—The pMSCVneoΔNIRF-3 retrovirus was generated by amplifying the sequence encoding ΔNIRF-3 from the pBSCMV-ΔNIRF-3 plasmid (42) by PCR (94 °C for 60 s, 55 °C for 60 s, 72 °C for 2 min, for 25 cycles) using Pfx DNA polymerase (Invitrogen) with the primers 5'-ACCTCCGATTCACTAGGGCCACGGCAGTG-3' and 5'-TCGACTGGACGGGACGGACCTT-3'. The cloning product was verified by sequencing, cleaved with EcoRI and XhoI (EcoRI and XhoI restriction sites were inserted within the primers), and cloned into pMSCVneo retrovector (Clontech), cut with the same enzymes. The Phoenix Amphotropic packaging cell line was transiently transfected with the plasmids pMSCVneo and pMSCVneoΔNIRF-3. At 48 h post-transfection, retrovirus-containing medium was harvested and used to infect rat VSMC. VSMC were infected twice at 24-h intervals in the presence (47). Briefly, 7.5% acrylamide gels (without SDS) were pre-polymerized by electrophoresis for 60 min at 25 mA. Immuno- 

Preparation of whole cell extracts (WCE) and analysis of IRF-3, TBK1, and IKK complex activities was accomplished as described previously (25). The phosphotransferase activity of ERK1, p38, and JNK2 kinase assays (KA) using immunocomplex and myelin basic protein (MBP), GST-ATF2, and GST-c-Jun as substrates, respectively.

were assayed by resuspending the beads in 40 μl of kinase buffer containing 1 μg of GST-IRF-3 or GST-IkBα respectively, 20 μM ATP, and 20 μCi of [γ-32P]ATP. The reactions were incubated at 30 °C for 30 min and stopped by the addition of 2× Laemmli sample buffer. The samples were analyzed by SDS-PAGE. After Coomassie staining, the gels were dried and exposed to a gel documentation device (Typhoon scanner 9410, Amaresham Biosciences) for imaging and quantification. In some experiments, the upper part of the gel was transferred electro- 

were added to a 96-well plate (2.0 × 10^4 cells/well) without antibiotics. After 24 h, cells were transfected with siRNA oligonucleotides using Lipofectamine 2000 (Invitrogen) according to the manufacturer's specifications. siRNA duplexes were used at a final concentration of 120 nM in transfections. Cells were harvested for analysis 72 h after transfection.

were selected. For selection of clones from both rat VSMC (Neo) and rat VSMC (ΔNIRF-3), cloning rings were used, and expression of the transgene was verified by immunoblot analysis.

RNA—Total RNA was isolated using TRizol (Invitro- 

RNase-treated (Ambion, Austin, TX). InVitrogen) polyacrylamide gel. After electrophoresis the gels were dried and exposed to a Typhoon scanner 9410 for imaging and quanti- 

Electrophoretic Mobility Shift Assays (EMSA)—For EMSA, 15 μg of WCE was incubated at 15 °C for 5 min in a buffer containing 20 mM HEPES, pH 7.0, 40 mM KCl, 20 mM NaCl, 10 mM NaF, 1 mM MgCl2, 10 mM dithiothreitol. TBK1 and IKK complex activities were assayed by resuspending the beads in 40 μl of kinase buffer containing 1 μg of GST-IRF-3 or GST-IkBα respectively, 20 μM ATP, and 20 μCi of [γ-32P]ATP. The reactions were incubated at 30 °C for 30 min and stopped by the addition of 2× Laemmli sample buffer.

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activity as measured by phosphorylation of the C-terminal region of IRF-3. The phosphotransferase activity started at 4 h postinfection (hpi) and was sustained for 20 hpi. Under our experimental conditions, IKK\(\beta\)/H9280 was not detected in TBK1-immune complexes (data not shown). To delineate which Ser/Thr residues of IRF-3 were phosphorylated by TBK1, in vitro kinase assays were accomplished with different GST-IRF-3 recombinant proteins. Fig. 2B shows that major phosphoacceptor sites targeted by TBK1 in vitro mapped to the second clustered Ser/Thr residues (see Fig. 2, B and C), an acidic region known to be involved in IRF-3 activation (31). We also addressed the level of activation of the canonical IKK complex after infection of primary HAOSMC with HCMV. Fig. 3A shows that TBK1 phosphotransferase activity was again strongly activated by HCMV and, to a lesser extend, the enveloped RNA virus SeV used as a positive control (compare lanes 4–5 and 8–9). When the same cellular extracts were used to verify the phosphotransferase activity of the IKK complex, a significant increase was observed when cells were infected with SeV (Fig. 3B, lanes 1–5). However, no activation of the IKK complex was detected in HCMV-infected HAOSMC in the first hours after the infection (Fig. 3B, lanes 6–9). Intriguingly, other studies have shown activation of the NF-\(\kappa\)B pathway in human SMC infected by HCMV (50, 52). Because degradation of the NF-\(\kappa\)B inhibitor I\(\kappa\)B\(\alpha\) is often used as a hallmark of the activation of the NF-\(\kappa\)B pathway, we decided to verify the expression level of the latter in our model. In a reproducible manner, another set of experiments showed no detectable phosphotransferase activity of the IKK complex as opposed to a significant activation of TBK1 at 4 hpi (Fig. 3C, compare a and b, lanes 4–6). Importantly, the inability of HCMV to induce the activation of the IKK complex correlated with a lack of in vivo phosphorylation of I\(\kappa\)B\(\alpha\) on Ser-32 (Fig. 3C, c). Nevertheless, the expression level of I\(\kappa\)B\(\alpha\) was reduced significantly upon virus infection (Fig. 3C, d). The apparent lack of IKK complex activation after HCMV infection could have been the result of a poor sensitivity.
of our in vitro kinase assay. However, after infection of HAOSMC with SeV, a specific phosphoserine 32 signal of endogenous IxBα followed the same kinetics of activation of the IKK complex (Fig. 3D). Moreover, quantitative analysis revealed that the phosphotransferase activity of the IKK complex was increased up to 13-fold after SeV infection (Fig. 3D) and 20-fold after a 5-min exposure of the cells to tumor necrosis factor-α (data not shown). Collectively, these data suggest that HCMV has the capability to induce the degradation of IxBα. Importantly, however, this seems to occur in an IKK-independent mechanism. Because the phosphotransferase activity of TBK1 was strongly induced in HCMV-infected cells, these findings suggest that TBK1 might play a more important role than the canonical IKK complex in HCMV pathogenicity. They also suggest that IRF-3 is likely activated in vivo by HCMV in primary HAOSMC.

Activation of IRF-3 by HCMV—Through biochemical evidence such as DNA binding activity and nuclear accumulation, previous reports have suggested the activation of IRF-3 in fibroblasts infected with HCMV (35, 36). However, these reports suggested a possible virus-induced protein with antagonistic activity toward IRF-3 because activation of the latter was partly or totally dependent on the presence of cycloheximide. Viral interference with the IRF-3 pathway might be dependent on the presence of the HCMV tegument protein pp65 because activation of the latter was weak in primary HAOSMC (Fig. 5A). A monoclonal antibody specific for IRF-3 included in the binding reaction specifically inhibited the formation of the complex (Fig. 5A, lanes 11 and 12). Fig. 5B shows the specificity of the binding signal in EMSA. An excess of homologous oligonucleotide inhibited complex formation by extracts of HCMV-infected primary HAOSMC (Fig. 5B, lanes 5 and 11), whereas an excess of a derivative oligonucleotide containing a single point mutation had no detectable effect (Fig. 5B, lanes 6 and 12). Even if the TBK1/IRF-3 pathway was strongly activated by HCMV in primary HAOSMC, we did observe a weak but significant effect of cycloheximide in these primary cells (Figs. 4B and 5A, compare lanes 3–5 with lanes 8–10). Given that LPS is a well characterized inducer of IRF-3 (27–30), there was the possibility that our observations were caused by contamination of the viral stock with minute quantities of endotoxin. To exclude this possibility, we heat-treated our HCMV preparation. Indeed, LPS exhibits thermostability (55), whereas most proteins are thermolabile. Heat treatment of our HCMV preparation completely blocked its ability to activate IRF-3 in primary HAOSMC. Moreover, LPS alone failed to induce IRF-3 under our experimental conditions (data not shown). Together these experiments, although partly confirming previous observations, nevertheless clearly demonstrate that HCMV has the intrinsic capability of activating the TBK1/IRF-3 pathway in permissive primary HAOSMC.

Both IKK-related Kinases Are Required for Full Activation of IRF-3 in HCMV-infected Primary HAOSMC—In addition to TBK1, IKKe is known to be part of the signaling cascade leading to IRF-3 activation (26–30). However, IKKe is expressed predominantly in immune cells and tissues, including peripheral blood leukocytes, spleen, and thymus (17). On the other hand, TBK1 is constitutively and ubiquitously expressed (19). Indeed, using an antibody that recognizes both isoforms, we observed that the expression level of IKKe was weak in...
primary HAOSMC as opposed to the U937 monocytic cell line used as a positive control (see Fig. 6A). However, TBK1 was equally expressed in both cell models. Despite this differential profile in their respective distribution, we decided to verify the contribution of these two isoforms in IRF-3 activation by using siRNA technology. Upon transfection of the indicated siRNA duplexes, the expression levels of both isoforms were down-regulated by 60–70% (see Fig. 6, B and C). When the isoforms were individually down-regulated, a net decrease (~50%) in HCMV-induced IRF-3 dimerization was observed (Fig. 6D, compare lane 2 with lanes 4 and 6). However, under conditions where both isoforms were knocked down, HCMV-induced IRF-3 dimerization was almost completely abrogated (see Fig. 6D, lanes 7 and 8). These data thus demonstrate that both kinases are required for full activation of IRF-3 in HAOSMC infected with HCMV.

**HCMV Infection of VSMC Results in the Induction of Proatherosclerotic Genes in an IRF-3-dependent Manner**—Chemokines are thought to play a major role in atherosclerosis development. Because the genes Cxcl10 (encoding for the chemokine IP-10) and Ccl5 (encoding for the chemokine RANTES) contain ISRE elements in their promoters and their transcription is induced by IRF family members such as IRF-3 (40, 56), we addressed whether these genes were induced in an IRF-3-dependent fashion in primary HAOSMC and rat VSMC infected by HCMV. Semiquantitative RT-PCR analysis revealed that both Ccl5 and Cxcl10 transcripts were induced by HCMV (Fig. 7A). This increase in these proatherogenic transcripts was followed by the detection of their related proteins in the supernatant of HCMV-infected HAOSMC (Fig. 7B). Remarkably, only IP-10, RANTES, and IL-6 were increased significantly in the supernatant of HAOSMC at 20 hpi. Activation of the TBK1/IRF-3 pathway was also observed in HCMV-infected rat VSMC (see Fig. 9F and data not shown). These cells were therefore used as a model for stable expression of a dominant-negative mutant of IRF-3 (ΔNIRF-3) (40). Different clones were isolated, amplified, and characterized (Fig. 8A). After HCMV infection, induction of transcripts for both Ccl5 and Cxcl10 was observed.

**Fig. 5.** DNA binding activity of IRF-3 in HAOSMC infected with HCMV. Cells were left untreated (−) or infected for different periods of time with HCMV in the presence or absence of 100 μg/ml cycloheximide (CHX). A, WCE were analyzed by EMSA using an ISG15-specific oligonucleotide as a probe. Anti-IRF-3 was added in lanes 11 and 12. B, EMSA were carried out on WCE in the absence (lanes C) or presence of a 100-fold excess of homologous oligonucleotide (lanes H) or a mutant oligonucleotide with a single bp difference from the probe (lanes M).

**Fig. 6.** Roles of IKKe and TBK1 in HCMV-induced IRF-3 activation in primary HAOSMC. A, 70 μg of WCE, prepared from primary HAOSMC and the U937 monocytic cell line, were subjected to immunoblot analysis using an antibody that recognized both IKK-related isoforms. B, RNA silencing (siRNA)-mediated knock-down of IKK-related kinase isoforms. HAOSMC were transfected with a siCONTROL Nontargeting silencing RNA (Control) or RNA duplexes designed to specifically target IKKe and TBK1 as indicated. 64 h post-transfection, cells were left uninfected (−) or infected with HCMV for the indicated time. WCE were prepared and analyzed for IKKe and TBK1 expression by immunoblotting as described in A (a). The membrane was stripped and reprobed with an anti-β-actin to verify equal loading of proteins (b). C, densitometry analysis of the blot shown in B. Each value was graphed as relative protein intensity normalized to β-actin. Closed bars, relative amount of IKKe; open bars, relative amount of TBK1. D, WCE from B were used in native-PAGE analysis to verify the dimerization state of IRF-3.
in the two clones of rat VSMC expressing only the neomycin cassette (clones neo 3 and 6; Fig. 8, B and C). However, the presence of a high level of \( /H9004 \) NIRF-3 in clone 3.3 markedly attenuated the induction of both \( Ccl5 \) and \( Cxcl10 \) mRNA by HCMV (Fig. 8, B and C). The clone expressing a moderate level of \( /H9004 \) NIRF-3 (clone 8.3) displayed only intermediate levels of induction of these transcripts. Together, these data clearly demonstrate that HCMV infection of vascular cells results in the production of proatherosclerotic chemokines in an IRF-3-dependent manner.

**Activation of the TBK1/IRF-3 Pathway by HCMV Is Independent of PTX-sensitive Ga-\( \alpha \) Proteins and Signaling through EGFR**—Studies have shown that binding of HCMV glycoprotein to host cell receptors activates cellular signaling pathways and cytokine production (for review, see Ref. 57). This was specifically demonstrated for the envelope glycoprotein B (gB) of HCMV, which alone was shown recently to induce IRAK-3 activation in the presence of cycloheximide (58). Interestingly, gB interacts, induces intracellular signaling, and allows viral entry through at least one type of host receptor, i.e. the EGFR (37). In addition, PTX-sensitive \( G \) proteins are involved in HCMV-induced activation of ERK, cPLA2, and NF-\( \kappa \)B in primary HAOSMC (50) and IL-6 production in fibroblasts (59). In light of these interesting findings, we thought to verify the involvement of these pathways in TBK1/IRF-3 activation by HCMV in HAOSMC as well as rat VSMC. As reported previously (41), treatment of rat VSMC with PTX, a potent inhibitor of \( G \) \( \alpha \) proteins (50), totally blocked LPA-induced ERK1 activation (Fig. 9A, lane 8). Pretreatment with the tyrphostin AG1478, a selective EGFR kinase inhibitor that effectively blocks HCMV-induced intracellular signaling and viral entry (37), inhibited the activation of ERK1 by both angiotensin II and LPA (Fig. 9A, lanes 6 and 9) as reported previously (41). However, in the presence of these inhibitors, IRF-3 was still activated by HCMV in HAOSMC when verified by a decrease of mobility in SDS-gel, the use of the phosphospecific antibody, the dimerization state and the DNA binding activity of IRF-3 (Fig. 9, B–E). These observations correlated with an activation of the phosphotransferase activity of TBK1 in rat VSMC over-expressing a dominant-negative version of EGFR (HERCD533) (41) (Fig. 9F) and the failure of PTX and AG1478 to inhibit HCMV-induced TBK1 activation in human primary HAOSMC (Fig. 9G).

**DISCUSSION**

Production of Proatherogenic Chemokines by HCMV Is, in Part, Mediated though the Activation of an IKK-related Pathway in Primary HAOSMC—The innate immune response and the process of inflammation are interconnected (for review see Ref. 60). Excessive and continuing cytokine production in re-
sponse to pathogens is thought to induce the development of an inflammation response leading to pathological conditions such as atherosclerosis (61). In addition to its effect on the cell cycle and apoptosis, VSMC migration, proliferation, and cholesterol uptake, we show here that infection of primary HAOSMC with HCMV induces strong production of RANTES and IP-10 chemokines in an IKK-related kinases/IRF-3 dependent manner. HCMV infection of HAOSMC also induces a marked degradation of the NF-κB inhibitor, IκBα (Fig. 3C, d), thus suggesting activation of the NF-κB pathway in HCMV-infected HAOSMC as also reported by others (50, 52). In this scenario, we propose that activation of NF-κB is most likely in part responsible for the presence of IL-6, a known NF-κB-regulated gene, in the

**Fig. 8.** IRF-3-dependent cytokine production by rat VSMC infected with HCMV. A, after infection with a retrovirus encoding a dominant-negative version of IRF-3 (ΔNIRF-3), stable selected clones were analyzed by immunoblotting for the expression of the transgene. B, after infection of rat VSMC with HCMV for different periods of time, total RNA was prepared and analyzed for Ccl5 and Cxcl10 gene induction by RT-PCR. C, quantification of the relative yields of PCR product was determined with a gel documentation device (Typhoon 9410). Each value was graphed as relative transcript intensity normalized to β-actin. Closed bars, relative amount of Ccl5 transcripts; open bars, relative amount of Cxcl10 transcripts.

**Fig. 9.** Role of EGFR and Gαs/o pathways in the activation of the TBK1/IRF-3 pathway by HCMV in VSMC. A, ERK1 kinase assay. Quiescent rat VSMC were pretreated for 16 h with 100 ng/ml PTX (P), 30 min with 250 nM AG1478 (A), or 0.01% vehicle dimethyl sulfoxide (D) and then stimulated for the indicated times with 100 nM angiotensin II (Ang II) and 20 μM LPA. WCE were prepared, and the phosphotransferase activity of ERK1 was measured by immune complex kinase assay using myelin basic protein (MBP) as substrate. B, C, and D, HAOSMC were pretreated with AG1478 and PTX as described in A and then infected with SeV (S; used as a positive control) or HCMV (H) for 6 h. WCE were prepared and IRF-3 activation was measured by immunoblotting (B and C) as well as native-PAGE (D). E, DNA binding activity of IRF-3. HAOSMC were left uninfected (−) or infected with HCMV for the indicated times. WCE were prepared and subjected to EMSA analysis using ISG15-specific oligonucleotide as a probe. DMSO, dimethyl sulfoxide. F, activation of TBK1 in rat VSMC. Parental and HERCD533 cells were infected with HCMV for the indicated time, and the activity of TBK1 was measured by immune complex kinase assay using GST-IRF-3 as substrate. G, activation of TBK1 in HAOSMC. Cells were pretreated for 16 h with 100 ng/ml PTX (P), 30 min with 250 nM AG1478 (A) or 0.01% vehicle dimethyl sulfoxide (D) and then left uninfected (−) or infected for 6 h with HCMV. The phosphotransferase activity of TBK1 was measured by immune complex kinase assay using GST-IRF-3 as substrate.

supernatant of HCMV-infected HAOSMC (Fig. 7). Because the canonical IKK complex was only poorly activated (depending on the lot of primary HAOSMC, a very weak increase in the phosphotransferase activity (1.5-fold) of the IKK complex was
sometime detected), it is unlikely that it is involved in HCMV-mediated IkBo degradation. Indeed, phosphorylation on Ser-32 of IkBo was not observed during the time course studied (Fig. 3C, c). We therefore propose that other intracellular pathways must be activated by HCMV and involved in IkBo degradation. On the other hand, because the IKK-related kinase TBK1 was significantly activated by HCMV, it could also have an effect on the induction of NF-κB-targeted genes through phosphorylation of p65 subunit of NF-κB on Ser-536 (21), thus influencing the recruitment of the basal transcriptional machinery (62). We have initiated the characterization of the pathways activated after HCMV infection of HAOSMC and leading to IkBo degradation as well as p65 phosphorylation. We do not have results explaining the weak induction of the IKK complex by HCMV in primary HAOSMC, but one can argue that it might be, in part, related to the presence of the HCMV tegument protein pp65 (33).

RANTES has been implicated in cardiac inflammatory disorders after organ transplantation or arterial injury (63, 64). RANTES has also been detected in plasma samples from patients suffering from cardiovascular diseases (65), and more recently, a study showed a reduction of atherosclerotic plaque formation in mice treated with a RANTES receptor antagonist (66). IP-10, like RANTES, is a chemoattractant for monocytes and lymphocytes. Its levels are increased in the carotid artery (66). IP-10, like RANTES, is a chemoattractant for monocytes in patients suffering from cardiovascular diseases (65), and more recently, a study showed a reduction of atherosclerotic plaque formation in mice treated with a RANTES receptor antagonist (66). IP-10, like RANTES, is a chemoattractant for monocytes and lymphocytes. Its levels are increased in the carotid artery (66). IP-10, like RANTES, is a chemoattractant for monocytes in primary HAOSMC, but one can argue that it might be, in part, related to the presence of the HCMV tegument protein pp65 (33).

Proatherosclerotic Effects of HCMV

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Recently Tontonoz’s group (34) demonstrated that only upon infection at high multiplicity of infection (i.e. when several pp65 molecules are delivered to the infected cell) would the HCMV tegument protein inhibit the activation of IRF-3.

Signaling Pathways Leading to TBK1/IRF-3 Pathway Activation by HCMV in VSMC—Transcriptionally inactive HCMV induces the activation of the innate arm of the immune system as well as cytokine production (70, 71). These observations suggest that structural components of the virion, such as gB from HCMV, could probably initiate these responses through activation of cell surface receptors on the host cells (51, 72). Accordingly, Wang et al. (37) demonstrated that EGFR was involved in HCMV-induced signaling and viral entry and, moreover, that gB was a ligand of EGFR. In addition, Compton’s group (58) showed that IRF-3 was activated by recombinant gB in the presence of cycloheximide. In our model system, however, it is unlikely that EGFR signaling contributes to TBK1/IRF-3 activation. The use of a highly characterized EGFR tyrosine kinase inhibitor (AG1478) did not affect IRF-3 activation by HCMV, and importantly, the phosphotransferase activity of TBK1 was even stronger in VSMC expressing a dominant-negative version of EGFR (see Fig. 9F). Another scenario suggests that EGFR could be used for docking of virions on the host cells prior to receptor clustering and activation of the innate immunity (73). However, we did not observe a significant reduction in the activation of IRF-3 when using a neutralizing antibody against EGFR that inhibits viral entry (data not shown) (37). A similar conclusion was reached with the use of the tyrphostin AG1478, which was also shown to inhibit HCMV entry in target cells (37). Because hematopoietic cells do not express EGFR but are efficiently infected by HCMV (74), the existence of other HCMV receptors, or other gB receptors in addition to EGFR, is to consider. Indeed, while this manuscript was under revision, Compton’s group showed that integrins are likely to play a key role in HCMV entry (75). The involvement of a Gαi2/γ12-coupled receptor is also unlikely because PTX treatment had no antagonistic effect on the intracellular pathways leading to IRF-3 activation in HAOSMC.

TLRs are major key players in pathogen recognition (for reviews, see Refs. 76 and 77). A recent study demonstrated that the induction of inflammatory cytokines by HCMV was mediated via a TLR-2-dependent activation of NF-κB (78). However, this study did not verify whether IRF-3 was activated in a TLR-2-dependent fashion by HCMV. So far, data in the literature suggest that this is unlikely because of the 10 human TLR members published to date, only TLR-3 and TLR-4 have been shown to induce type I interferon production through IRF-3 activation (for review, see Ref. 79). Thus, experiments are under way to try to identify other HCMV receptors or gB receptors that are responsible for the activation of the innate arm of the immune response through the TBK1/IRF-3 pathway.

Recently Tontonoz’s group (80) demonstrated a cross-talk between LXR and TLR signaling mediated by IRF-3, the latter acting as an antagonist of cholesterol metabolism through its effect on the ABCA1 transporter (80). Our discovery of a functional link between activation of TBK1 and IRF-3 in HCMV-infected smooth muscle cells and the work from Tontonoz’s group extends our knowledge on the possible role of IRF-3 in atherogenesis. More work is still necessary to appreciate the complex response of host cells to virus infection and more precisely how HCMV modulates atherogenesis.

Acknowledgments—We thank Drs. John Hiscott, Sylvain Meloche, Tom Maniatis, Rongtuan Lin, and Jeff Vieira for reagents used in this study. We also thank Drs. Pierre Moreau, Huy Ong, Sylvie Laquerre, and Guy Servant for helpful discussions.
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J. Biol. Chem. 2005, 280:7477-7486.
doi: 10.1074/jbc.M410392200 originally published online December 24, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M410392200

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