Transmissible gastroenteritis virus infection induces cell apoptosis via activation of p53 signalling

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Transmissible gastroenteritis virus (TGEV) infection induced apoptosis in several cell lines in vitro. Our previous studies demonstrated that TGEV could activate FasL- and mitochondria-mediated pathways to induce apoptosis in PK-15 cells. In this study, we investigated the regulation of p53 and p38 mitogen-activated protein kinases (MAPK) signalling pathways in the interaction of TGEV with host cells. We observed that TGEV infection decreased p300/CREB, downregulated MDM2 and promoted p53 phosphorylation at serines 15, 20 and 46, resulting in accumulation and activation of p53 in PK-15 cells. TGEV infection induced the transient activation of p38 MAPK in the early phase of inoculation and constant activation in the later phase of infection. However, UV-irradiated TGEV did not promote the activation of p53 and p38 MAPK in the later phase, whereas it only triggered the transient activation of p38 MAPK in the early phase. Blocking of p53 activation significantly inhibited the occurrence of apoptosis through suppressing the TGEV-induced FasL expression, Bcl-2 reduction, Bax and cytochrome c redistribution, while inhibition of p38 activity moderately blocked apoptosis induction and partly attenuated the accumulation and activation of p53. However, inhibition of p38 and p53 activity had no significant effects on viral gene transcription at 12 and 24 h post-infection. Taken together, these results demonstrated that TGEV infection promoted the activation of p38 MAPK and p53 signalling, and p53 signalling might play a dominant role in the regulation of cell apoptosis. These findings provide new insights into the function of p53 and p38 MAPK in the interaction of TGEV with host cells.

INTRODUCTION

Transmissible gastroenteritis virus (TGEV) is an enveloped single-stranded positive-sense RNA virus belonging to the family Coronaviridae in the genus Alphacoronavirus (Carstens, 2010; Lorusso et al., 2008). TGEV infection causes severe lethal watery diarrhoea and dehydration in piglets and results in serious economic loss to the pork industry worldwide (Enjuanes & Van der Zeijst, 1995). Consistent with in vivo pathological changes, TGEV infection induces morphological and biochemical changes in host cells and some porcine cell lines in vitro (Eléoué et al., 1998). Previous studies have reported cell pathways affected by TGEV infection and leading to apoptosis (Cruz et al., 2011; Ding et al., 2012; Eléoué et al., 1998, 2000; Sirinarumit et al., 1998; Zhong et al., 2012). However, these studies provide limited information regarding the signalling pathways leading to TGEV-induced apoptosis. Further study is needed to establish which upstream signalling pathways are activated and involved in the regulation of apoptotic signalling pathways following TGEV infection.

Tumour suppressor protein p53 is a major transcription factor for the control of cell cycle arrest, senescence and cell apoptosis (Gostissa et al., 2003). Under normal conditions, p53 is tightly modulated and maintained at low levels, but upon exposure to stress, p53 transiently accumulates and transfers into the nucleus in an active form to regulate transcription of target genes (Bálint & Vousden, 2001; Prives & Hall, 1999). Several studies have demonstrated that p53 is accumulated and activated to induce transcription of pro-apoptotic genes such as Bax and FasL and repress transcription of anti-apoptotic genes such as Bcl-2, which play important roles in the regulation of apoptosis in response to virus infection in host cells. These studies suggest p53 plays an important role in mediating apoptosis in response to virus infection (Everett & McFadden, 1999; Lin et al., 2009; Padhan et al., 2008). In addition, p38 is also induced in response to viral infections to enhance IFN-dependent antiviral activity independently of its functions as a pro-apoptotic and tumour suppressor gene (Muñoz-Fontela et al., 2008; Pampin et al., 2006; Takaoka et al., 2003). These findings not only demonstrate that p38 plays important roles in virus-infected cells to evoke apoptosis and IFN-dependent antiviral activity, but also suggest that p53 may have a broader and more complicated role in viral-host interaction.

The mitogen-activated protein kinases (MAPK) including p38 MAPK and c-Jun N-terminal kinase/stress-activated

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protein kinases (JNK/SAPK) have been shown to be activated to further phosphorylate p53 in response to virus infection, leading to cell apoptosis (Lin et al., 2009). Coronavirus infection has been shown to activate JNK and/or p38 MAPK pathways (Mizutani et al., 2004). Severe acute respiratory syndrome coronavirus (SARS-CoV) infection of permissive cells induces p38 MAPK signalling (Mizutani et al., 2004). Murine coronavirus mouse hepatitis virus (MHV) can activate p38 MAPK to regulate virus replication and cytokine production (Banerjee et al., 2002). These findings suggest that JNK and p38 MAPK pathways may be activated and play some roles in TGEV-infected cells.

In this study, we investigated the activation pattern and roles of p53, p38 MAPK and JNK in the TGEV induction of apoptosis and the relationship of these signalling pathways. We reported that TGEV induced the activation of p53 and p38 MAPK in a different pattern. Activated p53 signalling played a dominant role in triggering apoptosis occurrence through regulating the expression of apoptosis-associated genes. However, the activation of p38 MAPK is only partially responsible for the activation of p53 and contributed minimally to the TGEV-induced apoptosis. Our results demonstrated that compared with p38 MAPK signalling, p53 signalling plays a more important role in the interaction of TGEV with host cells.

RESULTS

**TGEV infection promotes the accumulation and transcriptional activity of p53 in PK-15 cells**

In a previous study, we have demonstrated that TGEV infection upregulated FasL and Bax to activate apoptotic signalling (Ding et al., 2012). A number of apoptosis-associated genes such as Bax, Bcl-2 and FasL, are regulated by p53 in some types of cells (Kuribayashi et al., 2011; Miyashita & Reed, 1995; Smeenk et al., 2011). To determine whether the upregulation of FasL and Bax were mediated by p53 in TGEV-infected PK-15 cells, we detected the expression and activation of p53 in TGEV-infected cells by Western blot analysis and luciferase reporter assay. The results showed that TGEV infection increased the protein level of p53 and resulted in phosphorylation of p53 at serine 15, 20 and 46 (Fig. 1a). The ratio of p-p53 : total p53 showed that after 12 h post-infection (p.i.), the changes in some p-p53 species appeared to plateau or decline a little. Despite some p-p53 species showing such a change over the period of TGEV infection, the level of p53 phosphorylation was still maintained at a higher level through the whole infectious time in TGEV-infected cells compared with that in mock-infected cells (Fig. 1b). To further test whether increased p53 protein subsequently resulted in concurrent increase in its transcriptional activity, PK-15 cells were transfected with a firefly luciferase plasmid that contains a p53 consensus transcription binding sequence. After 12 h transfection, the cells were infected with or without 10 m.o.i. of TGEV and luciferase activity was determined at the indicated times. The results showed that p53 transcriptional activity was increased as early as 4 h p.i., peaked by 12 h p.i., followed by a decline, but still maintained a higher level through the whole infectious time (Fig. 1c), suggesting that p53 is activated in TGEV-infected PK-15 cells.

**p53 plays a pivotal role in TGEV induction of cell apoptosis**

To further explore the roles of p53 activation in TGEV-induced apoptosis, we investigated the effects of pifithrin-α (PFT-α), a specific inhibitor of p53, on the expression of FasL, Bax and Bcl-2, caspase-3 activity and apoptosis induction. In TGEV-infected cells, addition of PFT-α inhibited TGEV-induced p53 translocation to the nucleus (Fig. 2a). As predicted, pre-incubation of PK-15 cells with PFT-α downregulated FasL, upregulated Bcl-2 in protein levels and evidently attenuated Bax and cytochrome c (Cyt c) redistribution in mitochondria and the cytosol in TGEV-infected PK-15 cells (Fig. 2b, c). In addition, caspase-3 activity was remarkably decreased in the TGEV-infected cells with PFT-α compared with that without PFT-α (Fig. 2d). Consequently, PFT-α treatment significantly decreased TGEV-induced apoptosis in PK-15 cells (Fig. 2e). To test whether the p53 inhibitor affects TGEV replication, we measured the effects of PFT-α on the transcription of TGEV structural genes. Results showed that mRNA levels of TGEV genes did not appear significantly different between inhibitor-treated cells and control cells at 12 and 24 h p.i. (Fig. 2f). These results suggest that p53 play a pivotal role in the process of TGEV induction of cell apoptosis.

**TGEV infection downregulates MDM2 and p300/CBP in PK-15 cells**

Since p53 is a potent inhibitor of cell growth as well as a potent inducer of cell death, its cellular activity must be held in tight control. MDM2 has been assigned as a major molecule in the regulation of p53 through forming a negative feedback loop with p53, acting as an E3 ubiquitin ligase to promote p53 ubiquitination and proteasomal degradation (Li et al., 2001; Mayo & Donner, 2002). TGEV infection increased the protein level of p53. Thus, we asked whether increased p53 protein is associated with the change of MDM2 in TGEV-infected cells. As expected, the protein levels of MDM2 decreased with the increase of infectious time in TGEV-infected cells (Fig. 3a), suggesting that in response to TGEV infection, a decrease in MDM2 protein levels might lead to increased p53 stabilization.

In addition to MDM2, the p300/CBP transcription coactivators play an important role in regulating MDM2 stability and cooperating with MDM2 to promote p53 degradation (Grossman et al., 1998; Shi et al., 2009). So it is interesting for us to further detect the levels of p300 and...
CBP in TGEV-infected cells. We observed that the levels of p300 and CBP were markedly decreased in PK-15 cells after infection with 10 m.o.i. of TGEV for 8 h, and progressively declined at subsequent time points (Fig. 3b). These results suggest that TGEV infection downregulates p300, CBP and MDM2, which might work together to cause an increase in steady-state p53 abundance and p53 half-life in infected cells.

**Fig. 1.** Activation of p53 by TGEV infection. (a) PK-15 cells were infected with 10 m.o.i. of TGEV. Cells were collected at the indicated times and subjected to Western blot analysis using antibodies against p53 and p53-specific phosphorylation sites. (b) Ratio of p-p53 : total p53. Representative densitometry of p-p53 relative to total p53 was calculated after being normalized to β-actin. Results are expressed as the mean ± SEM of three separate experiments. (c) Luciferase assay. PK-15 cells were co-transfected with p53 fiery luciferase reporter plasmid p53-TA-luc and pRL-TK- Renilla-luciferase plasmid. Then the cells were infected with 10 m.o.i. of TGEV for different times. Firefly luciferase activity was measured and normalized by Renilla luciferase activity at indicated times. The value of p53 relative activity in initial mock-infected cells at 0 h was defined as 1; other values are plotted as the number of folds above to it. Values are means ± SEM of three independent experiments. *P<0.05, ** P<0.01 versus mock infection or cells at 0 h p.i.
TGEV infection promotes p38 MAPK signalling activation

The MAPK cascades, especially p38 MAPK and JNK signalling pathways, have been previously reported to mediate the degradation of p300 (Li et al., 2012), the activation of p53 (Lin et al., 2009), or regulation of p53-mediated apoptosis signalling pathways (Lin et al., 2009; Wu, 2004). We therefore determined the effects of TGEV infection on p38 MAPK and JNK signalling pathways in PK-15 cells. The results showed that phospho-p38 increased in a time-dependent manner during the TGEV infection period, while the phosphorylation levels of the main isoforms of JNK, p46/JNK1 and p53 increased in a time-dependent manner during the TGEV infection period. The results also showed that the phosphorylation levels of the main isoforms of JNK, p46/JNK1 and p53 increased in a time-dependent manner during the TGEV infection period.
p54/JNK2, did not appear to obviously increase (Fig. 4a), suggesting that the p38 MAPK pathway is activated during TGEV infection. Next, we tested whether signalling of p38 MAPK and JNK was involved in TGEV-induced p53 activation and apoptosis. As predicted, addition of SB203580 (a specific inhibitor of p38 MAPK) attenuated TGEV-induced phosphorylation of p38 (Fig. 4b). Incubation with SP600125 (a specific inhibitor of JNK) attenuated cisplatin-induced phosphorylation of JNK (Fig. 4c). This showed that the inhibitors work in our system. The inhibitor of p38 downregulated FasL and Bax, upregulated Bcl-2 in protein levels and partly prevented TGEV-induced apoptosis in TGEV-infected PK-15 cells (Fig. 4d, e). However, the inhibitor of JNK failed to block FasL and Bax upregulation, Bcl-2 downregulation induced by TGEV infection, and to protect cells from TGEV induction of apoptosis in infected cells (Fig. 4d, e). It has been reported that SP600125 could induce p38 phosphorylation in macrophages (Stempin et al., 2008). To test whether this side effect occurs in PK-15 cells, we detected the phosphorylated p38 in the presence of 20 μM of SP600125. Results showed that SP600125 did not affect p38 phosphorylation in PK-15 cells (Fig. 4d). To test whether the p38 MAPK inhibitor affects TGEV replication, the effects of SB203580 on the transcription of TGEV genes were measured. Results showed that mRNA levels of TGEV genes did not appear to be significantly different between SB203580-treated cells and control cells at 12 and 24 h p.i. (Fig. 4f). These results suggest that p38 MAPK signalling might be activated and involved in the regulation of apoptosis in TGEV-infected PK-15 cells.

**Activation of p53 is partly mediated by p38 MAPK activation upon TGEV infection**

To determine the temporal relationship between p53 and p38 activation in TGEV-infected cells, we tested phospho-p38 and phospho-p53 levels during the earlier phase of TGEV infection. As shown in Fig. 5(a), phosphorylation of p53 at serines 15, 20 and 46 was observed as early as 4, 4 and 8 h p.i., respectively, whereas phospho-p38 rapidly increased from 0.25 through to 1 h when TGEV were inoculated into cells, followed by a decrease at 1.5 and 3 h p.i., then increased secondarily at 4 h p.i. and subsequently steadily increased in TGEV-infected cells. These results suggested that the interaction between TGEV and cells in the early phase could promote the transient activation of p38 MAPK, and that p38 MAPK signalling might be activated prior to p53 signalling in TGEV-infected cells.

Next, we tested whether the activation of p38 MAPK signalling is involved in the regulation of the abundance, stability and activation of p53. We examined the effect of a p38 MAPK-specific inhibitor on the levels of p300, CBP and MDM2 and the phosphorylation of p53 in TGEV-infected cells. The results showed that SB203580 treatment increased the levels of MDM2, p300 and CBP, and attenuated phosphorylation of p53 at serines 15, 20 and 46 (Fig. 5b, c). Correlating with decreased phospho-p53 by SB203580, inhibition of p38 MAPK signalling reduced the abundance and transcriptional activity of p53 in TGEV-infected cells as expected, but did not completely abolish them (Fig. 5b, d), suggesting that activation of p53 signalling is at least partly dependent on p38 MAPK activation in TGEV-infected cells. However, the inhibition of p53 transcriptional activity by PTF-α did not appear to have a marked effect on the phosphorylation of p38 MAPK in TGEV-infected cells (Fig. 5e). These results suggest that p38 MAPK activation is partly involved in the regulation of p53 stability and activation in TGEV-infected cells.

**Constant activation of p53 and p38 MAPK require virus replication**

To further determine whether p53 and p38 MAPK activation induced by TGEV is also associated with viral replication, we used UV treatment to abrogate replication of viruses partly or completely and to investigate the capacity of UV-treated TGEV to promote p53 and p38 phosphorylation. Results showed that no virus progeny were produced when cells were infected with 3000 J m⁻² UV-inactivated virus (Fig. 6a). UV-inactivated TGEV lost the ability to induce p53 accumulation and p53 phosphorylation, and to downregulate the levels of MDM2, p300 and CBP in PK-15 cells at 24 h p.i. (Fig. 6b),
Fig. 4. The activation of p38-MAPK signalling by TGEV (10 m.o.i.) infection. (a) PK-15 cells were mock infected or TGEV infected for different time periods. Western blot analysis was performed using the indicated antibodies to detect phosphorylated p38 and JNK. (b) Effect of p38-MAPK specific inhibitor (SB203580) on the phosphorylation of p38. PK-15 cells were infected with TGEV for 24 h in the presence or absence of different concentrations of SB203580 (5, 10 and 20 µM). Cells were collected and subjected to Western blot analysis. (c) Effect of JNK specific inhibitor (SP600125) on the phosphorylation of JNK. PK-15 cells were treated with cisplatin (50 µg ml⁻¹) for 24 h in the presence or absence of different concentrations of SP600125 (5, 10 and 20 µM). Cells were collected and subjected to Western blot analysis. (d) Effects of SB203580 or SP600125 on the expression of FasL, Bcl-2 and Bax. PK-15 cells were infected with TGEV for 24 h in the presence or absence of SB203580 (20 µM) or SP600125 (20 µM), and the proteins were detected by Western blot analysis. (e) Effect of SB203580 or SP600125 on TGEV-induced apoptosis. PK-15 cells were infected with TGEV for 24 h in the presence or absence of SB203580 (20 µM) or SP600125 (20 µM), and the apoptotic ratio was analysed by flow cytometry. Values are shown as mean ± SEM of three independent experiments. *P<0.05 versus TGEV infection without inhibitors. (f) Effects of SB203580 on the transcription of TGEV genes. PK-15 cells were pretreated with DMSO or SB203580 (20 µM) for 1 h and then infected with TGEV (10 m.o.i.) for 1 h. After removal of the inoculum, the cells were continuously treated with DMSO or 20 µM SB203580 for 12 and 24 h. Cells were collected and total cellular RNA was isolated. The relative TGEV genes mRNA levels were detected by qRT-PCR. Data are means ± SEM of three independent experiments.
suggesting that TGEV-induced p53 accumulation and activation require viral replication in cells. Unlike p53, however, p38 MAPK was activated during the earlier phase of TGEV inoculation. To determine whether earlier activation of p38 MAPK just requires the interaction between TGEV and the cell surface, we tested phospho-p38 levels in the earlier phase of UV-inactivated TGEV infection. The results showed that phospho-p38 rapidly increased from 0.25 through to 1 h when UV-inactivated TGEV were inoculated into cells. This was followed by a decrease at 1.5 h p.i., which was similar to the change in TGEV-inoculated cells, but UV-inactivated TGEV were not
able to promote phosphorylation of p38 MAPK secondarily after 4 h p.i. (Fig. 6c), suggesting that TGEV can bind to a cell-surface receptor to trigger the transient activation of p38 MAPK in the early phase of inoculation, while the constant activation of p38 MAPK requires viral replication in the later phase of infection in cells.

**DISCUSSION**

TGEV has also been shown to induce cell apoptosis through the activation of FasL/Fas and mitochondria-mediated caspase-dependent pathways in our previous study (Ding et al., 2012). In response to viral infections, induction of infected cell apoptosis can prevent viral replication, viral dissemination or persistent viral infection of the cell, and be regarded as a predominant event in viral diseases pathogenesis. The regulation of cell apoptosis actually involves the activation of many cell signalling pathways (Barber, 2001). Accumulating evidence has begun to support the notion that either the p53 or the p38 MAPK pathway or both may have a critical role in viral-induced apoptosis (Austin et al., 2012; Mizutani et al., 2004; Padhan et al., 2008), viral replication (Rahaus et al., 2004; Zhou et al., 2012), as well as host antiviral defence (Desloges et al., 2008; Muñoz-Fontela et al., 2008; Regan et al., 2009). In this study, we observed that TGEV induced the activation of p53 and p38 MAPK in a different pattern. Activated p53 signalling played a dominant role in triggering the occurrence of apoptosis through regulating the expression of apoptosis-associated genes, whereas the activation of p38 MAPK was only partially responsible for the activation of p53 and contributed minimally to the TGEV-induced apoptosis.

p53 and p38 MAPK signalings are involved in different essential cellular events or functions, such as the cell cycle, cell differentiation, apoptosis or inflammation in specific cell types upon different stresses (Gostissa et al., 2003; Zarubin & Han, 2005). In coronaviruses, (i) MHV can activate p53 to induced G0/G1 cell cycle arrest (Chen et al., 2004) and activate p38 MAPK to regulate virus replication and cytokine production (Banerjee et al., 2002); (ii) feline infectious peritonitis virus can activate p38 MAPK to regulate pro-inflammatory cytokine production (Banerjee et al., 2002); and (iii) SARS-CoV 3a protein causes p38 MAPK-mediated upregulation of p53, which plays an important role in 3a protein-mediated apoptosis (Padhan et al., 2008). In this study, TGEV infection activated p53 and p38 MAPK pathways in PK-15 cells. TGEV infection promoted the accumulation and activation of p53 through decreasing p300/CBP, downregulating MDM2 and inducing

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**Fig. 6.** The activation of p38 MAPK and p53 in the cells treated with UV-inactivated TGEV. (a) Determination of the viral titres of UV-inactivated TGEV using PK-15 cells. Virus was treated with different UV doses, cells were infected and then virus titres were measured. (b) TGEV were treated with different doses of UV, and then infected PK-15 cells for 24 h. Western blot analysis of p53, p53 phosphorylation, p38 phosphorylation, MDM2, p300 and CBP in the cells infected with UV-inactivated TGEV. (c) PK-15 cells were infected with 10 m.o.i. of UV-TGEV inactivated by 3000 J m\(^{-2}\). Western blot analysis of phosphorylation of p38 at different infection times.
p53 phosphorylation at serine 15, 20 and 46 in PK-15 cells. In TGEV-infected cells, the total p53 and phosphorylated p53 protein continuously increased or maintained a higher level from 4 to 36 h p.i., while the transcriptional activity of p53 measured by the luciferase-reporter-containing p53 response element increased from 8 h p.i. and peaked by 12 h p.i. This was followed by a steady decline from 12 to 36 h p.i., which might be due to the different roles and effects played by virus and host cells at different regulation sites in the p53 signalling pathway, suggesting that p53 regulation is a complex process in virus-infected cells. Nonetheless, p53 signalling was continuously activated in the TGEV-infected cells after 8 h p.i. whereas TGEV infection induced the transient phosphorylation of p38 MAPK in the early phase of inoculation (0.25–1 h p.i.) and constant phosphorylation in the later phase of infection (4 h p.i.). In TGEV-infected cells, phospho-p38 was detected earlier than phospho-p53 and showed two peaks, but phospho-p53 was not detected synchronously with phospho-p38 in the early phase (0.25–1 h p.i.) and did not follow the same trend as p38 MAPK. In the cells treated with UV-inactivated TGEV, phospho-p38 also could be detected in the early phase (0.25–1 h p.i.) but not at subsequent times, whereas phospho-p53 could not be detected at any of the times we tested. These results demonstrate that p53 and p38 MAPK are activated via different ways in cells following TGEV infection. TGEV triggers the activation of p38 MAPK through binding to a cell-surface receptor or virus replication, whereas TGEV-induced p53 activation is dependent on virus replication, at least on viral gene expression. In addition, we noted that phosphorylated p38 at early times (after 1 h p.i.) did show a different decrease rate between TGEV infection (Fig. 5a) and UV-inactivated TGEV infection (Fig. 6c). One reason for this difference is that PK-15 cells were infected with 10 m.o.i. of TGEV in Fig. 5(a), but with UV-inactivated TGEV in Fig. 6(c). Another reason for this difference is that the infection methods were different in these two experiments. In the experiments of Fig. 5(a), PK-15 cells were infected with 10 m.o.i. of TGEV. After 1 h of TGEV adsorption, the virus inoculum was removed, then cells were washed with PBS and fresh basal medium containing 2% serum was added. However, in the experiments of Fig. 6(c), PK-15 cells were infected with UV-inactivated TGEV. The virus inoculum was not removed after 1 h of TGEV adsorption.

Previous studies have demonstrated a regulatory role of p38 MAPK in p53 activation (Lin et al., 2009; Padhan et al., 2008; Perfettini et al., 2005). Activated p38 MAPK signalling can phosphorylate p300 and induce proteasome-mediated p300 degradation (Chen & Li, 2011), and phosphorylate p53 at serine 46, which is involved in selectivity of certain apoptotic target genes (Perfettini et al., 2005; Smeenk et al., 2011). Although p53 and p38 MAPK pathways are activated in a different way, a possible role of p38 MAPK pathways in the activation of p53 has been suggested in this study. In TGEV-infected cells, inhibition of p38 MAPK by SB203580 could partly attenuate p53 activation including total p53, p53 phosphorylation, p53 transcriptional activity, and MDM2 and p300 loss, whereas inhibition of p53 by PFT-α had almost no effect on the phospho-p38. Although these results suggest that p38 MAPK might regulate p53 activation, p38 MAPK might contribute minimally to the p53 activation in TGEV-infected cells. Taken together, we concluded that p38 MAPK is only partially responsible for the activation of p53 in TGEV-infected cells.

The different activation pattern between p53 and p38 MAPK signalling pathways suggest this deserves special attention to establish whether these two pathways are involved in different intracellular events, or play a different role in the same intracellular event in TGEV-infected cells. Our results presented here also provide evidence for the different role of p53 and p38 MAPK signalling pathways in TGEV-induced apoptosis. TGEV-induced phosphorylation of p38 was partially inhibited by the p38 inhibitor SB203580 at 10 μM and completely inhibited at 20 μM, but the inhibition of apoptosis and apoptosis-associated genes by SB203580 was minimal (though statistically significant) and was not dose dependent, suggesting that activation of p38 signalling minimally contributes to TGEV-induced apoptosis. However, inhibition of p53 signalling by PFT-α significantly inhibited apoptosis-associated gene expression and activation, and almost completely inhibited the occurrence of apoptosis. These results demonstrate that p53 signalling plays a more powerful role than p38 signalling in regulating apoptosis-associated genes and the occurrence of apoptosis, and also suggest that virus–host interaction is a complex process.

In conclusion, we have provided evidence that p53 and p38 MAPK also function as key regulators of intracellular activity in the interaction of TGEV with host cells. Our study revealed a different pattern between the p53 and the p38 MAPK pathways following TGEV infection, provided enough evidence for the role of p53 in mediating TGEV-induced apoptosis, and analysed the possible interaction between p53 and p38 MAPK pathways in TGEV-infected cells. These findings suggest a significant contribution of p53 in cell apoptosis regulation, with potential implications in the pathogenesis of TGEV infection.

**METHODS**

**Antibodies, inhibitors, cells and virus.** mAbs against total p53, JNK1/2, p-JNK1/2, p38, p-p38, p300, MDM2, CBP, cytochrome c (Cyt c), Bax, Bcl-2, Fasl, β-actin and Cox4 were purchased from Santa Cruz Biotechnology. p-p33 (Ser15), p-p33 (Ser20) and p-p33 (Ser46) were purchased from Cell Signalling Technology. Anti-histone H3 antibody and PTF-α were obtained from Sigma. HRP-conjugated secondary antibody was purchased from Pierce, and SB203580 and SP600125 were from Merck. PK-15 cells (ATCC, CCL-33) were grown in Dulbecco’s minimal essential medium (Gibco), supplemented with 10% heat-inactivated FBS (Gibco), 100 IU penicillin and 100 μg streptomycin ml⁻¹ (Gibco), at 37 °C in humidified 5% CO₂. The TGEV Shaanxi strain was isolated from intestinal tract contents of TGEV-infected piglets in Shaanxi Province of China and propagated in PK-15 cells (Ding et al., 2012). Virus titres were determined by TCID₅₀ (Reed & Muench, 1938).
Luciferase assay. p53-TA-luc (Beyotime Biotechnology) is a cis-reporting system containing the firefly luciferase reporter gene, which is controlled by a synthetic promoter that contains the transcription recognition sequences for p53. First, PK-15 cells (90% confluent in a 96-well plate) were co-transfected with p53 firefly luciferase reporter plasmid p53-TA-luc plasmid (200 ng) and pRL-TK-Renilla-luciferase plasmid (20 ng) (Promega). The cells were then infected with 10 m.o.i. of TGEV 24 h after transfection. Firefly luciferase activities were measured by the Dual-Glo Luciferase Assay System (Promega) according to the manufacturer’s instructions. Data were normalized for transfection efficiency by dividing firefly luciferase activity by that of Renilla luciferase.

Caspase activity assay. Caspase-3 activity was measured by colorimetric assay kit (BioVision) according to the manufacturer’s instructions as described previously (Ding et al., 2012).

Western blot analysis. Cell extracts were prepared as described previously (Ding et al., 2012). Isolation of nuclear and cytosolic proteins was performed using the Nuclear/Cytosol Fractionation kit (BioVision). Protein concentrations were measured using BCA Protein Assay Reagent (Pierce). Equivalent amounts of proteins were subjected to Western blot analysis as described previously (Ding et al., 2012).

Flow cytometry analysis. Annexin V-FITC Apoptosis kit (BioVision) was used for apoptosis detection according to the manufacturer’s protocol as described previously (Li et al., 2012).

Quantitative real-time PCR (qRT-PCR). Total RNA extraction, reverse transcription and cDNA amplification by qRT-PCR were performed and analysed as described previously (Ding et al., 2012). The first-strand cDNA (10 ng) was subjected to 45 cycles of real-time PCR using primers 5’-GGGTGTACCGTCTGGGAAAC-3’ and 5’-AAGCCACTAA GTAGCTGCTAGTAAT-3’ and 5’-GAGCTTGCCTTCTAGGCTCC-3’ for TGEV-S; 5’-GAGCTTGGGCTTCTAGGCTCC-3’ and 5’-GCAACA CACCATGCTAGCT-3’ for TGEV-E; 5’-GTACAGCCTGATTGATGATG-3’ and 5’-AAT GCCATACAGGAGAA-3’ for TGEV-M; and 5’-ACGAAGG-CTGGAGAGAAGAC-3’ and 5’-GGTCTGTA ACACAGATGGACAC-3’ for TGEV-N. The reference gene, β-actin, was amplified with primers (5’-GGACTTCTGAGCAGAGATG-3’ and 5’-AGGAAGG-AGGGCTGGAAGAG-3’). The relative quantification of gene expression was analysed by the two-ddCt method after all qRT-PCR results were normalized according to β-actin.

Statistical analysis. Data are means ± SEM of three independent experiments. Results were analysed by one-way ANOVA. A value of \( P<0.05 \) was considered significant.

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REFERENCES


