Resistance to viral infection of super p53 mice

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Induction of expression of the tumor suppressor p53 after interferon treatment has been recently demonstrated (Takaoka *et al.*, 2003), suggesting an antiviral activity of the protein. In addition, a direct correlation between p53 levels and tumor resistance has been addressed by generating mice with an extra copy of p53 ('super p53' mice) (Garcia-Cao *et al.*, 2002). Here, we show that vesicular stomatitis virus replication in mouse embryo fibroblasts derived from 'super p53' mice is impaired as a result of apoptosis induction via p53 activation. These findings unequivocally demonstrate an antiviral activity of p53, a process that may contribute to inhibit the spread of the virus *in vivo*.

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Interferons (IFNs) are cytokines responsible for conferring an antiviral state on cells and with a role in rejection of malignancies (reviewed by Clemens, 2003). These effects are mediated by the gene products activated or induced following the interaction of IFNs with their cognate receptors(s), involved in a variety of processes, including inflammation, cellular stress-response programmes and apoptosis (reviewed by Samuel, 2001).

p53 acts as a tumor suppressor through its capacity to induce cell cycle arrest and apoptosis in response to a variety of cellular stresses (reviewed by Levine, 1997) like DNA damage, transcription inhibition, depletion of nucleotide pools, oncogene expression and heat shock (Ljungman, 2000; Pluquet and Hainaut, 2001). A recent paper by Takaoka *et al.* (2003) demonstrated that IFN treatment of the cells induced p53 gene expression and p53-mediated apoptosis, which translated in antiviral protection. The antiviral activity of this tumor suppressor is reinforced by the fact that it is frequently targeted

by viral proteins. In this sense, it is known that the products of some viruses such as papillomavirus E6 protein, simian virus 40 large T antigen, hepatitis B X antigen, adenovirus 5 and 12 E1b protein, adenovirus E4orf, Epstein–Barr virus EBNA-5 or the human cytomegalovirus IE84 protein, all target p53 (Lane and Crawford, 1979; Linzer and Levine, 1979; Kao *et al.*, 1990; Scheffner *et al.*, 1992; Feitelson *et al.*, 1993; Szekely *et al.*, 1993; Speir *et al.*, 1994; Dobner *et al.*, 1996).

Since accessibility of the tumor to a therapeutic agent is a major limitation of a successful antitumor therapy, oncolytic viruses provide an attractive tool for cancer treatment due to their ability to replicate selectively within the tumor and to kill neighboring cancer cells by lysis and after secondary infections (Kirn *et al.*, 2001). One of the oncolytic viruses that is currently developed as a vector for tumor gene therapy is vesicular stomatitis virus (VSV). VSV is a negative-stranded RNA virus, prototypic member of the Rhabdoviridae family, and has several advantages as a vector for tumor destruction. The virus is extremely sensitive to the antiviral action of IFNs (Wagner and Rose, 1996), exhibits potent oncolytic activity both *in vitro* and *in vivo* (Balachandran and Barber, 2000; Stojdl *et al.*, 2000), replicates rapidly to high titers in virtually all transformed cells, has no transforming potential, is non-hazardous to humans and has been extensively characterized immunologically (Balachandran *et al.*, 2001). Besides, the malleability of the virus genome makes VSV an attractive vector for the delivery of proapoptotic and immunostimulatory genes into the tumor, thus potentially enhancing its oncolytic activity (Giedlin *et al.*, 2003).

Cancer resistance can be enhanced by a genetic modification, as shown with 'super p53' mice carrying supernumerary copies of the p53 gene in the form of large genomic transgenes (Garcia-Cao *et al.*, 2002). We have taken advantage of this experimental system to further challenge the proposed antiviral role of p53. In particular, the antiviral role of p53 has been so far demonstrated by comparing the response of wild-type (WT) and p53-null cells (Takaoka *et al.*, 2003). This comparison, although highly suggestive, is nonetheless limited by the fact that the absence of p53 is likely to have large pleiotropic effects that may affect indirectly

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the antiviral response. To unequivocally test the implication of p53 in antiviral defence, without the above-mentioned confounding effects, we have compared the behavior of WT and 'super p53' cells in response to viral infection.

Primary MEFs derived from p53^{-/-}, WT or 'super p53' mice were infected with 10 plaque-forming units (PFU)/cell of VSV and virus titers in supernatants, after all cells died as a result of the infection, were determined. The virus yield in WT-infected cells was around 10-fold higher than in 'super p53' MEFs and 10-fold lower than in p53^{-/-} MEFs (Figure 1a). The magnitude of the increase in the amount of virus produced from p53^{-/-} MEFs compared to control WT MEFs was similar to that reported by Takaoka *et al.* (2003). The decrease in virus yield in the 'super p53'

mice-derived MEFs indicated that 'super p53'-derived fibroblasts are significantly less permissive to VSV infection. In order to investigate if the introduction of the transgene also contributes to restrict the replication of a different viral agent, we carried out similar experiments using instead a DNA virus. As shown in Figure 1b, virus yields obtained after vaccinia virus (10 PFU/cell) infection of WT MEFs was slightly higher than that obtained in transgenic MEFs and clearly lower than in p53^{-/-} cells. However, the differences found between WT and 'super p53' MEFs were not statistically significant, indicating that the levels of p53 protein did not affect vaccinia virus infection in cultured cells. We interpret that the increased replication of vaccinia virus in p53^{-/-} cells is an indirect consequence of the absence of p53. In contrast, the results obtained with VSV unequivocally implicate p53 as a direct regulator of the antiviral response elicited by this virus.

The tumor suppressor p53 has the ability to trigger proliferation arrest and cell death upon the occurrence of a variety of stresses such as DNA damage, oncogenic stress or viral infection. The viral agent VSV also triggers apoptosis of the infected cells (Kopecky *et al.*, 2001; Gadaleta *et al.*, 2002; Sur *et al.*, 2003). In order to determine if apoptosis induced by VSV infection in MEFs derived from the transgenic mice correlated with lower viral yields in these cells, MEFs from WT, p53^{-/-} or 'super p53' mice were infected with 1 PFU/cell of VSV, and 24 h after infection, cells were processed to determine apoptosis induction. Analysis of the cells derived from the transgenic mice revealed that 89% of the cells were apoptotic. In contrast, only about 55% of the WT MEFs and 32% of the MEFs derived from the p53^{-/-} mice infected under the same conditions were apoptotic (Figure 2a). In addition, the apoptosis induced by VSV infection correlated with the activation of the proapoptotic p53 target gene Puma (Figure 2b). The higher apoptotic level observed in WT MEFs in comparison with p53^{-/-} cells was similar to previous findings (Takaoka *et al.*, 2003). These results indicate that 'super p53' mice are more sensitive to apoptosis induced by VSV infection. In addition, these observations point to apoptosis as the mechanism responsible for the decreased virus replication in cells derived from transgenic mice. In contrast, analysis of the MEFs infected with vaccinia virus revealed no statistically significant differences in the levels of apoptosis induction among the different p53 genotypes (data not shown). This is probably related to induction of vaccinia virus genes known to counteract *in vitro* the host response at both extracellular and intracellular levels (reviewed by Smith *et al.*, 1997), inhibiting the induction of apoptosis of the infected fibroblasts (Li and Beg, 2000).

The results obtained with MEFs (Figures 1 and 2) predict that 'super p53' mice should be more resistant to VSV infection compared to WT mice. In order to examine *in vivo* the higher resistance to VSV infection of the 'super p53' mice, WT or transgenic mice were intranasally inoculated with VSV (1×10^4 PFU) and the survival of these mice was monitored daily. Statistical

Figure 1 Correlation between p53 levels and the extent of VSV replication. Virus titers of the supernatant of the p53^{-/-}, WT and super p53 MEFs after the viability of all the cells was lost after VSV (a) or vaccinia virus (b) infection (10 MOI) were determined by plaque assay formation. Indicated values are means of triplicate experiments. The standard deviation of the measurements was within 15% in each assay. The results for each assay were separately conducted with at least three independent clones of MEFs derived from the different mice

Figure 2 p53-dependent apoptosis in VSV-infected cells. (a) Correlation between p53 levels and percentage of apoptotic cells in VSV-infected cells. p53^{-/-}, WT and super p53 MEFs were infected with VSV (1 MOI), and 24 h after infection, the apoptotic response was analysed by propidium iodide staining and flow cytometry and was measured as fractions with less than G1 DNA content. Percentage of apoptosis in uninfected cells is also shown (white bars). Results shown represent the average of at least three independent experiments ($\pm$ s.d.). (b) Activation of proapoptotic p53 target gene Puma by VSV infection. p53^{-/-}, WT and super p53 MEFs were infected with VSV (1 MOI) and cell lysates were analysed by immunoblotting with specific antibodies against Puma (Abcam Ltd, Cambridge, UK) and actin (MP Biomedicals, Aurora, OH, USA)

analysis of survival rate comparison between groups was performed using the nonparametric Mann–Whitney test. We observed a slight delay in the time of death of the 'super p53' mice compared with WT animals. However, as shown in Figure 3a, all mice succumbed to VSV infection within 7 days after inoculation and the results did not show statistical differences. This result is in discordance to the significant differences in the sensitivity to VSV infection found between WT and p53^{-/-} mice reported by Takaoka *et al.* (2003). These differences may be related with the intravenous route of infection used in the former studies and suggest that the route of inoculation might be critical for the antiviral action of p53. In addition, to further examine the susceptibility of 'super p53' mice to vaccinia virus, WT or transgenic mice were intranasally inoculated with vaccinia virus (5×10^6 PFU) and the weight loss was calculated at different times after infection. Mann–Whitney test of the percentage of weight loss at 1 week after infection revealed statistically significant differences between both groups ($P=0.049$) (Figure 3b). Vaccinia virus infects a broad range of host cells in

Figure 3 Susceptibility of 'super p53' mice to viral infection. (a) Survival rate of WT and 'super p53' mice after VSV infection. Five WT C57BL/6 and five 'super p53' mice of the same genetic background C57BL/6 were intranasally infected with 10^4 PFU VSV in $20 \mu\text{l}$ at the age of 3 months and mice were daily monitored for survival. Comparison between both groups of mice of the survival rate was performed using the Mann–Whitney U test. (b), Analysis of the percentage of weight loss at one week after vaccinia virus infection. Six WT and six 'super p53' mice were intranasally infected with 5×10^6 PFU vaccinia virus in $20 \mu\text{l}$ at the age of 3 months and the weight of these mice was monitored daily. Comparison between both groups of mice was performed using the Mann–Whitney U-test and represented by a box-and-whisker plot. The results obtained were statistically significant different ($P=0.049$)

culture, and usually causes their lysis. Although infection of fibroblasts with vaccinia virus *in vitro* does not induce apoptosis, this virus can trigger an apoptotic response after infection *in vivo* and in macrophages (Gonzalo *et al.*, 1994; Humlova *et al.*, 2002). These differences might explain the variations observed between the *in vivo* and *in vitro* experiments after vaccinia virus infection.

Overall, our results reveal that increased levels of p53 might affect the resistance of the cells to be infected by specific viral agents. These results have relevance on the potential clinical utility of p53. In particular, the use of oncolytic viral agents expressing p53 might be counter-productive because it could decrease viral infection and spreading through the tumoral cells. The evaluation of the viral vector to be used for gene therapy in which normal p53 is introduced back into tumour cells has to be carefully considered.

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