p53 as a retrovirus-induced oxidative stress modulator

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Infection of astrocytes by the neuropathogenic mutant of Moloney murine leukemia virus, ts1, exhibits increased levels of reactive oxygen species (ROS) and signs of oxidative stress compared with uninfected astrocytes. Previously, we have demonstrated that ts1 infection caused two separate events of ROS upregulation. The first upregulation occurs during early viral establishment in host cells and the second during the virus-mediated apoptotic process. In this study, we show that virus-mediated ROS upregulation activates the protein kinase, ataxia telangiectasia mutated, which in turn phosphorylates serine 15 on p53. This activation of p53 however, is unlikely associated with ts1-induced cell death. Rather p53 appears to be involved in suppressing intracellular ROS levels in astrocytes under oxidative stress. The activated p53 appears to delay retroviral gene expression by suppressing NADPH oxidase, a superoxide-producing enzyme. These results suggest that p53 plays a role as a retrovirus-mediated oxidative stress modulator.

Reactive oxygen species (ROS) are constantly produced during normal cellular processes and cells engage in a variety of ways to regulate or modulate ROS levels to maintain redox homeostasis to control cell fate. Increasing evidence suggests that intracellular ROS may have various cellular consequences depending on their concentration. Therefore, it is important to understand the mechanisms by which cells channel ROS to accomplish the desired outcomes (Bensaad & Vousden, 2005; Rhee, 2006). It is well known that the tumour suppressor p53 restricts abnormal cell proliferation through either growth arrest or apoptosis in response to oxidative and genotoxic stresses (Olovnikov et al., 2009; Purvis et al., 2012). However, in recent years, it has been shown that p53 protects the genome from oxidation by decreasing intracellular ROS (Bensaad & Vousden, 2005; Bensaad et al., 2006; Hu et al., 2010; Sablina et al., 2005; Suzuki et al., 2010). The absence of p53 exposed cells constantly to higher levels of ROS and treatment with antioxidants extended the life span of cancer-prone p53−/− mice, suggesting that p53 plays important roles in suppressing ROS upregulation (Sablina et al., 2005).

Although upregulation of p53 accompanied by that oxidative stress is a feature of several viral infections of the central nervous system (CNS), most of these studies have assumed that p53 activation by retrovirus is associated with cellular apoptosis (Ehsan et al., 2000; Garden et al., 2004; Jayadev et al., 2007; Jones et al., 2007; Mukerjee et al., 2008; Nardacci et al., 2005; Noorbakhsh et al., 2010). We have observed that upregulation of p53 in the CNS occurred in ts1-infected mice. However, p53-positive astrocytes in the ts1-infected mouse brain did not exhibit the characteristic morphology of apoptotic cells suggesting that p53 might not be involved in ts1-mediated apoptosis (Kim et al., 2002).

Recently, we have reported that ROS upregulation during the early phase of retrovirus infection plays an important role in viral establishment in the host cell (Kim & Wong, 2013). To investigate whether ROS increases p53 phosphorylation, we treated C1 astrocyte cells with 100 μM H₂O₂, a dose that does not cause cell death. ATM, the protein kinase encoded by the ataxia-telangiectasia (A-T) mutated gene (Atm), is a p53 upstream kinase and is crucial in regulating cellular redox status. We and others have previously observed that ATM activation by ROS appeared to be a separate event from the DNA damage response (Bagley et al., 2007; Guo et al., 2010; Kim & Wong, 2009a, b; Schubert et al., 2004). To assess the activation of ATM and p53, Western blotting was performed using the following antibodies: mouse anti-ATM (Novus Biological), mouse anti-phospho ATM (serine 1981, Cell Signalling), rabbit anti-p53 (Cell Signalling), rabbit anti-phospho p53 (serine 15, Cell Signalling), HRP-conjugated anti-mouse and anti-rabbit antibodies (Bio-Rad). As shown in Fig. 1(a), H₂O₂ activated ATM, resulting in phosphorylation of p53 at serine 15. To verify ATM phosphorylates p53 in response to ROS, we treated C1 cells with 5 mM Ku55933, an ATM inhibitor. As shown in Fig. 1(b), the H₂O₂-induced p53 phosphorylation was inhibited by Ku55933 treatment. We have previously shown that ts1 infection enhances intracellular ROS levels during the early phase of infection (4–8 h post-infection, p.i.) and this ROS upregulation is a separate event from the mitochondria-dependent cell death (after 48 h p.i.) in C1 cells (Kim & Wong, 2013). We suspected...
that ATM and p53 might be activated as a consequence of the early ROS upregulation after ts1 infection. Virus titres were determined by a modified direct focus-forming assay as described previously (Wong et al., 1985) and C1 astrocytes were infected with ts1 at an m.o.i. of 5. As shown in Fig. 1(c), ATM activation occurred at 4 h p.i. This ATM activation preceded p53 phosphorylation at 8 h p.i. and treatment with Ku55933 prevented p53 activation, suggesting ATM is an upstream kinase for p53 (Fig. 1d). The development of ts1-mediated neuropathogenesis is caused by accumulation of the misfolded viral precursor envelope protein (gPr80env), which accumulates in the endoplasmic reticulum leading to unfolded protein response (UPR) (Kuang et al., 2010). We treated C1 cells with 0.5 μg tunicamycin ml⁻¹ for 8 h to cause UPR without viral infection. As shown in Fig. 1(e), UPR did not activate p53. To verify UPR, Western blotting was performed using a rabbit anti-BiP antibody (Cell Signalling).

As mentioned before, ts1 infection causes two distinct phases of ROS upregulation; one involved in the establishment of viral infection and the other involved in ts1-mediated cell death (Kim & Wong, 2013). To investigate whether p53 upregulation is associated with ts1-mediated cell death during the late phase, 2-day-old mice were inoculated intraperitoneally with 2 x 10⁶ infectious units of ts1 virus as described previously (Kim & Wong, 2013). We differentiated disease progression into four stages: stage I, hindlimb drop; stage II, tremor; stage III, loss of mobility; stage IV, paralysis. The mice were sacrificed at stage IV. Animal care was in accordance with The University of Texas M. D. Anderson Cancer Center guidelines for animal experiments. As shown in Fig. 2(a), the absence of p53 did not prevent ts1-mediated neurodegeneration. We also cultured primary astrocytic cultures (PACs) from p53+/+ and p53−/− mouse brains by a method described previously and infected them with ts1 at an m.o.i. of 20 (Lin et al., 1997). As shown in Fig. 2(b), the absence of p53 did not prevent ts1-induced cell death. This raises the question of what is the function of p53 in ts1-infected astrocytes? Previous studies by other researchers demonstrated that expression of p53 increased intracellular antioxidant levels (Bensaad et al., 2006; Hu et al., 2010; Sablina et al., 2005; Suzuki et al., 2010). By the same token, the lack of p53 could hamper anti-oxidant defences in ts1-infected astrocytes. Intracellular ROS levels in PACs were determined by loading with 20 μM CM-H₂DCFDA, as described previously (Kim & Wong, 2013). As shown in Fig. 2(c), levels of intracellular ROS were higher in p53−/− PACs compared to those in p53+/+ PACs. Furthermore, when both cells were treated with the same 100 μM H₂O₂, levels of intracellular ROS were more elevated in p53−/− cells compared to in p53+/+ cells (Fig. 2d). We noticed that intracellular ROS levels in immortalized C1 cells were raised more than 250% (Kim & Wong, 2013) whereas those in PACs increased much less after the same 100 μM H₂O₂ treatment (Fig. 2d), suggesting a tighter ROS defensive mechanism in PACs compared to immortalized

**Fig. 1.** ATM activates p53 in response to intracellular ROS upregulation and ts1 infection. To access activation of ATM and p53, C1 cell lysates were harvested and subjected to immunoblotting using anti-ATM and anti-p53 antibodies after the following treatment: (a) 100 μM H₂O₂ treatment, (b) 100 μM H₂O₂ treatment with or without 5 mM Ku55933, (c) ts1 infection, (d) ts1 infection with or without 5 mM Ku55933 [C1 astrocytes were infected with ts1 (m.o.i. 5) as described previously (Kim & Wong, 2013)], (e) 0.5 μg tunicamycin ml⁻¹. To verify unfolded protein response, immunoblotting was performed using anti-BiP antibody.
We also observed that C1 cells. We also observed that ts1 infection upregulated intracellular O$_2^-$ levels higher in p53$^{-/-}$ cells measured by dihydroethidium as described previously (Fig. 2e) (Behrens et al., 2007). This indicates that p53 is involved not only in regulating intrinsic ROS levels but also in mitigating oxidative stress, particularly the stress caused by O$_2^-$. The nature of retrovirus-mediated cell death could be either direct or indirect. In the case of human immunodeficiency virus type 1 (HIV), retrovirus failed to integrate into the host genome in cells lacking ATM. Due to the absence of ATM, DNA repair is dysfunctional. Thus, Atm deficient cells were susceptible to retrovirus-induced cell death (Daniel & Pomerantz, 2005; Lau et al., 2005). Studies by other researchers demonstrated that only a small portion of cells that are productively infected with HIV died via caspase-3 mediated apoptosis, whereas the majority of cells died via pyroptosis triggered by caspase-1 (Doitsh et al., 2014). In our ts1 model, neurons are not infected by ts1; however, the infected astrocytes appear to affect neurons to cause neurodegeneration. We suspected the reason for which ts1-infected mice did not exhibit any significant differences between p53$^{++}$ and p53$^{-/-}$ mice could be that neurodegeneration might not directly be associated with virus-infected cell death. To investigate whether p53 influences effective retrovirus infection, we compared the expression of viral proteins in p53$^{++}$ and p53$^{-/-}$ PACs after ts1 infection. Western blotting was performed using rabbit anti-cleaved caspase 3 (Cell Signalling), goat anti-gp70env, rabbit anti-p30capsid and goat anti-gp91phox antibodies (Santa Cruz Biotechnology). As shown in Fig. 3(a), expression of viral proteins appeared earlier in p53$^{-/-}$ cells compared to p53$^{++}$ cells when
infected at an m.o.i. 20. Furthermore, p53−/− PACs showed higher levels of activated caspase 3 than p53+/+ PACs following ts1 infection (Fig. 3b). It remained unclear how p53 could suppress this intracellular ROS upregulation during the early phase of infection. Previously, we have demonstrated that NADPH oxidase was activated by ts1 infection and the inhibition of NADPH oxidase decreased viral integration into the host genome (Kim & Wong, 2013). As shown in Fig. 3(c), the catalytic subunit of NADPH oxidase (gp91phox) expression level was downregulated in ts1-infected p53+/+ PACs, whereas the suppression of NADPH oxidase expression did not occur in p53−/− PACs. This suggests that astrocytes may have a feedback mechanism to suppress levels of NADPH oxidase to attenuate further ROS generation although we do not know whether the suppression of NADPH oxidase is regulated by p53 directly or indirectly at present.

Many cancer cells show increased levels of intracellular ROS (Szatrowski & Nathan, 1991), and normal cells exposed to low levels of H2O2 show increased proliferation (Burdon et al., 1995; Ranjan et al., 2006). ROS levels oscillate with every cell division in various cell lines and ROS upregulation appears to play an important role in cell cycle because proliferative cells undergo transient arrest and fail to progress from G1 to S phase during treatment with various antioxidants (Deng et al., 2003; Havens et al., 2006; Iiyama et al., 2006; Sattler et al., 1999). We suspect that host cell status could be altered by the retrovirus in order to create the optimal environment for retrovirus to establish itself in host cells. HIV-1 gene expression was markedly increased by progression of cell cycle beyond the G1/S phase checkpoint (Foli et al., 2007). Cell cycle progression and viral replication in cells were efficiently suppressed with the use of cyclin-dependent kinase inhibitors (de la Fuente et al., 2003). Previously, we observed that astrocytes that survived from chronic ts1-infection (C1-ts1S) in culture were proliferating slower and producing fewer viruses than normal C1 cells. The ability of these cells to survive from ts1 infection was associated with lower levels of intracellular ROS, and higher levels of glutathione and cysteine, compared with those in acutely infected cells or with those in uninfected cells (Qiang et al., 2006). Up until now, whether entry of retrovirus into the cell could promote proliferative signalling pathway has not been established. We have speculated that retroviral infection causes intracellular ROS upregulation to promote early

Fig. 3. p53 plays a role in suppressing NADPH oxidase expression resulting in delayed viral protein expression. (a–c) PAC were isolated from 1 to 2-day-old newborn mouse pups at an m.o.i. of 20 by a method described previously (Jiang et al., 2006). p53+/+ and p53−/− PACs lysates harvested after ts1 infection and subjected to immunoblotting using indicated antibodies described in the text. gpr80env, gpr70env and p30capsid are the precursor and mature form of the envelope, and capsid protein. gp91phox is the catalytic subunit of NADPH oxidase complex. Each band represents 1, 2, 3, 4, 5 and 6 days p.i.
retroviral establishment in the host cells (Kim & Wong, 2013). The interaction between retrovirus and host cell is a mutual reaction; retrovirus is prompt in establishing its genes in the host genome and the host cells may attempt to protect themselves from viral invasion. We have hypothesized that regulating the levels of ROS can be a key determinant of viral latency and replication in astrocytes (Qiang et al., 2004). In this study, we demonstrated that the low level of ROS upregulation activated ATM, which phosphorylated p53 during the early phase of ts1 infection. Our finding is consistent with studies by other researchers showing that the exposure of cells to HIV pseudoparticles activates p53 at 4 h and Ku55933 treatment inhibits p53 activation (Lau et al., 2005). Our study here attempts to reveal the role of p53 activation during the early phase of ts1 infection. We have reported that NADPH oxidase was activated in ts1-infected cells (Kim & Wong, 2013). NADPH oxidase activation also occurs in HIV infection (Olivetta et al., 2009, 2005; Salmen et al., 2010), and overexpression of superoxide dismutase, which converts O$_2^–$ to H$_2$O$_2$, delays neurodegeneration in a HIV mouse model (Louboutin et al., 2007). Here we showed that lack of p53 compromised intracellular ROS levels in astrocytes and this ROS upregulation appears to facilitate viral gene expression in the host cells compared to wild-type cells (Fig. 3a). As shown in Fig. 3(b), we also observed that ts1 infection causes caspase 3-mediated cell death in the p53 deficient astrocytes, suggesting more efficient retroviral infection in p53 deficient astrocytes than wild-type astrocytes (Doitsh et al., 2014). This observation further supports our hypothesis that low levels of ROS upregulation in p53 deficient astrocytes can facilitate retroviral gene expression. In addition, we observed suppression of NADPH oxidase expression in the presence of p53 during the process of ts1 infection (Fig. 3c). Based on our observations, we hypothesize that suppression of the ROS upregulation may provide an effective strategy for preventing retroviral establishment in the host genome and p53 plays a role in suppressing virus-mediated ROS upregulation. We propose that a better understanding of retrovirus life cycle and its interaction with host cells may lead to improved treatment strategies for retrovirus infection-mediated neurodegeneration.

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References


