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EXPRESSION OF THE *p53* TUMOUR SUPPRESSOR GENE PRODUCT IS A DETERMINANT OF CHEMOSENSITIVITY

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SUMMARY. Many cytotoxic agents act by causing DNA damage, and the *p53* tumour suppressor gene is known to be involved in the cellular response to DNA damage. Since inactivation of *p53* is common in many tumours, we wondered if this would affect the sensitivity of cancer cells to cytotoxic agents. We have shown that this is indeed the case in transformed mouse cell lines with and without a mutated *p53* gene; *p53* "knockout" mouse fibroblasts; and normal human skin fibroblasts treated with an anti-sense *p53* oligonucleotide. In addition, we have demonstrated a correlation between p53 protein expression in human breast cancer specimens and their chemosensitivity. The results show that inactivation or mutation of p53 renders cells more sensitive to those cytotoxic drugs whose primary mechanism of action is DNA damage.

Although the use of cytotoxic drugs has produced a dramatic improvement in the outlook for some types of cancer, resistance to these drugs is either constitutive or rapidly acquired in many patients. Most currently available drugs act by selectively interfering with processes critical to progression through the cell cycle, many by causing DNA damage (1). The *p53* tumour suppressor gene is involved in the cellular response to DNA damage (2-8) and is abnormal in many tumours (9). Often point mutation of one allele is accompanied by loss of the remaining wild type allele (6,9). Mutant p53 protein has an increased half-life within the cell (10) and can be readily detected by immunocytochemistry (10-11). Inactivation of p53 can also occur by deletion of both alleles, or by binding of wild-type protein to other oncoproteins (12-13). In some instances it appears that the growth suppression function of wild type p53 can be bypassed by activation of the *ras* and *myc* oncogenes together (14).

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The p53 molecule has been described as both a regulator of DNA replication (12,15) and a transcriptional regulator (16). These possibilities need not be mutually exclusive. The amino terminal domain of p53 has transcriptional regulatory activity, while the carboxy terminal domain is responsible for the oligomerisation of p53; a process essential for DNA binding (17-18). The level of expression of wild type p53 is increased following DNA damage by irradiation (UV or gamma), actinomycin D, or UV mimetic drugs (2-3). In fibroblasts, such treatments result in arrest of the cell cycle in G1, an effect not seen in cells lacking wild type p53 (2). The physiological role of p53 may therefore be to act as a switch leading to growth arrest in G1, blocking progression to mitosis following DNA damage and thereby maintaining genomic stability (2,6). Recently it has been shown that p53 may be able to drive cells with excessive DNA damage into apoptosis as an alternative response which would also maintain genomic stability and prevent the proliferation of potentially oncogenic cells (4-5,19). It might therefore be expected that p53 status will affect the outcome of cellular exposure to DNA-damaging cytotoxic drugs.

In this study, we have assessed chemosensitivity *in vitro* using a recently developed luminescence assay which measures the adenosine triphosphate (ATP) content of cells cultured for 6-7 days with cytotoxic drugs at pharmacologically relevant doses by the luciferin-luciferase reaction (20).

Materials and Methods

Cell Lines and Tumours: Murine reconstituted prostate cell lines β.gal and R2 (14,24), and heterozygous and homozygous *p53* deletion fibroblasts in which disruption of a p53 allele within these cells has completely abrogated p53 expression (22) were grown in 25cm^3 flasks (Greiner Labortechnik, Dursley, UK) in RPMI 1640 with 10% newborn calf serum (NCS, Gibco BRL, Paisley, Scotland) and antibiotics (Penicillin + Streptomycin (Gibco)). 822A human fibroblasts were obtained from Dr M Faed, Department of Pathology, Ninewells Hospital, Dundee and cultured in DMEM + antibiotics and 10% NCS (Gibco). Primary breast adenocarcinoma samples were assayed consecutively using material which was not required for histological or oestrogen receptor examination. Ethical permission was obtained from the Tayside Medical Research Ethics Committee.

Tumour Chemosensitivity Assay: TCA-100 luminescent assays (BATLE LE Inc, Fort Lauderdale, USA) were performed as previously described (20) with the following modifications for the cell lines used in this study. Murine prostatic and fibroblast (human/mouse) cell lines were plated at 3,000 cells/well in flat bottom polystyrene microplates (Greiner Labortechnik). The results are expressed as %inhibition of ATP content measured by the luciferin-luciferase reaction after 7 days in culture at 37 °C in 5% CO_2 against concentration of drug or oligonucleotide. In experiments with oligonucleotides, NCS was heated for one hour prior to use at 60 C to reduce endogenous nuclease activity. HPLC purified oligonucleotides 15 bases long (anti-sense 5'-CTGCGGCTCCTCCAT-3', sense 5'-ATGGAGGAGCCGCAG-3', Genosys) with two phosphothiorated terminal linkages (23) were added at serial dilutions from 200μg/ml 24 hours before the addition of drug to standard TCA-100 assays. Oligonucleotide-containing assays were extracted for ATP measurement at 4 days.

p53 ELISA: p53 levels in cytosols prepared for oestrogen receptor estimation were measured by ELISA using a method which has previously shown good correlation with the results of immunocytochemistry (11). Breast tumour cytosols and cytosols prepared from an A431 vulval carcinoma cell line (from Dr L Newman, Department of Surgery) which expresses mutant p53 (used as control) were standardised for protein content by use of the Bradford dye binding assay (25). Extracts from breast tumours were stored at -20 °C until assay. The degree of inhibition in the TCA-100 assay is represented by a simple index (700 - sum[%inhibtion at each concentration tested]) which decreases as sensitivity increases.

Data Analysis: Data from TCA-100 assays was analysed as previously descibed (20). The results of p53 ELISAs and TCA-100 assays were compared by simple linear regression using Statgraphics (STSC, California, USA) software.

Results

In initial studies, the chemosensitivities of two well-characterised murine prostatic epithelial cell lines (14) with either wild type *p53* (β.gal) or *p53* mutation with activated *ras* (R2) were compared. Both cell lines were otherwise isogenic. The R2 cell line showed greater sensitivity to cisplatin (Fig. 1a). Similar results were also obtained with 4-hydroperoxycyclophosphamide (4-HC, an active metabolite of cyclophosphamide), doxorubicin, 5-fluorouracil (5-FU), methotrexate and vincristine (data not shown).

To study the effects of abnormal p53 expression in isolation, we tested the chemosensitivity of fibroblasts from homozygous germline *p53*-disrupted mice and heterozygotes (22). There was no difference in sensitivity to 5-FU, and methotrexate (data not shown) which have indirect effects on DNA. However homozygous cells were consistently found to be more sensitive to 4-HC than those from heterozygotes (Fig. 1b) despite their slightly lower growth rate in culture. Similar results were obtained with doxorubicin and cisplatin (data not shown). The *p53*-disrupted cells showed increased sensitivity to vincristine (data not shown), a commonly used drug which prevents spindle formation during mitosis.

In further experiments, we exposed 822A skin fibroblasts to a phosphothiorated anti-sense oligonucleotide encoding the initial five codons of the p53 mRNA (23), and a sense control, in the presence and absence of 4-HC or vincristine. Vincristine was used as an internal control, since neither of the oligonucleotides would be expected to affect chemosensitivity to spindle-active agents. The same oligonucleotide sequences were used successfully by Hara *et al* (23) at a concentration of 200μg/ml, and we therefore used this level in our experiments, but made doubling dilutions since we observed some activity of the sense oligonucleotide in initial assays. The results (Fig. 2) show a clear increase in the chemosensitivity of cells exposed to 0.75μg/ml 4-HC in the presence of an anti-sense oligonucleotide over a range of 100 - 12.5μg/ml in comparison with controls to which either sense or no oligonucleotide had been added (Fig. 2a). The sense oligonucleotide results overlap those of the no oligonucleotide results over most

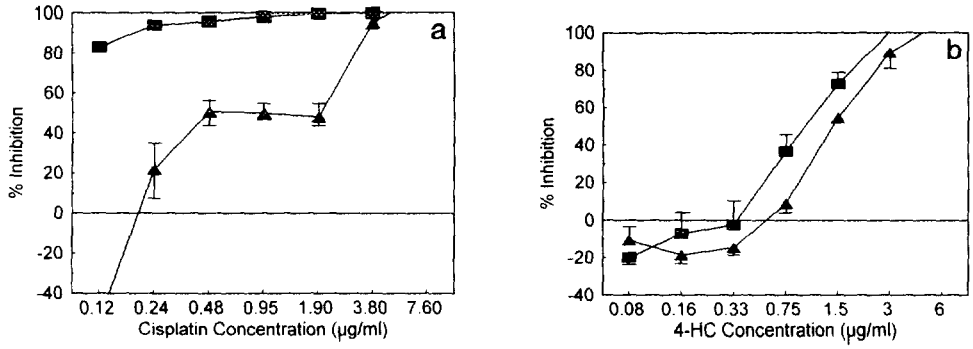

Figure 1. The effect of *p53* mutation or deletion on chemosensitivity to 4-HC and cisplatin assessed by TCA-100 assay. Each value for growth inhibition represents the mean of three independent assays: the error bars indicate the range of growth inhibition obtained from duplicate wells in the three experiments. *(a)* Cisplatin chemosensitivity of murine reconstituted cell lines β.gal and R2. The R2 line (■) shows greater sensitivity to cisplatin at serial dilutions from 7.6µg/ml than β.gal (▲) at all except the highest concentrations of cisplatin. The R2 cell line grew more rapidly that the β.gal cell line in the absence of drugs (R2: 57ng/ml ATP, β.gal: 29ng/ml). *(b)* 4-HC sensitivity in heterozygous (▲) and homozygous (■) germline *p53* disrupted fibroblasts from transgenic inbred mice. The homozygous disrupted fibroblasts show greater chemosensitivity, despite the higher growth rate of the heterozygous cells in wells without drugs (heterozygous cells 56ng/ml, homozygous cells 32ng/ml). The apparent negative inhibition observed at low drug levels in both experiments is probably due to slight overgrowth of the cells in the MO wells leading to reduced ATP levels, rather than stimulation of growth by low drug concentrations.

of this range. In contrast to the results with 4-HC, there was no effect of the addition of anti-sense or sense oligonucleotides on sensitivity to 0.1µg/ml vincristine.

The role of p53 in determining chemosensitivity in patients was studied by comparison of p53 levels measured by ELISA (11) with an index of chemosensitivity of human breast

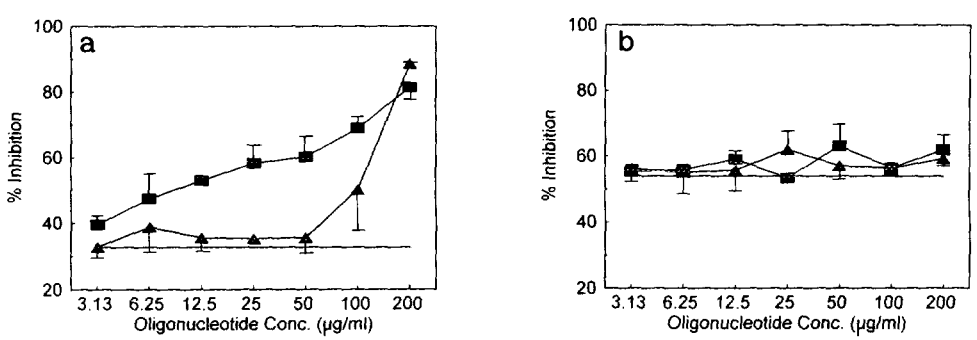

Figure 2. Effect of *p53* anti-sense and sense oligonucleotides on the chemosensitivity to 4-HC and vincristine of 822A human fibroblasts. The results are expressed as the mean percentage inhibition of ATP content from triplicate wells measured by TCA-100 assay: the error bars represent the range of values obtained. Growth of cells without added 4-HC or vincristine was similar whether oligonucleotide (sense or anti-sense) was present or not (No oligo = 2.063 ng/ml ATP, sense = 2.003 ng/ml ATP, anti-sense = 1.985ng/ml ATP). *(a)* The sense oligonucleotide (▲) shows some enhancement of chemosensitivity to 4-HC at 0.75µg/ml above 50µg/ml oligonucleotide, but it is clear that the anti-sense (■) has a significantly greater effect, particularly at lower concentrations in comparison with the sense oligonucleotide. *(b)* Neither oligonucleotide showed activity with vincristine at 0.1µg/ml.

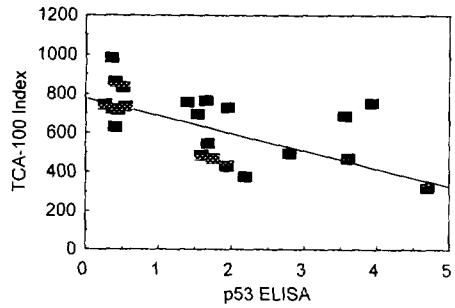

Figure 3. Correlation of chemosensitivity to 4-HC at serial dilutions from 6.0μg/ml of 23 primary human breast adenocarcinomas of infiltrating ductal type assessed by TCA-100 assay with p53 level measured by ELISA(11).There is strong negative correlation of p53 levels and 4-HC chemosensitivity ($r=-0.57$, $p<0.007$, Spearman rank sum correlation) showing that there is greater sensitivity (lower index) with higher levels of p53 protein.

adenocarcinomas (20), determined *in vitro* using the same assay system as that used for the cell lines. Clear correlations (Spearman Rank) were obtained between p53 level and 4-HC (Fig. 3) and 4-HC containing drug combinations, including CMF (cyclophosphamide + methotrexate + 5-FU) and CAF (cyclophosphamide + doxorubicin + 5-FU) ($r=-0.467$, $p<0.04$ and $r=-0.536$, $p<0.02$). No correlations were found with chemosensitivity results for 5FU, vincristine, or methotrexate (data not shown). Thus the lowest indices (greatest sensitivity) were seen with the highest, presumed mutant, p53 levels with those drugs or combinations whose primary mechanism of action is DNA damage.

Discussion

In this study, several different systems have been used to demonstrate both causal and correlative relationships between p53 expression and chemosensitivity. The R2 cell line, which has a mutant *p53* gene and an activated *ras* gene, showed greater sensitivity to alkylating agents than the β.gal cell line which has a wild-type *p53* gene and an activated *ras* gene. However R2 cells grow much faster than the β.gal cell line in our culture conditions. The increased chemosensitivity observed in the first series of experiments may therefore be due to the higher proportion of cells in the growth cycle in the R2 cell line, as well as any direct effect of p53 on chemosensitivity. Sklar (21) suggested that *ras* activation produces resistance to cisplatin in murine fibroblasts. Here we have shown that the combination of *ras* activation and *p53* mutation confers increased sensitivity to cisplatin (Fig. 1a), although the differences in cell growth rates make interpretation difficult.

Homozygous *p53*-disrupted fibroblasts show marginally greater chemosensitivity to 4-HC than heterozygous cells despite their lower growth rate. We were surprised to see

increased sensitivity of the *p53*-disrupted cells to vincristine, a commonly used drug which prevents spindle formation during mitosis. However, *p53* null cells have a high rate of karyotypic abnormality in comparison with normal cells (7) and may therefore have a higher rate of mitotic failure in the presence of spindle-active agents.

The use of oligonucleotides to inhibit genes in cells is controversial due to problems associated with interpretation of the results and the difficulties of providing adequate controls within the experiments. Nevertheless, it was necessary to confirm the results from the previously described experiments in human cells. The observed enhancement by *p53* anti-sense oligonucleotides of sensitivity to 4-HC in comparison with vincristine is striking and suggests that interference with wild type p53 function in human fibroblasts increases their chemosensitivity to alkylating agents. The lack of vincristine sensitivity in these experiments is to be expected since there would be insufficient time for the oligonucleotide-exposed cells to accumulate the karyotypic abnormalities which may be responsible for the increased vincristine sensitivity observed in multiply-passaged mouse fibroblasts which lack p53. There may also be a species difference in the rate of accumulation of karyotypic abnormalities in p53 deficient fibroblasts (7-8).

The correlation observed between p53 level and chemosensitivity index in breast tumours is remarkably strong, since many other factors must also be involved. All of our results suggest that alterations of p53 expression are strongly associated with changes in chemosensitivity to DNA-damage agents, both in tumour samples and in cell lines. This is consistent with the model of p53 function in a G1/S cell cycle checkpoint for DNA damage (6). In this model, lack of p53 function will lead to an inability to arrest cell cycle progression following DNA damage by cytotoxic drugs and to the stabilisation of mutations in clones of cells within the tumour. Recently, a p53-dependent pathway for apoptosis following DNA damage has been described in thymocytes (4-5,19). Therefore, in contrast to the enhanced sensitivity which may be associated with G1/S arrest, the complete absence of p53 function may increase survival following drug-induced DNA damage in thymocytes and perhaps in other cell types.

This model will be of particular clinical importance for drugs whose primary mechanism of cytotoxicity is DNA damage. Although *p53* mutation may confer clinical sensitivity to alkylating agents in the short term, previous work (7-8) suggests that such sensitivity may not last as the increased genomic instability caused by *p53* mutation leads to the enhanced development of genomic mechanisms of resistance. The p53 status of some human tumours, including breast carcinoma, may therefore be one factor of importance in determining the success or failure of a chemotherapeutic regimen, perhaps including the common phenomenon of short-term sensitivity and subsequent recurrence with drug resistance which is seen clinically in many human solid tumours.

The circumvention of mechanisms of drug resistance represents an important goal for experimental oncology. The discovery that biochemical determinants of drug sensitivity in tumours also exist may have considerable implications for the design of chemotherapeutic regimens and new agents.

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