Regulation of p53 protein expression in human breast cancer cell lines

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INTRODUCTION

High levels of p53 protein are a common feature of many human neoplasias (Bartek et al., 1991; Cattoretti et al., 1988; Crawford et al., 1984; Iggo et al., 1990). They are associated with poor prognosis in several tumour types including breast and gastric cancer (Martin et al., 1992; Thor et al., 1992). The molecular mechanisms underlying these elevated levels of p53 protein are therefore of great practical and theoretical interest. While in many cases a close correlation has been shown between mutation of the p53 gene and accumulation of high levels of p53 in tumours (Bartek et al., 1990b; Bennett et al., 1991; Davidoff et al., 1991; Iggo et al., 1990; Rodrigues et al., 1990) it is now clear that this is not the only mechanism responsible for the enhanced expression of the protein. The normal protein can also occur at elevated levels in cells exposed to DNA damaging agents (Hall et al., 1992; Lu et al., 1992), cells transformed by papovavirus (Oren et al., 1981; Sarnow et al., 1982) and adenoviruses, and in the normal cells of certain cancer family patients (Barnes et al., 1992). Elevated levels of normal p53 have also recently been demonstrated in certain non-virally transformed cells. These include cells transformed by the ras and myc oncogenes (Lu et al., 1992) and certain cell lines derived from spontaneous tumours (Casey et al., 1991). In both these cases the pattern of expression of the normal p53 protein shows striking cellular heterogeneity, a phenomenon also noted in some human tumours (Midgley et al., 1992). Transfection of cells that express either mutant p53 (Baker et al., 1990; Casey et al., 1991; Goyette et al., 1992; Mercer et al., 1990; Mercer et al., 1991) or no p53 at all (Chen et al., 1990) with wild-type p53 can profoundly suppress their growth, but tumour cells that express high endogenous levels of the wild-type protein are apparently resistant to the growth inhibitory effects of transfection with the wild-type gene (Casey et al., 1991; Lu et al., 1992).

In order to understand the basis for these important effects we selected two well characterised breast cancer cell lines, T47D, which expresses high levels of mutant p53 and whose growth is inhibited by wild-type p53 and MCF7, which shows heterogeneous expression of wild-type p53 and whose growth is not inhibited by transfection with a plasmid encoding the wild-type gene (Bartek et al., 1990b; Casey et al., 1991). We transfected these cells with a very well characterised mouse temperature-sensitive p53 mutant gene (Michalovitz et al., 1990). This allowed us to use species-specific antibodies to examine the expression of the exogenous and endogenous genes in the same cells. Our results strongly support the concept that cellular environment rather than mutation per se is the critical regulator of p53 protein expression.

SUMMARY

Mutation of the p53 gene is a common occurrence in human breast cancers but is by no means universal. However, even in tumours where the gene is not mutated altered levels of p53 protein are often detected. This is also observed in cell lines derived from human breast cancers. By transfecting such cell lines containing either wild type or mutant p53 genes with a temperature-sensitive mutant mouse p53 gene we have established that the cellular environment plays a critical role in the regulation of p53 protein expression. The results suggest that tumours that aberrantly express wild-type p53 may have lost the normal growth regulatory response to the protein and thus be functionally similar to those expressing the mutant protein.

Key words: p53, breast cancer, p53 expression

MATERIALS AND METHODS

Cell culture
Breast cancer cell lines MCF7 and T47D were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum (FBS). For some experiments, the exponentially growing cells were infected with SV40 virus at 32°C or 37°C and cultured for a further 60 h before harvesting.

Antibodies
MAB DO-1 (Vojtěšek et al., 1992) is a monoclonal antibody specific for human p53; PAB 242, PAB 246 and PAB 248 (Yewdell...
et al., 1986) are all monoclonal antibodies specific for mouse p53.
PAb 419 (Harlow et al., 1981) is a monoclonal antibody to SV40
large T antigen. The anti-β-galactosidase monoclonal antibody
BG-2 was used as negative control.
CM-1 is a rabbit polyclonal antibody raised against pure human
p53 (Midgley et al., 1992). Polyclonal antisera to SV40 large T
antigen was prepared by immunising rabbits with pure T antigen
(Simans and Lane, 1985).

Transfection
Cells were plated at 70-80% confluency in a 25 cm² flask, and
cotransfected with 5 µg/ml MSVcl-val plasmid DNA (Michalovitz
et al., 1990) (encoding a temperature-sensitive mutant mouse p53)
and 1 µg/ml of the pSV2 neo plasmid DNA using a calcium phos-
phate method (Glover, 1985). Following exposure to DNA in cal-
cium phosphate for 4 h, cells were treated to a 15% glycerol shock
for 2 min. After a 24 h incubation at 37°C cells were trypsinized
into 4 dishes (5 cm in diameter) and drug selection applied after
a further 24 h incubation. Both breast cancer cell lines were
exposed to 600 µg/ml G418 (Gibco). Colonies arose after 14
d and were expanded for further study.

Immunocytochemistry
The indirect immunoperoxidase staining method was performed
on tissue culture cells grown on glass coverslips prefixed for 8
min in a mixture of cold methanol and acetone (1:1 by volume).
Cells were then incubated overnight at 4°C with monoclonal anti-
body supernatants and after incubation the cells were washed in
phosphate-buffered saline (PBS). Three changes of PBS were used
for all washings between applications of the staining reagents. For
indirect immunoperoxidase staining procedures, peroxidase-con-
jugated rabbit anti-mouse immunoglobulin antisera (Dako, Den-
mark) diluted 1/50 was used as the second antibody with 3,3-
diaminobenzidine in 0.03% nickel sulphate as chromagen.

Immunoprecipitation
Cells were lysed in 150 mM NaCl, 50 mM Tris, pH 8.0, 5 mM
EDTA, 1% NP40, 1 mM polymethanesulphonyl fluoride for 30
min on ice and the cell extract was centrifuged at 14,000 r.p.m.
for 30 min to remove cell debris. Immunoprecipitation of the p53
or SV40 large T proteins from the cell extract was performed
essentially as described previously (Gannon et al., 1990) with
mouse monoclonal antibodies using Protein G Sepharose beads
(Pharmacia) for both pre-absorption of the lysates and isolation
of the antibody-protein complexes.

Gel electrophoresis and immunoblotting
Proteins immunoprecipitated as above were separated by SDS-
polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel
and transferred onto nitrocellulose membrane in a Bio-Rad Mini
Trans-Blot Electrophoretic Transfer Cell for 4 h, at 4°C and 150
mA in transfer buffer (25 mM Tris, 190 mM glycine and 20%
methanol; Harlow and Lane, 1988). Prestained molecular mass
markers (Bio-Rad) were run in parallel.
The blots were blocked in 0.1% Tween-20 in PBS for 1 h,
probed overnight at 4°C with either rabbit anti-p53 antisera CM-
1 or rabbit anti-large T antisera (see above) and washed in 0.1%
Tween-20 in PBS. The blots were then incubated for 1 h at room
temperature in Dako horseradish peroxidase (HRP)-conjugated
swine anti-rabbit immunoglobulin antiserum diluted 1 in 100, and
washed again in 0.1% Tween-20 in PBS. The peroxidase activity
was visualised with chloronaphthol (Sigma).

ELISA assay
A sandwich immunoassay was used for quantitation of the p53
proteins performed essentially as described by (Gannon et al.,
1990) using mouse monoclonal antibodies that recognize either
human or mouse p53 protein as the solid-phase reagents and poly-
clonal rabbit antisera to p53 protein (CM-1) to detect the captured
proteins. Falcon 96-well microtitre plates were coated overnight
at room temperature with 50 µl per well of 20 µg/ml purified
mouse monoclonal antibody, blocked for 2 h in 3% bovine serum
albumin (BSA) in PBS and rinsed in PBS. 
Cell extract was prepared as for immunoprecipitation, 50 µl of
serial twofold dilutions in lysis buffer were added to each well
and incubated for 3 h at 4°C. The plates were washed once in
PBS, twice in 0.1% NP40 in PBS, once in PBS again and appro-
priate rabbit antiserum (diluted 1:1000 in 1% BSA in PBS with
0.1% NP40) was added and the incubation continued for 3 h. The
plates were washed as above and peroxidase-conjugated swine
antisem to rabbit immunoglobulin (Dako, diluted 1:1000) was
added for 2 h, bound enzyme was detected with the tetramethyl-
benzidine substrate and the results monitored in an automatic
ELISA plate reader (Harlow and Lane, 1988).
Assay points were determined in duplicate. The specificity con-
trols included wells coated with irrelevant monoclonal antibodies,
or normal rabbit serum used instead of CM-1.

RESULTS
Expression of endogenous p53 in MCF7 and T47D
cells
Using a panel of monoclonal antibodies to p53 we con-
firmed our earlier results on the expression of human p53
in T47D and MCF7 cells. T47D cells, which contain a
single mutant allele of the p53 gene (194 Leu→Phe) show
strong nuclear staining in the majority of the cells in cul-
ture (Fig. 1A). The staining typically spares the nucleoli
and is excluded from condensed chromatin in mitotic cells. This kind of staining pattern is typical of that seen in about 20-30% of human breast cancers. The staining pattern of the wild-type p53 protein expressed by MCF7 cells is far more complex. Typically intense nuclear staining is seen in 5% of the cells (Fig. 2A) while the remainder show very weak though clearly positive staining. The intensely positive cells are not uniformly morphological distinguishable from the other cells. It is noticeable that they do not often occur in pairs or clusters and positive post-mitotic pairs have not been seen. The fraction of positive cells is quite stable and is preserved in all subclones of the line. This remarkable staining pattern is seen in some primary breast cancers and is reminiscent of the pattern of expression seen in normal cells exposed to low levels of DNA damaging agents and in the cells transformed by the ras and myc oncogenes studied by Lu et al. (1992). This pattern is not seen in normal primary human keratinocytes or fibroblasts in which all cells are uniformly negative or extremely weak for p53 staining.

Expression of a temperature-sensitive murine p53 protein in MCF7 and T47D cells

Cultures of MCF7 cells and T47D cells were co-transfected with the MSVcl-val plasmid encoding a temperature-sensitive mutant mouse p53 and the pSV2 neo plasmid. After drug selection surviving cells were grown and examined for expression of the introduced p53 gene using murine-specific anti-p53 antibodies PAb242 (Figs 1D, 2D), 246 and 248 and for expression of the endogenous human p53 gene using the human-specific anti-p53 antibody DO-1 (Figs 1C, 2C). In the T47D cells the murine p53 protein is expressed at moderate levels in the majority of the cells (Fig. 1D) at both the permissive and non-permissive temperature. Interestingly, the protein was clearly nuclear in location at both temperatures, in contrast to the temperature dependence of its nuclear location seen in rat embryo fibroblasts transformed by this mutant p53 plus an activated ras gene (Gannon and Lane, 1991; Ginsberg et al., 1991; Martinez et al., 1991). Both the human p53 protein and the murine p53 protein can be readily detected in extracts of the transfected T47D cells by immunoprecipitation with species-specific antibodies and western blotting (Fig. 3A). In the MCF7 cells the murine mutant p53 showed a different pattern of expression since, like the endogenous gene (Fig. 2A,C), it showed tremendous heterogeneity of expression with only 5% of cells showing clear nuclear staining (Fig. 2D). In these cells the low levels of murine p53 were undetectable by immunoprecipitation and western blotting whereas the low levels of wild-type human p53 were just detectable by this method (Fig. 3B).

The effect of SV40 virus infection on p53 expression

The heterogenous expression of exogenous genes has been noted before in transfected cells (Hanahan et al., 1980) and may result from sporadic loss of the plasmid. Alternatively the heterogenous expression of the p53 protein could reflect the fact that the exogenous protein is being regulated by the same mechanism that causes the heterogenous

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![Fig. 2. Immunocytochemical detection of p53 in MCF7 breast cancer cell line growing at 32°C. (A) MCF7 cells stained with the human p53-specific MAb DO-1. (B) MCF7 cells stained with the mouse p53-specific PAb 242. (C) MCF7 cells transfected with mouse temperature-sensitive mutant p53 and stained with MAb DO-1. (D) MCF7 cells transfected with mouse temperature-sensitive mutant p53 and stained with PAb 242. x144.](image)

![Fig. 3. Immunoblot analysis of p53 protein from breast cancer cell lines MCF7 and T47D transfected by temperature-sensitive mouse p53 and growing at 32°C. (A) immunoprecipitation of p53 from cell extract of transfected T47D cells with human p53-specific monoclonal antibody DO-1 (lane 1), mouse p53-specific antibodies PAb 242 (lane 2), PAb 248 (lane 3), PAb 246 (lane 4) and an irrelevant negative control antibody BG-2 (lane 5). (B) Immunoprecipitation of p53 from cell extract of transfected MCF7 cells with monoclonal antibodies MAb DO-1 (lane 1), PAb 242 (lane 2), PAb 248 (lane 3), PAb 246 (lane 4) and an irrelevant negative control antibody BG-2 (lane 5). Molecular mass standards (kDa) are shown on the left.](image)
expression of the normal wild-type p53 gene in the MCF7 cells. To distinguish between these two alternatives we examined the effect of SV40 infection on the expression of the endogenous and exogenous p53 proteins. When the parent transfected MCF7 cells are infected with SV40 virus there is a dramatic increase in p53 protein expression with the majority of the cells now staining intensely for p53 (Fig. 4A,B). Immunoprecipitation of extracts of these cells with anti-p53 and anti-T antibodies followed by immunoblotting of the immunoprecipitates with CM-1 anti-p53 polyclonal sera showed that human and mouse p53 were expressed in the infected cells at elevated levels (Fig. 5). The same immunoprecipitates were analysed on a separate gel by immunoblotting with an anti-T polyclonal serum to confirm the presence of large T in the immunoprecipitates and thus the presence of a complex between p53 and large T proteins (Fig. 5). Differences between p53 protein level before and after infection with SV40 virus could be seen in quantitative ELISA (Fig. 6A,B,C). Infection of the transfected cells clearly demonstrated the same effect for both the murine and human p53 proteins. Thus, in the uninfected cells the murine p53 gene is present in all the cells but the expression of murine p53 protein mirrors that of the endogenous gene.

**DISCUSSION**

The very high mutation rate in the p53 gene found in human cancers combined with the high tumour rate in mice and man that have inherited deficiencies in the gene strongly suggest that the normal function of this gene plays a critical role in protecting individuals and cells against neoplastic change. Recent studies have suggested that this might in part be explained by a model in which p53 acts as part...
of a cell cycle checkpoint control that is invoked by DNA damage (Hartwell, 1992; Kuerbitz et al., 1992; Lane, 1992). If this is the case then it is probable that multiple gene products will be involved in this pathway and that inactivation of other components of the pathway may also predispose to neoplasia. Recent support for this idea has come from the observation that induction of the p53 pathway may be defective in certain cases of Ataxia Telangiectasia (Kastan et al., 1992) and that altered expression of p53 without mutation is found in the normal cells of some cancer susceptible families (Barnes et al., 1992). The finding that both viral gene products and host gene products that bind p53 and inactivate its function (Momand et al., 1992) can act as dominant oncopgenes also supports this idea.

On such a model one can predict that there will be gene products that act both upstream and downstream of p53. Upstream products might act to sense DNA damage and downstream products might act to mediate the growth response to p53. In human breast cancer 20-30% of primary cancers examined show point mutations in the p53 gene. Typically these tumours have lost the remaining wild-type allele and are expressing high levels of a point missense mutant protein (Bartek et al., 1990a; Bartek et al., 1990b; Davidoff et al., 1991). In addition to this group, however, immunohistochemical analysis identifies another large group with obviously aberrant expression of high levels of p53 without obvious mutation. The pattern of expression is often very heterogeneous showing a moderate to low level in most cells of the population with 5-15% of the cells showing very intense staining (Midgley et al., 1992). The basis of this aberrant expression of the normal protein is unclear. It is notable in a model system of cells transformed by the ras and myc oncogenes that retain wild-type p53 genes that the intensely staining p53-positive cells frequently show gross signs of mitotic disorders such as micronuclei. This suggests that the elevated p53 levels seen may be related to the elevation of p53 seen after exposure of cells to DNA damaging agents (Lu et al., 1992).

We have examined the regulation of p53 protein expression in two breast cancer cell lines. In addition to examining the expression of the endogenous gene we undertook to introduce an exogenous p53 gene and examine the expression of its product. We were able to do this by using species-specific monoclonal antibodies. We used a temperature-sensitive (ts) mutant mouse p53 as our reporter because the product of this mutant gene shows wild-type activity at 32°C and mutant activity at 37°C and would therefore allow us to examine the expression of both forms of the protein.

The ts mutant mouse p53 protein is expressed uniformly and at high levels in the T47D cells that express an endogenous mutant p53 protein both at 32°C and at 37°C. However, the same gene product is expressed at very low levels at both temperatures in the MCF7 cells that express an endogenous wild-type gene. Thus the mutant p53 protein is only stable in a cellular environment where the endogenous p53 gene is stable. The instability of mutant p53 proteins in the normal cells of Li-Fraumeni patients is consistent with this hypothesis (Srivastava et al., 1990). An earlier study using a murine model also found differences in the stability of mutant p53 dependent on cellular background (Reihaus et al., 1990). The result is underscored by the finding that the few MCF7 cells that did express high levels of wild-type p53 also expressed high levels of the mutant mouse gene. Thus whatever mechanism results in the aberrant expression of the wild-type gene in the MCF7 cells can exert the same effect on the exogenous gene product. Since the endogenous gene and the exogenous gene are under the control of completely different promoters and enhancers, this effect is unlikely to be regulated at the transcriptional level. It is most probably a post-translational mechanism of the type that results in high levels of p53 in cells exposed to UV (Maltzman and Czyzyk, 1984) or SV40 (Oren et al., 1981). Careful examination of the positively stained cells in the population did not reveal any obvious morphological signs of DNA damage, unlike that seen in the model system of Lu et al. (1992). However, it was noticeable that no mitotic or immediately post-mitotic cells showed this staining pattern. Infection of these cells with SV40 virus created an environment in which both the ts mutant mouse protein and the endogenous wild-type p53 protein accumulated to high levels as judged not only by immunohistochemistry but also by quantitative techniques. Interestingly Depert and his colleagues (Depert and Steinmeyer, 1989; Depert et al., 1989) found that SV40 infection alone was not sufficient to stabilise p53 in primary rodent cells. Thus the activities that control instability in the MCF7 cell line may be more limited in their effect than those seen in primary cells. This is consistent with the relatively high endogenous level of p53 in these cells compared to primary cells.

The relative instability of wild type and mutant p53 in MCF7 cells may explain why they are resistant to the growth inhibitory effect of transfection with exogenous wild-type p53, since they may not accumulate sufficient protein to exert this regulatory function. Alternatively one can imagine that they have lost the downstream response to p53 and that this is in some way related to their heterogeneous expression of the protein. In either case it is clear that the cellular environment and, by implication, other gene products, play a critical role in determining p53 protein levels and that mutations in the p53 gene itself are not a sufficient explanation of the elevated levels of p53 seen in human tumour cells lines. This model system, which is readily manipulated and shows a good correlation with in vivo observations, may provide a sound basis for determining the nature of the regulatory factors that control p53 protein expression in tumours.

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