Regulation of the Mdr1 isoforms in a p53-deficient mouse model

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Both p53 and multidrug transporters play important roles in chemoresistance. A transcriptional dependence of the Mdr1 gene promoter by p53 was first established a decade ago, and despite intense study, the p53-Mdr1 relationship still remains vague in vivo. The general model proposes that wild-type p53 down regulates, while mutant p53 up regulates, the Mdr1 promoter. Given that many studies have utilized cancer cell lines, minimal promoters and non-specific cDNA expression for in vitro experiments, we first sought to confirm the model using dermal fibroblasts isolated from the p53-null (p53−/−) mouse. We show that the gene products of the mouse Mdr1 homologue (Mdr1a and Mdr1b), namely P-glycoprotein (P-gp), appear upregulated at both the protein and mRNA levels in p53−/− fibroblasts compared with p53+/− cells. We demonstrate that transient transfection of a mouse p53WT expression plasmid into short-term primary p53−/− fibroblasts can revert P-gp overexpression. The difference in P-gp levels has functional significance in that p53−/− fibroblasts are more resistant to the P-gp inhibitor, verapamil. Furthermore, we demonstrate that in kidney, spleen and testis, P-gp expression is elevated in the absence of p53. In contrast, other organs such as heart, liver, lung, brain, thymus and skeletal muscle, show no difference in expression between p53+/− and p53−/− mice. Thus, our data shows a tissue-specific regulation of P-gp isoforms by p53 in the context of a p53-null mouse model.

Introduction

During cancer progression, tumour cells often establish resistance to a broad spectrum of drug substrates frequently mediated by members of the ABC transporter superfamily. These are large transmembrane proteins localized to apical surfaces of cells that actively efflux xenobiotics out of the cell interior through hydrolysis of ATP (1). A common mechanism for the multidrug resistance (MDR) phenotype of cancer is due to overexpression of the Mdr1 (HUGO designation, ABCB1) gene product, P-glycoprotein (P-gp). In humans, P-gp is encoded by two loci, Mdr1 and Mdr2 (ABCB4) but in rodents encoded by a small three-member gene family, Mdr1a, Mdr1b and Mdr2 (2,3); however, to date, a role in multidrug resistance has not been attributed to either Mdr2/3 for humans or the Mdr2 gene product for rodents.

Numerous studies over the past decade have confirmed transcriptional control of the Mdr1 promoter by p53 (reviewed in ref. 4). The traditional model proposed that normal p53 could trans-repress Mdr1 transcription through mechanisms acting independently of a p53 consensus binding sequence while mutants of p53 would activate transcription of both human and rodent Mdr1 promoters (5,6). This has been demonstrated in multiple mammalian cell lines using a variety of promoter constructs, reporter genes and p53 versions in many different genetic backgrounds (i.e. wild-type, mutant or nullizygous for p53 and drug-sensitive or resistant for P-gp). Unfortunately, few studies have established a correlation with endogenous P-gp protein levels and thus functional relevance is questionable (7). More recently, new data has sought to refine the model by further validating that mutant p53 may interact with other transcription factors such as ETS to up regulate Mdr1 transcription (8). A unique orientation of the conventional p53 DNA-binding sequence was identified in the human Mdr1 promoter to serve as the repressive element (9). Another study asserts that p53 potentially interacts with histone deacetylases via an adaptor protein, mSin3a, and the TATA binding protein to mediate transcriptional repression of other p53-repressed genes (10).

Despite the multiple levels at which p53 seems to control P-gp-induced drug resistance, few studies have addressed this relationship in vivo. The complexity is further amplified, particularly in rodents, considering that there are two P-gp isoforms, Mdr1a and Mdr1b, with extensive homology of >90% (3,11) and that they are co-expressed in some tissues yet also show tissue specificities (12). Furthermore, variations in the temporal distribution of the isoforms have been determined (13) including a developmental conversion from Mdr1al/Mdr1b to predominantly Mdr1a within intestinal epithelium (14). Although alternative splicing has not been adequately confirmed for the mouse Mdr1al/Mdr1b genes, it is implicated for human Mdr1 (15) and possibly hamster pgpl (16). Clearly, a complex regulatory mechanism exists. Remarkably, however, the endogenous tissue expression of the P-gp isoforms has not been established in vivo within the context of a p53-null background for multiple tissues. Therefore, we have utilized the p53-null mouse model as a means to gain further insight into the expression regulation of P-gp by p53.

We isolated primary dermal fibroblasts from neonatal mice (mFbs) to show that both Mdr1a and Mdr1b mRNA levels are dramatically increased in the p53−/− cells compared with p53+/− cells (Figure 1A). More significantly, we show that this is recapitulated at the protein level (Figure 1B). In an attempt to demonstrate that we can get reversion of the molecular phenotype, we transfected into early passage p53−/− fibroblasts, a wild-type mouse p53 expression plasmid and then assessed P-gp protein levels after 48 h. We found a clear reduction in the amount of P-gp expression in the transfected...
Fig. 1. (A) RT–PCR of Mdr1a and Mdr1b isoforms in dermal fibroblasts from p53+/+ and p53−/− mice and NIH 3T3 cells. Total RNA was extracted with Trizol (Invitrogen, Mississauga, ON) and quantified before making first-strand cDNA with Reverse Transcriptase as described previously (17). Equal amounts of cDNA (1 µl) was used for PCR with an annealing temperature of 55°C for the indicated cycles and specific primers: Mdr1a forward, 5'-CCCATCATGGCATAGCTGG-3'; Mdr1a reverse, 5'-TCCAACATATCCGGCTTAGGC-3'; Mdr1b forward, 5'-TGCTTATGGATCCC-AGAGTGAC-3'. Mdr1b reverse, 5'-TTGTGAGGATCTCTCGGCT-3'; GAPDH forward, 5'-CTCTGACCAGTCATGCCATCATC-3'; GAPDH reverse, 5'-CTCTTTCCACCCTTCTTGATGTC-3'. (B) Total protein was isolated from fibroblasts grown in 60 mm plates and run on 10% SDS–PAGE gels for western blotting as described previously (18) and probed with antisera to P-gp that recognizes both Mdr1a and Mdr1b isoforms or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). (C) Early passage murine fibroblasts were grown to 50% confluency in 60 mm plates and transiently transfected by Effectene (Qiagen, Mississauga, ON) with either a normal mouse p53 cDNA from the pMR plasmid or an N-terminal deletion mutant mouse p53 cDNA from the pMR53 plasmid (a kind gift of Dr S. Benchimol, University of Toronto). The transfection rate was estimated to be ~40% by co-transfection with a pEGFP-N2 plasmid (BD Clontech, Palo Alto, CA). Cells were harvested, protein extracted and quantified as above for western blotting. (D) Early passage mFbs were transfected with either a CAT control plasmid, WT-Mdr1b::CAT, or MUT-Mdr1b::CAT (kind gifts of Dr M. Raymond, McGill University). Cell lysates were prepared, quantified, and then used to detect CAT expression by a CAT ELISA method as per manufacturer’s instructions (Roche, Laval, QC). These results represent the mean of duplicate experiments.

cell line versus the control p53−/− fibroblasts transfected with a GFP expression vector to evaluate our transfection efficiency (Figure 1C). In a reciprocal experiment, we were able to overexpress a mutant mouse p53 into early passage p53+/+ fibroblasts and show an increase of endogenous P-gp expression compared with the control cells perhaps by interfering with wild-type p53 function (Figure 1C).

Using a reporter construct containing a 5′-flanking sequence of the Mdr1b promoter (~1165 to +84) linked to a CAT reporter gene (WT-Mdr1b::CAT) or another version in which the p53 consensus sequence has been mutated (MUT-Mdr1b::CAT), we expressed these plasmids in p53−/− fibroblasts (19). After standardizing to a pCAT control plasmid containing a generic promoter, surprisingly, there was a modest reduction in CAT expression in p53−/− fibroblasts transfected with the WT-Mdr1b reporter while in contrast, there was a dramatic increase in CAT expression in p53+/+ cells (Figure 1D). This is contrary to previous results showing that human p53 activates a rat Mdr1b promoter (11). Interestingly, we see a comparable increase over the control in both cell lines with the MUT-Mdr1b reporter. This may suggest some p53-independent basal CAT activation from other cis-acting regulatory elements not mutated on this construct (19,20).

These results support a generalized model that the absence or dysfunction of p53 can promote increased P-gp expression. We further confirm this hypothesis with functional assays determining survival after either doxorubicin (DOX) or vincristine (VIN) treatment. As DOX and VIN are known substrates of P-gp, we treated both p53−/− and p53+/+ fibroblasts of equal passage with increasing doses of these drugs for 48 h then quantified the relative survival. There is an obvious difference in the resistance mechanism associated with P-gp overexpression, determining survival after either doxorubicin (DOX) or vincristine (VIN) treatment. As DOX and VIN are known substrates of P-gp, we treated both p53−/− and p53+/+ fibroblasts of equal passage with increasing doses of these drugs for 48 h then quantified the relative survival. There is an obvious difference in the susceptibility of the different cell genotypes. The p53−/− fibroblasts are dramatically more resistant to DOX and VIN than the p53+/+ control cells (Figure 2A and B). The strong resistance in the p53−/− fibroblasts could be substantially reversed in the presence of the P-gp inhibitor, verapamil (VER) (Figure 2C).

The organ-specific differences for the murine P-gp isoforms may be due to the nature of the polypeptide structures and thus they may have different yet overlapping substrate specificity (21). Alternatively, their respective promoters may
contain specific response elements that provide for tissue-specific expression. Although the tissue distribution of the Mdr1a and Mdr1b isoforms has been established, we found that there was a clear increase in the P-gp levels in certain organs of the p53-knockout samples over the wild-type tissues. Specifically, we detected higher expression in kidney, spleen and testis of p53<sup>−/−</sup> mice. This was evaluated at both the message and protein levels (Figures 3A and B, denoted by * in Figure 3A). In contrast, we found no difference for P-gp expression in brain, heart, liver, lung, muscle or thymus, although variable levels were evident among the different organs. Our RT–PCR results demonstrate that Mdr1a, but not Mdr1b, predominates in brain tissue supporting the findings by Demeule et al. (22); however, overamplification after additional cycles (40 cycles) produced a detectable band of Mdr1b that was more intense in the p53<sup>−/−</sup> tissue (Figure 3A). Finally, we confirm our western blot and RT–PCR results with in situ immunohistochemical staining in selected tissues. Comparable P-gp expression is seen in mouse heart from p53<sup>−/−</sup> and p53<sup>−/−</sup> mice. However, in kidney samples there is considerably stronger P-gp staining of the proximal tubules in the knockout tissue than the p53<sup>−/−</sup> tissue consistent with the results from western analysis (Figure 3D).

We feel that establishing the tissue-specific differences for the P-gp multidrug transporter in the context of the p53-knockout mouse could have insightful ramifications on the study of tumour treatment and in vivo carcinogenesis. Most functional studies ascertaining the regulatory relationship between p53 and increases in drug transporters are based on promoter/reporter control of multidrug resistant-related genes by p53 mutants in long-established cell lines. Very few studies have determined the actual transporter protein expression and fewer utilized primary cells as we have done. Therefore, in those p53-null tissues with higher basal levels of Mdr1a/Mdr1b transcripts and P-gp, a repressional rather than activational role for p53 is implicated which has particular significance for the Mdr1b isoform. This is confounded by recent data from Schuetz et al. (11) as well as Kuo et al. (23). However, we find several shortcomings with the previous reports. The in vitro activation of the rat or mouse Mdr1b promoter was performed using human normal and mutant p53 cDNAs frequently in human (SAOS-2, p53-null and Rb-inactivated) or rat (H-4-II-E) tumour cell lines with very minimal promoter constructs. We would caution that this may be a very synthetic system. The core domain of mouse and human p53<sup>−/−</sup> are known to differ by 15% at base residues. As well, there are mutational differences between the homologues, for example, UV-induced mutations do not occur at exactly the same positions, which may have functional manifestations on the protein (24).

The discovery that p53 activates Mdr1b in a sequence-specific DNA-binding manner apparently does not correspond with high throughput genomic screens using microarray (25) or SAGE (26) techniques to identify p53-responsive targets for which to our knowledge, Mdr1b was not identified. Other genes with authentic p53-binding sites can be repressed such as the anti-apoptotic gene, Survivin (27) and the interferon-inducible, p202 gene (28). Finally, in mouse liver, Lecureur et al. (11) show by RNase protection assay that Mdr1b could have higher basal expression in the p53<sup>−/−</sup> versus the p53<sup>−/+</sup> liver tissue. We detected very high levels (after 30 PCR cycles) in Figure 3A). In contrast, we found no difference for P-gp expression in brain, heart, liver, lung, muscle or thymus, although variable levels were evident among the different organs. Our RT–PCR results demonstrate that Mdr1a, but not Mdr1b, predominates in brain tissue supporting the findings by Demeule et al. (22); however, overamplification after additional cycles (40 cycles) produced a detectable band of Mdr1b that was more intense in the p53<sup>−/−</sup> tissue (Figure 3A). Finally, we confirm our western blot and RT–PCR results with in situ immunohistochemical staining in selected tissues. Comparable P-gp expression is seen in mouse heart from p53<sup>−/−</sup> and p53<sup>−/−</sup> mice. However, in kidney samples there is considerably stronger P-gp staining of the proximal tubules in the knockout tissue than the p53<sup>−/−</sup> tissue consistent with the results from western analysis (Figure 3D).

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The body of literature overwhelmingly substantiates that loss of p53 whether as an early or late event in tumourigenesis contributes to cancer severity and directly to chemoresistance through a combination of properties including inhibition of
Fig. 3. Organ-specific expression of P-gp protein and *Mdr1a* or *Mdr1b* mRNA. (A) Twelve week old *p53*+/+ (+) and *p53*−/− (−) male mice (Taconic, Germantown, NY) were killed and the organs removed for protein extraction in lysis buffer as described previously (17). (B and C) Mouse organs were homogenized with a microhomogenizer and RT–PCR performed as described earlier (Fig. 1A) except for the additional PCR cycles from the magnified region. (D) Immunohistochemistry was performed on paraffin-embedded blocks cut at 6 µm sections of biopsies from 12-week-old male mice. Briefly, tissues were dewaxed by heating at 55°C for 30 min followed by three 5 min washes in xylene. Samples were re-hydrated by washing for 5 min in 100, 90 and 70% ethanol and 30 min in PBS. Endogenous peroxidase activity was quenched in a 0.3% H2O2 solution for 10 min and unmasking performed by microwaving samples for 4.5 min in a sodium citrate solution (pH 6.0). Samples were blocked for 20 min in non-specific rabbit serum and immunolabelled using the polyclonal anti-P-gp rabbit antibody (Santa Cruz Biotechnology) with the ImmunoCruz staining kit (Santa Cruz Biotechnology). Signals were developed using a DAB substrate in a hydrogen peroxide buffer (Vector Laboratories, Burlington, ON). Experiments were repeated in organs from three animals and similar results were obtained.

apototic mechanisms and increases in endogenous drug transporters. We show that the absence of p53 directly causes an up regulation of a mouse *Mdr1b* promoter in *p53*−/− fibroblasts and that several distinct tissues show increased expression of *Mdr1a* and *Mdr1b* isoforms. The fact that P-gp appears to be expressed at different levels in some tissues of *p53*+/+ and *p53*−/− origin and similar levels in others under non-inducible conditions is intriguing. Our results imply that basal expression of P-gp isoforms is repressed either directly or indirectly by p53. This has particular ramifications on tumourigenesis and potential treatment modalities. As p53 is mutated in over half of all human cancers (29), and has a clear involvement in maintaining genomic stability, the absence of normal p53 may by default increase endogenous P-gp isoform expression and thus create a multidrug-resistant cell. While formally, the *p53*−/− mouse fibroblasts we used may be resistant as a result...
of ampalation in the Mdr locus, we find this less likely because they are early passage cells, thus, we suspect the increased resistance to be dependent on p53 loss.

The incidence of tumours may largely be determined by the genetic background on which mutations and biochemical pathways are evaluated. Therefore, knowing that genetic background on which mutations and biochemical increased resistance to be dependent on p53 loss.

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