Regulation of the \textit{Mdr1} isoforms in a \textit{p53}-deficient mouse model

Jason A. Bush and Gang Li

Division of Dermatology, Department of Medicine, Vancouver Hospital and Health Sciences Centre, University of British Columbia, Vancouver, British Columbia V6H 3Z6, Canada

1To whom correspondence should be addressed at Jack Bell Research Centre, 2660 Oak Street, Vancouver, BC, V6H 3Z6 Canada

Email: gangli@interchange.ubc.ca

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

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

Despite the multiple levels at which \textit{p53} seems to control P-gp-induced drug resistance, few studies have addressed this relationship \textit{in vivo}. The complexity is further amplified, particularly in rodents, considering that there are two P-gp isoforms, \textit{Mdr1a} and \textit{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 \textit{Mdr1a} to \textit{Mdr1b} predominantly \textit{Mdr1a} within intestinal epithelium (14). Although alternative splicing has not been adequately confirmed for the mouse \textit{Mdr1a/Mdr1b} genes, it is implicated for human \textit{Mdr1a} (15) and possibly hamster \textit{pgp1} (16). Clearly, a complex regulatory mechanism exists. Remarkably, however, the endogenous tissue expression of the P-gp isoforms has not been established \textit{in vivo} within the context of a \textit{p53}-null background for multiple tissues. Therefore, we have utilized the \textit{p53}-knockout mouse model as a means to gain further insight into the expression regulation of P-gp by \textit{p53}.

We isolated primary dermal fibroblasts from neonatal mice (mFbs) to show that both \textit{Mdr1a} and \textit{Mdr1b} mRNA levels are dramatically increased in the \textit{p53}\textsuperscript{−/−} cells compared with \textit{p53}\textsuperscript{+/+} 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 \textit{p53}\textsuperscript{−/−} fibroblasts, a wild-type mouse \textit{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

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 \textit{Mdr1} (HUGO designation, \textit{ABCB1}) gene product, P-glycoprotein (P-gp). In humans, P-gp is encoded by two loci, \textit{Mdr1} and \textit{Mdr2} (\textit{ABCB4}) but in rodents encoded by a small three-member gene family, \textit{Mdr1a}, \textit{Mdr1b} and \textit{Mdr2} (2,3); however, to date, a role in multidrug resistance has not been attributed to either \textit{Mdr2} or \textit{Mdr1b} gene product for rodents.

Abbreviations: DOX, doxorubicin; P-gp, P-glycoprotein; Vin, vincristine.
cell line versus the control p53<sup>+/−</sup> 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<sup>−/−</sup> 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<sup>+/+</sup> and p53<sup>−/−</sup> fibroblasts (19). After standardizing to a pCAT control plasmid containing a generic promoter, surprisingly, there was a modest reduction in CAT expression in p53<sup>+/−</sup> fibroblasts transfected with the WT-Mdr1b reporter while in contrast, there was a dramatic increase in CAT expression in p53<sup>−/+</sup> 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 overexpression, we treated both p53<sup>+/+</sup> and p53<sup>−/−</sup> 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<sup>−/+</sup> fibroblasts are dramatically more resistant to DOX and VIN than the p53<sup>+<//+</sup> control cells (Figure 2A and B). The strong resistance in the p53<sup>−/−</sup> 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...
organ of the $p53$-knockout samples over the wild-type tissues. Specifically, we detected higher expression in kidney, spleen and testis of $p53^{-/-}$ 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 $p53^{+/-}$ tissue (Figure 3C). 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 $p53^{+/-}$ and $p53^{-/-}$ mice. However, in kidney samples there is considerably stronger P-gp staining of the proximal tubules in the knockout tissue than the $p53^{+/-}$ 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 $p53$ 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 $p53^{+/-}$ versus the $p53^{+/-}$ liver tissue. We detected very high levels (after 30 PCR cycles) of both the $Mdr1a$ and $Mdr1b$ transcripts by RT–PCR in liver and therefore were not able to quantitatively determine a difference (data not shown).

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 µ 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 xylanes. 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.

apoptotic mechanisms and increases in endogenous drug trans-
porters. 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 amplification 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 p53 status is a factor in a wide variety of cancers (30,31), utilizing genetically engineered mice with p53 perturbations is an invaluable tool (32). With the ever-expanding list of ABC transporters involved in the multidrug resistance phenomenon, the p53-deficient mouse is a useful model system for determining organ-specific chemotherapeutic regimes to better target a particular cancer type. Moreover, it can be used to estimate the potential inherent resistance for certain substrates based on the predominance of P-gp or in fact other multidrug transporter isofoms. More precisely, we believe our observations have particular importance for understanding chemoresistance susceptibility for individuals with predisposing p53 germline mutations such as Li-Fraumeni syndrome (33,34). Given the differences between mouse and human Mdr promoters, experiments on primary human cells with null or mutant p53 backgrounds will further clarify the p53 regulation of Mdr expression.

Acknowledgements

We wish to thank K-John Cheung, Eric Campos and Paulo Lin for excellent technical assistance. This work is supported by the Canadian Dermatology Foundation and Canadian Institutes of Health Research. J.A.B. is a recipient of Roman M. Babicki Fellowship and Winton Fund Fellowship. G.L. is a Research Scientist of the National Cancer Institute of Canada supported with funds provided by the Canadian Cancer Society.

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