Tumour Growth and Cell Kinetics in Variants of a Human Endometrial Adenocarcinoma Expressing Either Wild-Type or Mutant *p53*

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We have compared the baseline cell proliferation and tumour growth in two variants of a human endometrial adenocarcinoma grown in nude mice. One of these tumour variants expressed wild-type *p53* whereas the other had mutations of the *p53* gene at codon 175 in both alleles and at codon 248 in one allele. There was no difference in growth rate between the tumour variants. Cell proliferation parameters, such as labelling index and S-phase fraction, were significantly increased in the tumour with mutated *p53* and consequently there was a significantly lower proportion of cells in the G1-phase, proposing an at least partial loss of suppressor function in this tumour. Semi-quantitative analysis of the p53 and bcl-2 proteins showed a significant overexpression of p53 and a decreased expression of the bcl-2 protein in the *p53* mutated tumour variant compared with the variant with wild-type *p53*. We conclude that wild-type p53 protein acts as an active suppressor in the regulation of the baseline growth and cell kinetics of this tumour and could be linked through a p53–bcl-2 system in human endometrial adenocarcinomas.

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Growth of experimental tumours is determined by variations in both growth fraction and cell loss factor (CL) (1, 2). Spang-Thomsen et al. (2, 3) and Rofstad et al. (4) have previously suggested that no single proliferation parameter is sufficient to characterize the growth of individual tumours. Rather, cell loss is the dominant factor.

From our own experiments we have noted that an increased baseline (i.e. spontaneous) cell proliferation is not necessarily accompanied by faster tumour growth in some human endometrial adenocarcinomas (5). In the same work, we discussed the possibility of a connection between mechanism(s) regulating cell proliferation, cell loss and tumour growth. We suspected an important role for p53 expression in this potential connection. Recently we found that oestradiol may influence not only p53 but also bcl-2 expression, and thereby affecting the cell loss factor through apoptosis (6, 7) (p53 and bcl-2 are reviewed in: (8–15)). These experiments, however, did not give information about the role of the *p53* gene product in the regulation of the baseline (i.e., spontaneous) proliferation and tumour growth.

However, one of our nude mouse-borne human endometrial adenocarcinomas, expressing wild-type p53 protein, has recently been established in vitro, and the p53 gene has mutated (16). Having performed subcutaneous retransplantation of this in vitro cell line, we were able to compare tumour growth and cell proliferation in these 2 variants of the same tumour. Such a comparison could give important information about effects of lost p53 function, and indirectly, about growth regulating mechanism(s) in these tumour variants.

The aim of the present study was thus to compare tumour growth, cell kinetics, p53 and bcl-2 expression in variants of this human endometrial adenocarcinoma, expressing wild-type p53 and its counterpart, the mutant p53.

MATERIAL AND METHODS

Tumour characteristics

One tumour variant consisted of a moderately differentiated oestrogen receptor (ER) positive (190 fmol/mg protein), progesterone receptor (PgR) negative human endometrial adenocarcinoma (called TuWt) grown in nude mice for several years. The growth phenotype was characterized as oestradiol (ovarian) independent but responsive (inhibited), i.e., the tumour was capable of growing in the absence of oestradiol but its growth could be inhibited by

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the hormone (17). Sequencing of exons 5, 6, 7 and 8 of the p53 gene did not reveal any mutations. Immunohistochemical analysis, using the mutant-specific antibody Pab 240, was also negative, suggesting that the tumour expressed wild-type p53 protein (16).

From this tumour we established an in vitro cell line as described previously (16). Cells from the in vitro culture were then injected subcutaneously into nude mice. This tumour, growing in vivo, was subsequently transplanted through 3 passages prior to the present experiment (second variant, called TuM).

In the third passage this tumour was poorly differentiated and was ER and PgR negative. The growth phenotype was oestradiol (ovarian) independent and resistant. Sequencing of the *p53* gene showed 2 mutations: one was localized in exon 5, codon 175 with a G–A mutation on both alleles, resulting in an arginine to histidine change, and the other mutation, found in exon 7 at codon 248 on one allele, was also a G–A mutation resulting in an arginine to glutamine change (Fig. 1). Compared with TuWt this tumour markedly overexpressed the p53 protein.

Tumour transplantation procedures

Grafts (3 × 3 × 3 mm) from one original tumour were transplanted subcutaneously on the dorsal surface of female BALB/c nude (nu/nu) mice, one graft per animal. To minimize any effect of endogenous oestradiol, only oophorectomized animals were used. Oophorectomy was performed before tumour transplantation. During the transplantation procedure (day 0 of the experiment), the tumour grafts were kept in sterile 0.9% NaCl at room temperature for about 30 min. The surgical procedures were performed under general anaesthesia. At the end of the experiment, the animals were sacrificed and the tumours analysed.

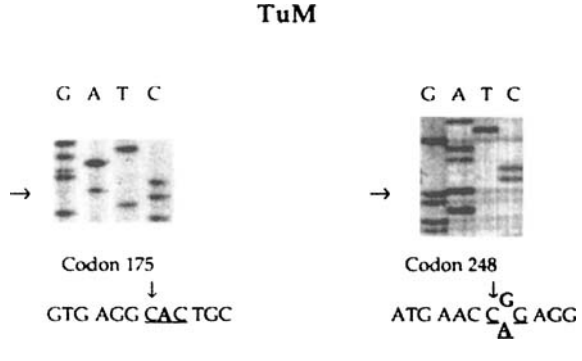

Fig. 1. p53 gene was amplified by PCR in both TuWt and TuM, and the PCR product was used for sequence analysis. TuM showed 2 mutations at codon 175 and 248.

Tumour growth measurement

Tumour volume was calculated from diameter measurements, according to the formula: volume = (width^2 × length)/2 as described previously (18). Tumour size was measured twice weekly during the experiment, and the logarithmic values of the calculated volumes plotted against time after transplantation. For the statistical analysis, the volume increase/day was used and a mean tumour volume doubling time (TVDT) value was calculated for each variant. Body weight was measured weekly, and animals manifesting a weight loss of more than 15% were excluded from the analysis.

Study design

Ten tumour grafts from TuWT and 10 grafts from TuM were transplanted into 20 nude mice at day 0 of the experiment. Tumour growth was followed for 32 days in TuWt and for 25 days in TuM, at which time the tumour bearing animals were treated with bromodeoxyuridine intraperitoneally as described below. Six hours later the animals were sacrificed and the tumours excised for analysis. To minimize the known tendency to central necrosis of TuM its follow-up period was shortened by 7 days.

5-bromo-2-deoxyuridine (BrdU)-labelling

On the last day of the experiment, BrdU (Sigma Chemical. St. Louis, USA) was injected intraperitoneally at a dose of 100 mg/kg (10 mg/ml in 0.9% NaCl). Tumour samples were excised 6 h after treatment, immediately fixed in 70% ethanol and stored at −20°C until analysis.

Flow cytometric analysis (FCM)

The principles of preparation and staining procedures have been described previously (19), and further modified by Wilson et al. (20). A Cytoron Absolute flow cytometer (Ortho Diagnostic Systems Inc. NJ, USA) was used to analyse approximately 20 000 nuclei per sample. The SPF was calculated with the Multicycle software package (Phoenix Flow Systems, CA, USA). For evaluation of cell data, 3 regions were set according to the bivariate BrdU versus DNA cytogram. One was set around the total cell population, the second around the undivided BrdU-labelled cells and the third around the divided BrdU-labelled cells. The LI was determined as the percentage of BrdU-labelled cells, corrected for cell division. The relative movement (RM, i.e., the increase in DNA content with time) of the undivided BrdU-labelled cells (fluorescence labelled, FL) relative to the DNA contents of G1 (FG1) and G2 (FG2) cells was calculated according to Begg et al. (21): RM = FL − FG1/FG2 − FG1. DNA synthesis time (Ts) was calculated from the following formula: Ts = (0.5/RM − 0.5) × t, where t is the time between BrdU injection and tumour excision. Tumour potential doubling time (Tpot) was determined taking no account of cell loss with

the equation Tpot = Ts × lambda/LI where lambda is a correction factor for the non-linear distribution of cells through the cell cycle and was set to 0.8. CL were calculated according to the formula CL = 1 − Tpot/TVDT.

Western blot analysis

Proteins were analysed by Western blotting. Briefly, frozen tumour tissue material (150 mg) was homogenized at +4°C and then completely dissolved by boiling in strong detergent solution (5% sodium dodecyl sulfate, SDS). The homogenate was centrifuged and the protein concentration of the supernatant measured. Equal amounts of tumour protein (150 μg) were separated by electrophoresis through polyacrylamide gels under denaturing and reducing conditions (22). Resolved proteins were then electroblotted onto nitrocellulose membranes. Membranes were pre-blocked by incubation in fat-free milk in TBS-T (20 mM Tris buffered saline with 0.02% Tween 20) followed by incubation with mouse monoclonal antibody directly conjugated with horseradish peroxidase (HRP) to detect p53 protein. We used the DO-1 monoclonal antibody, which reacts with an amino terminal epitope of human wild-type and mutant p53 (Santa Cruz Biotechnology, Inc.,USA). For detecting bcl-2 protein a rabbit polyclonal antibody (Santa Cruz Biotechn. Inc. USA) was used in combination with a secondary antibody conjugated with HRP.

After washing, the membranes were incubated in Amersham enhanced chemiluminescence (ECL) reagents for one minute, exposed to ECL hyperfilm and after 30–60 min developed as usual for autoradiography or x-rays.

A software package (Quantity One, pdi, NY, USA) in The Discovery Series Densitometric Systems (Pharmacia, Sweden) was used for semiquantitative measurement of oncoproteins. In this computer program the area of the bands was scanned and calculated in mm, and the optical density (OD) of each detected band was measured. Quantitation was done using the formula OD × area, resulting in values expressed in OD × mm^2. To minimize technical errors, all results obtained in the same Western blot analysis (all tumours in a given experiment), were scanned and quantified together.

Statistical analysis

Differences between the different groups with respect to p53 and bcl-2 protein amounts and cell kinetic variables were analysed with the Student's t-test. The relative increase in tumour volume (volume increase/day) during the experiment was analysed with log-linear regression. The mean value of TVDT was calculated by the formula TVDT = ^{10}log2/r where r is the regression coefficient.

RESULTS

Tumour growth

Growth curves are shown in Fig. 2. The calculated TVDT for TuWt variants was 5 +/− 0.8 days whereas for TuM it was 4 +/− 1 days. The difference was not significant.

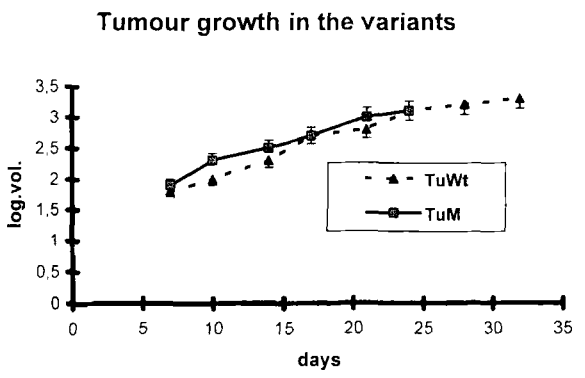

Fig. 2. Ten tumour grafts were from TuWt and TuM transplanted to oophorectomised nude mice on day 0 of the experiment. Tumour volumes were measured twice a week, and a mean TVDT value was calculated for each variant. The growth of TuWt was followed for 32 days and the growth of TuM 25 days, at which time the animals were sacrificed and the tumours excised for analysis.

Cell kinetics

We observed a significantly lower proportion of cells in the G1 phase in TuM than in TuWt (p = 0.0001) (Table 1) suggesting a loss of an active suppressor function of the *p53* gene in the TuM tumour. The mean values of the cell proliferation parameters S-phase fraction (SPF) and labelling index (LI) were significantly increased in tumour TuM (p = 0.0001 for both). The mean value of CL, the most important parameter for tumour growth, was increased in tumours with mutant *p53* (p = 0.02) compared with TuWt tumours. Ts was increased in tumour TuM (p = 0.0001) whereas the Tpot was significantly decreased (p = 0.002). We also observed a slightly lower proportion of cells in the G2 phase (p = 0.005). Thus, the results

Table 1

Cell kinetic parameters in tumours expressing either wild-type or mutant p53

Measured parameter	TuWt (n = 10)	TuM (n = 10)	
TVDT, days	5 (0.8)	4 (1.0)	n.s.
LI%	10 (2)	26 (4)	p = 0.0001
SPF%	13 (2)	42 (4)	p = 0.0001
G0/G1%	80 (2)	53 (3)	p = 0.0001
G2/M%	6 (1)	4 (0.7)	p = 0.005
Ts h	7 (0.7)	15 (1)	p = 0.0001
Tpot h	64 (10)	46 (9)	p = 0.002
CL%	42 (14)	59 (8)	p = 0.02

Results are presented as mean (SD)
p-values represents comparison between TuWt and TuM

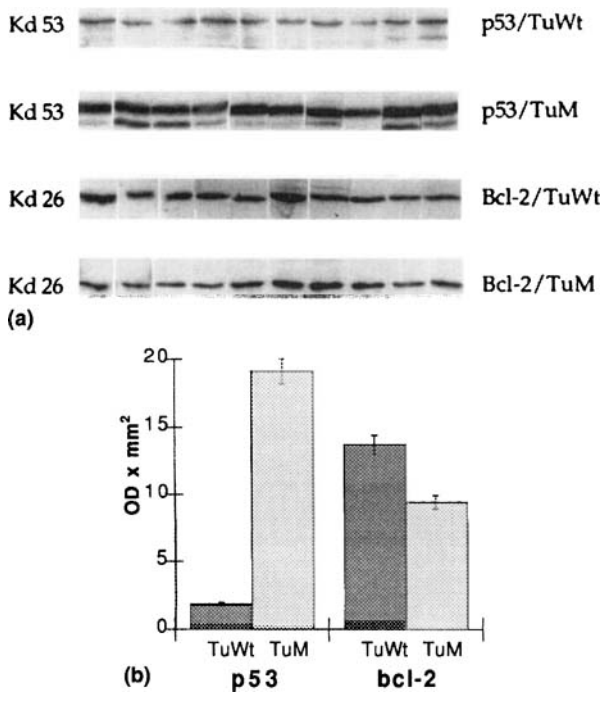

Fig. 3. a) Ten tumours of both variants were analysed for p53 and bcl-2 expression using Western blotting. The p53 protein was significantly overexpressed while the bcl-2 protein was significantly less abundant in TuM variants (the membranes of TuM tumour were exposed only for 1 min to make a comparison with bands of TuWt possible). b) Expression of p53 and bcl-2 in TuWt and TuM. Data were obtained by measuring Western blots and each column represent OD × mm^2 ± SD.

showed both a markedly increased cell proliferation (growth fraction) and an increased cell loss factor in the tumour variant with mutant (TuM) compared with tumours with wild-type p53 (TuWt). This could explain why no increase in growth rate was observed in the TuM variant.

p53 and bcl-2 expression

Semiquantitative analysis of the p53 protein showed significant overexpression in TuM compared with the expression in TuWt ($p = 0.0001$), while bcl-2 expression was lower in tumours containing mutant p53 than in tumours with wild-type p53 ($p = 0.03$) (Fig. 3). The decreased bcl-2 expression in TuM tumours may in turn explain an increased apoptosis, measured in the present experiment as increased CL.

DISCUSSION

In the present study we have found that cell proliferation, measured as SPF and LI, was significantly higher in TuM variants than in TuWt tumours. As a consequence, and since the difference between G2 phases was small, there were significantly fewer cells in the G1 phase in variants expressing mutant as compared with wild-type *p53*.

Our results thus suggest that the *p53* gene acts as an effective suppressor through blocking cells in the G1 phase in TuWt tumours expressing wild-type protein and, moreover, that mutations at codon 175 and 248 in the gene resulted in impaired suppressor function in this human endometrial adenocarcinoma.

The wild-type *p53* gene acts as a checkpoint control in normal cells. In DNA-damaged or stressed cells the half-life of the protein increases, i.e., the suppressor function activates, thereby switching off replication to allow extra time for DNA repair mechanisms to act (12). However, in accord with reports by Chang et al. (23) the increased SPF and LI and decreased number of G1 cells in TuM tumours in the present study suggest, that the wild-type p53 protein could act as a permanently active suppressor in malignant cells even in those without acute DNA damage triggered through radiation or cytotoxic agents. If this is true, p53 protein was also overexpressed in TuWt variants compared with that in normal endometrium.

One can only speculate that this suppressing function may vary depending, for example, on co-existing mutations in other proto-oncogenes or, more generally, on the cell type (origin) of the tumour. Therefore, one should be cautious in interpreting that positive immunohistochemical staining represents *p53* gene mutation, as has also been suggested by others (24).

The mean Ts was twice as long in TuM compared with TuWt variants. One explanation for this could be the fact that when higher numbers of cells with altered DNA progress into the S-phase, more time is required for DNA synthesis. Another explanation for our finding could be that the p53 suppressor function also includes control of the DNA synthesis time, which is lost when the gene is mutated at codon 175 and 248. No published results, however, provide an explanation for this finding.

In this study we found a significantly lower mean proportion of cells in the G2 phase in TuM than in TuWt tumours. The difference is small, however, and the results do not explain whether this effect is a direct or an indirect consequence of the p53 mutations.

Another important observation was the significantly lower bcl-2 expression in TuM tumours as compared with TuWt tumours, suggesting that overexpression of p53 protein probably down-regulated bcl-2 expression. This is in accordance with observations in which similar down-regulation of bcl-2 expression by increased p53 amounts was found (9, 25). Moreover, Haldar et al. (26) reported that most p53 mutations, excluding those at codon 280 or 285, may down-regulate bcl-2 expression in cancer cells. The significantly increased CL observed in our study could thus be a result of increased (wt p53-independent) apoptosis caused by decreased bcl-2 expression in the TuM tumours.

Taken together, our results indicate that the cell loss factor and cell proliferation could be linked through p53

and bcl-2 expression. Our findings also suggest that, through p53–bcl-2 linkage, the experimental tumours maintained a balance between cell proliferation and volume growth even when p53 has lost its wild-type function. This balance might be genetically determined and be characteristic for each tumour, reflected in their baseline (i.e. spontaneous) growth.

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