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**Ana-Maria Bamberger · Lutz Riethdorf
Karin Milde-Langosch · Christoph M. Bamberger
Imke Thuneke · Insa Erdmann · Heinrich M. Schulte
T. Löning**

Strongly reduced expression of the cell cycle inhibitor p27 in endometrial neoplasia

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Abstract In the present study we investigated the expression of the cell cycle inhibitor p27 in endometrial neoplasia using immunohistochemistry with a p27-specific antibody. Expression of p27 in endometrial carcinomas was compared with expression in the normal endometrium throughout the cycle. Normal endometrial cells showed strong nuclear expression of p27. Expression was present throughout the cycle and was stronger during the secretory phase. We found strongly reduced or abolished expression of p27 in endometrial carcinoma (85.3% of cases). The 41 tumours analysed were classified according to p27 staining intensity and percentage of positive cells into the following categories of p27 expression: negative/very low (56.0%); low (29.3%); moderate (14.7%) and high (0.0%). All the p27-positive tumours were well-differentiated endometrioid carcinomas of malignancy grade G1. Comparison with the p53 status showed that all tumours with strong p53 expression had low/negative p27 staining, while those that were positive for p27 had negative/low p53 staining. Reduced or absent p27 levels were also observed by Western blot analysis both in tumour samples and in HEC-1B endometrial adenocarcinoma cells. It thus seems that p27 expression is essential for the control of normal endometrial proliferation, and reduced or absent p27 expression may be an important step in endometrial carcinogenesis.

Introduction

Progression through the cell cycle and cellular proliferation is under the control of a series of cyclins and cyclin-dependent kinase (CDK) complexes (for reviews see [9, 28]). In mammalian cells, the cyclin D–CDK4 and cyclin E–CDK2 complexes are active during late G1 phase and are implicated in G1/S progression. p27 is one member of a group of proteins identified as CDK inhibitors, which cause G1 arrest when overexpressed in transfected cells (reviewed in [28, 29]). CDK inhibitors are classified into two families: the Cip/Kip family members (p21, p27 and p57) possess the ability to inhibit a variety of cyclin–CDK complexes and share partial structural similarity; the Ink4 family members (p15, p16, p18 and p19) are CDK4/CDK6-specific inhibitors [28, 29]. p27 (Kip1) is associated predominantly with cyclin D–CDK4, but shows the ability to inhibit a variety of cyclin–CDK complexes in vitro [21, 22, 32]. It has been suggested that p27 mediates G1 arrest induced by transforming growth factor-β (TGFß), contact inhibition and serum deprivation epithelial cells [21], which indicates that normal levels of p27 might be important in controlling cellular proliferation and opposing tumour progression. This role is sustained by the recent findings that p27 expression is reduced in malignancies of the breast, colon and stomach [2, 13, 15, 24, 31]. Decreased or absent p27 expression has been implicated as a powerful negative prognostic factor in these malignancies. We used immunohistochemistry to investigate expression of p27 in normal human endometrium throughout the cycle, and also in endometrial hyperplasia and endometrial carcinomas. This study was prompted by the clinical importance of endometrial cancer and by the unexplained observation that p27 knockout in female mice results in endometrial hyperplasia [5, 17], a frequent precancerous lesion of endometrial adenocarcinoma.

A.-M. Bamberger (✉) · L. Riethdorf · K. Milde-Langosch
T. Löning
Institute of Pathology, Department for Gynaecopathology,
University Hospital Eppendorf, Martinistrasse 52,
D-20246 Hamburg, Germany
Tel.: +49-40-47173162, Fax:+49-40-47174961

C.M. Bamberger · I. Thuneke · I. Erdmann · H.M. Schulte
Institute for Hormone- and Fertility Research,
University of Hamburg, Grandweg 64,
D-22529 Hamburg, Germany

Materials and methods

HEC-1B endometrial adenocarcinoma and SKUT-1B mixed mesodermal tumour cells were purchased from ATCC (Rockville, Md.). HEC-1B cells were cultivated in DMEM and SKUT-1B cells in a 1:1 mix of Ham's F12 and DMEM (all from Gibco, BRL), with 10% FCS and antibiotics, and passaged twice weekly.

For protein preparations, fresh human endometrium and endometrial tumours were obtained immediately after surgery and stored in liquid nitrogen. Each sample was also assessed histologically.

For immunohistochemistry, surgical specimens that had been routinely fixed in 4% buffered formalin and embedded in paraffin were used. Tissue samples included normal endometrium (n=21, proliferative phase 12, secretory phase 9) and endometrial carcinomas (n=41). The tissue material was selected following histological review from the files of the Department of Gynaecopathology, University Hospital Eppendorf, Hamburg. All lesions were classified according to the most recent WHO criteria.

For p27 immunohistochemistry, serial sections 4–6 µm thick were cut from the paraffin blocks and mounted on APES-coated slides, deparaffinized in xylene and rehydrated in graded alcohol to tris-buffered saline TBS: 50 mM Tris, 150 mM NaCl, pH 7.4). The slides were microwaved for 5×2 min in 10 mM citrate, pH 6.0. After cooling for 20 min, the slides were washed in TBS, blocked for 30 min at room temperature with normal goat serum (Dako, Glostrup, Denmark), diluted 1:20 in TBS and incubated with p27 mouse monoclonal antibody (Novocastra Laboratories, Newcastle-upon-Tyne, UK) at a dilution of 1: 150 in TBS for 24 h. Nonimmune murine serum (Dako) at the same dilution was used for negative control. Slides were then reacted with biotin-labelled anti-mouse IgG and incubated with preformed avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, Calif.). Diaminobenzidine (DAB) substrate was then added. Sections were counterstained with haematoxylin, dehydrated and mounted.

For p53 immunohistochemistry, deparaffinized tissue sections were washed in phosphate-buffered saline and preincubated in 0.5% blocking serum for 20 min. The slides were incubated overnight at 4°C using the mouse monoclonal p53 antibody Ab-6 (Oncogene Science, Dianova, Hamburg, Germany) diluted at 1:150 in 1% bovine serum albumin (Sigma, Deisenhofen, Germany). For detection, biotinylated rabbit anti-mouse secondary antibody (1:100; Amersham, Braunschweig, Germany) and streptavidine-alkaline phosphatase conjugate (1:1000; Gibco-BRL, Eggenstein, Germany) were used, each applied for 1 h at 37°C. After colour development with NBT/BCIP 8NBT: 0.3 mg/ml; BCIP: 0.2 mg/ml; Gibco-BRL, Eggenstein, Germany) for 15 min in the dark, the slides were mounted in glycerin gelatin without counterstaining. Using this method, 35 carcinoma samples had been previously analysed for p53 expression [26].

Histological and immunohistochemical evaluation were performed independently by two pathologists. Every tumour was assessed and given a score, obtained by multiplying the intensity of the staining (no staining=0; low level=1; medium staining=2; strong staining=3) by the percentage of cells stained (0%=0; under 10%=1; 10–50%=2; 51–80%=3; over 80%=4). With this system, the maximum score is 12 (over 80% of the cells showing strong staining).

For Western blot analysis, frozen tissue was minced and cells were lysed in ice-cold sample buffer b1 (50 mM Tris pH 6.8, 1% SDS, and 10% sucrose). Protein concentration was determined following standard protocols and using BSA protein standards diluted with sample buffer b1. Samples were diluted with a 1:1 mixture of sample buffer b1 and b2 (containing 50 mM Tris pH 6.8, 3% SDS, 10% sucrose, 10% β-mercaptoethanol, and 0.01% bromphenol blue) to give a final volume of 100 µl and a final protein concentration of 300 µg/ml. For electrophoresis, an equal amount of protein (40 µg) of each sample was loaded in each well. Electrophoresis was performed in a 10% polyacrylamide separating gel and a 3% stacking gel. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon P, Millipore, Eschborn, Germany). Membranes were stained with Ponceau S to determine transfer efficiency and homogeneity of protein lanes. After destaining with TBS (20 mM Tris/HCl, pH 7.6, 137 mM NaCl) for 5 min, membranes were incubated overnight at 4°C in blocking solution (0.1 M maleic acid, pH 7.5, 0.15 M NaCl, 0.005% thimerosal, and 1% blocking reagent; Boehringer Mannheim, Germany). The membranes were washed for 10 min in TBST (TBS plus 0.05% Tween 20) and incubated with anti-p27 mouse monoclonal antibody (Novocastra Laboratories) in a dilution of 1:400. Dilution was performed with 9:1 TBST/blocking solution. Blots were incubated for 1 h at room temperature, washed for 3×10 min in TBST and incubated with the second antibody for 1 h at room temperature. The second antibody was visualized by enhanced chemiluminescence (ECL) reagents (Amersham, Braunschweig, Germany) and Fuji RX 400 films.

Results

Immunohistochemistry was used to investigate expression of p27 protein in normal human endometrium and endometrial carcinomas. Every sample was assessed independently by two pathologists and given a score reflecting both the intensity of p27 staining and the percentage of cells stained. The maximum score using this system is 12 (over 80% of the cells showing strong staining).

In the normal human endometrium, p27 expression was observed throughout the menstrual cycle, that is to say in both the proliferative phase (Fig. 1a) and the secretory phase (Fig. 1b). The 21 samples analysed included none in the negative/very low (0–3) score category for p27 expression. Of the proliferative phase samples (n=12), 66.7% showed strong p27 expression (score 7–12), while 33.3% had a score of 4–6. Expression was even stronger in the secretory phase, 88.9% of these samples having a high level of expression (scores of 7–12).

A series of 41 endometrial carcinomas was analysed (Table 1). Reduced or absent p27 staining (score categories negative/very low and low combined) was observed in 35 (85.3%) of the 41 tumours (Table 1, Fig. 1c). Table 2 presents data on grading, invasiveness, histology and p53 status of the tumours analysed. For this analysis, the tumours were been divided into two categories, one with low p27 expression (score 0–6) and one with moderate/high p27 expression (score 7–12). It is interesting to observe that the p27-positive tumours were all well-differentiated endometrioid carcinomas of malignancy grade G1 (Table 2). There was no observable trend toward low p27 expression with invasion depth and/or high staging (Table 2). The p53 status of these tumours was also analysed (35 cases as part of a previous study focused on p53 expression; see [26]). Comparison with the p27 expression levels in these tumors showed that all those with strong p53 expression had low/negative p27 staining, while those that were positive for p27 had negative/low p53 staining (Table 2).

Table 1 p27 Staining intensity scores of the tumours analysed

p27 Staining	No. of tumours (total 41)	
intensity score	n	%
0–3 (Negative/very low)	23	56.0%
4–6 (Low)	12	29.3%
7–9 (Moderate)	6	14.7%
10–12 (High)	0	0.0%

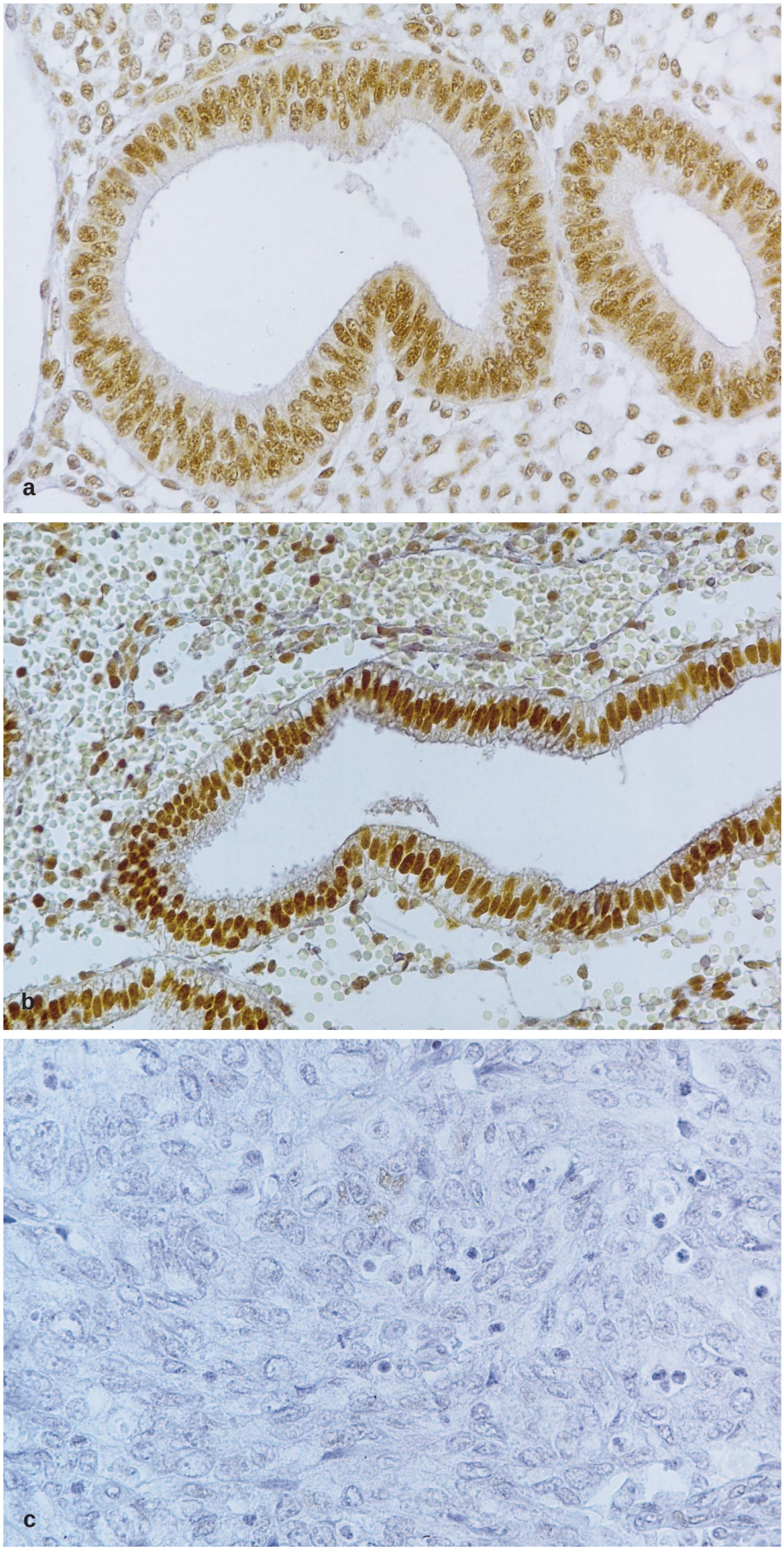

Fig. 1a–c Immunohistochemical detection of p27 in normal endometrium and endometrial carcinomas. **a**, **b** Normal endometrium: **a** proliferative phase and **b** secretory phase, showing strong nuclear staining for p27. **c** Endometrial adenocarcinoma, showing very low/absent expression of p27; this tumour had a score of 2. (×400)

Table 2 Expression of p27 in endometrial carcinomas – correlation with grading, invasiveness, histological type and p53 protein expression

Parameter	Score 0–6 (very low and low)		Score 7–12 (moderate and high)	
	n	%	*n*	%
1. Grading (*n* = 41)				
G1 (*n* = 21)	15	71.4%	6	28.6%
G2 (*n* = 15)	15	100.0%	0	0.0%
G3 (*n* = 5)	5	100.0%	0	0.0%
2. Invasiveness (n = 41)				
FIGO Ia (*n* = 8)	7	87.5%	1	12.5%
FIGO Ib (*n* = 21)	18	85.7%	3	14.3%
FIGO Ic (*n* = 6)	5	83.3%	1	16.7%
FIGO II (*n* = 6)	5	83.3%	1	16.7%
3. Histology (*n* = 41)				
Endometrioid, G1 (*n* = 21)	15	71.4%	6	28.6%
Endometrioid, G2 + G3 (*n*=16)	15	93.8%	1	6.2%
Nonendometrioid, G3 (*n* = 4)	4	100.0%	0	0.0%
4. p53 Status (*n* = 41)				
Negative (*n* = 16)	14	87.5%	2	12.5%
Very low/low (*n*=18)	13	72.2%	4	27.7%
Moderate/high (*n*=7)	7	100.0%	0	0.0%

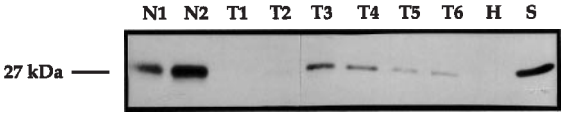

Fig. 2 Western blot analysis. Strong expression of p27 in normal endometrial samples (*N1*, *N2*) reduced or absent expression in tumour samples (*T1–T6*); abolished p27 expression in HEC-1B endometrial adenomacarcinoma cells (*H*) and strong p27 signal in SKUT-1B mixed mesodermal tumour cells (*S*). An equal amount of protein (40 µg/well) was loaded for each sample

Western blot analysis using the p27 antibody used for immunohistochemistry was performed on two normal and six tumor samples for which fresh-frozen material could be obtained. The results are presented in Fig. 2. This analysis confirms strong p27 expression in the normal endometrium (samples N1 and N2) and abolished or reduced expression in tumours (samples T1–T6) (Fig. 2). In addition, two tumour cell lines were analysed: the HEC-1B endometrial adenocarcinoma cell line, which is of epithelial origin and was negative for p27, and the SKUT-1B mixed mesodermal tumour cell line, which showed strong p27 expression (Fig. 2).

Discussion

In this study we used immunohistochemistry with a specific monoclonal antibody to investigate expression of the cell cycle inhibitor p27 in the normal human endometrium throughout the cycle, in endometrial hyperplasia and in endometrial carcinomas. Our data show that p27 is expressed in nuclei of normal endometrial cells throughout the menstrual cycle, and that expression is higher during the secretory phase. Of the 41 tumours analysed, 85.3% revealed diminished or absent expression of p27, indicating that loss of p27 expression plays an important part in the pathogenesis of endometrial carcinoma. The p27-positive tumours were all well-differentiated endometrioid carcinomas of malignancy grade G1, which indicates that a certain level of p27 expression might still be protective. However, in most (71.4%) of the G1 tumours p27 expression was already low/absent, which suggests that loss of p27 expression is probably an early event in the development of endometrial carcinoma. Comparison with p53 expression levels revealed that tumours with strong p53 expression had low/absent p27 staining. An interesting observation in this context is that p53 alterations (indicated by strong expression and/or mutations) have more frequently been associated with malignancy grade G3 and/or with nonendometrioid (serous–papillary) histology in previous studies [26]. In our study, G3 tumours (both endometrioid and nonendometrioid) showed low/absent p27 expression in addition to p53 alterations, indicating that both loss of p27 expression and p53 alterations may be implicated in progression to G3 or the development of a more aggressive nonendometrioid (serous–papillary) histology.

Western blot analysis using the same monoclonal antibody as had been used for immunohistochemistry showed that this antibody specifically recognizes a single band of 27 kDa molecular weight in normal endometrium, which we used as a positive control (Fig. 2). Compared with the p27 expression in the normal endometrium samples, in the tumour samples analysed p27 expression was low or absent (Fig. 2). Analysis of p27 protein levels in two uterine tumour cell lines with different origins, the HEC-1B endometrial adenocarcinoma cell line, which is p27 negative, and the SKUT-1B mixed mesodermal tumour cell line, which is p27 positive, indicates that abolished p27 expression might be an important specific step in the pathogenesis of adenocarcinoma.

There is increasing evidence that the molecules involved in cell cycle control are also involved in oncogenesis. One example is cyclin D1, which has been shown to be amplified in a subset of human cancers, including malignancies of the breast, ovary and endometrium [4, 7, 8, 18, 30]. Since they regulate cyclin–CDK complex activity, CDK inhibitors are potential tumour suppressors, and loss of their expression is possibly an important step in promoting tumour growth. Several mechanisms of inactivation have been described for CDK inhibitors, one of which is mutation and/or deletion in the human *p15*/*p16* locus. These are frequently found in human malignancies [3, 12, 14, 25, 27, 33]. However, this mechanism does not seem to play a part in loss of p27 expression; despite an extensive search for molecular aberrations, no significant alterations of the *p27* gene have been reported [1, 10, 16, 20, 23]. One mechanism involved in controlling p27 levels is the degradation of p27 protein by the ubiquitin–proteasome pathway [19]. Compared with proliferating cells, quiescent cells have been shown to have a smaller amount of p27 ubiquitinating activity, which results in a marked extension of p27 half-life in these cells [19]. Furthermore, colonic carcinomas with low or absent p27 have been shown to possess enhanced proteolytic activity specific for p27 [13], suggesting that aggressive tumours might result from clones lacking p27, because of increased degradation of the protein.

Female mice in which the *p27* gene had been knocked out had abnormal endometrial proliferation [17]. The cause for this abnormal proliferation of the endometrium is not known, and a possible hormonal cause, such as high production of oestrogen from hyperplastic ovarian follicles, has been suggested but not proven. Our data indicate that the phenomenon might be explained by the importance of p27 as an intrinsic regulator of normal proliferation of the endometrium. In addition, p27 null female mice were infertile [5, 11, 17], and it has been suggested that the cause for their infertility might be an inadequate uterine environment [5]. Superovulation was not sufficient to induce successful pregnancy and there was deficient implantation in the majority of knockout mice [5]. It will therefore be interesting to determine whether deregulated expression of p27 also has a role in human fertility. At this point, p27 seems to play an essential role in the physiology of normal endometrium in both rodents and humans and its reduction or absence seems to result in aberrant proliferation and tumour formation.

Reduced levels of p27 expression have also very recently been described in malignancies of other organs, such as the breast, the colon and the stomach [2, 13, 15, 24, 31]. Very recently, Fero et al. showed that both p27 nullizygous and p27 heterozygous mice are predisposed to tumours in multiple tissues when challenged with carcinogens or γ-irradiation [6]: p27 is, then, a multiple-tissue tumour suppressor. Loss of even one allele predisposes mice to tumour formation. Interestingly, lesions in female reproductive organs were also more frequent in p27-deficient mice and included endometrial adenocarcinoma, thus confirming the possibility of an important role for p27 in the pathogenesis of endometrial neoplasia. Therefore, at this point p27 seems to be a key molecule in processes regulating normal tissue growth and a reduction of its level to be a critical step in tumour development. The importance of p27 in regulating normal proliferation processes and its reduction in pathologic proliferative processes make it an interesting target for therapy of such states.

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