Decreased level of the cell cycle regulator p27 and increased level of its ubiquitin ligase Skp2 in endometrial carcinoma but not in normal secretory or in hyperstimulated endometrium

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p27 is a cyclin-dependent kinase (CDK) inhibitor whose specific late G1 destruction allows progression of the cell across the G1/S boundary. The protein is ubiquitinated by S-phase kinase-interacting protein-2 (Skp2) following its specific phosphorylation, and is subsequently degraded by the 26S proteasome. There is a direct relationship between low level of p27 and rapid proliferation occurring in several benign states and in many malignancies. In the glandular cells of the normal endometrium, the level of p27 is exceedingly low during the proliferative phase, whereas it is markedly increased during the secretory phase. The expression of p27 in endometrial carcinoma is very low but has been found to increase following treatment with progesterone. However, estrogen exposure is considered as a major risk factor in developing endometrial cancer. The implications of the high dose of estrogen and progesterone induced during IVF treatment are still unknown. We have examined the expression of p27 and Skp2 as well as of Ki67 proliferation marker by using endometrial extracts and cells from normal endometrium, from ovarian hyperstimulated patients, and from endometrial carcinoma patients. The expression of p27, Skp2 and Ki67 was found to be similar in both normal secretory endometrium and endometrium from ovarian hyperstimulated patients. In striking contrast, p27 is significantly lower while Skp2 and Ki67 are significantly higher in the endometrial carcinoma and in endometrium from the proliferative phase compared with their normal secretory counterpart tissue.

Key words: endometrium/IVF/ovarian hyperstimulation/p27/ubiquitin

Introduction

The ubiquitin proteolytic pathway plays key roles in regulating the levels of many proteins, which are involved in diverse cellular processes. Proteins targeted for degradation are first tagged by a poly-ubiquitin chain in a three-step cascade mechanism involving ubiquitin activation (catalysed by the ubiquitin activation enzyme, E1), ubiquitin transfer (catalysed by a ubiquitin carrier protein, E2) and ubiquitin ligation (catalysed by a specific ubiquitin protein ligase, E3). Ubiquitin ligases determine the high specificity and selectivity involved in the recognition of their target protein substrates. Tagged proteins are then degraded by the 26S proteasome complex (Glickman and Ciechanover, 2002), and then the free reusable ubiquitin is recycled.

p27Kip1 is a cell cycle regulator. It binds and negatively regulates the activity of CDK2–cyclins A and E. Its level is high in quiescent cells, but following mitogenic stimuli, it is rapidly degraded by the ubiquitin system, allowing the CDK–cyclin complexes to drive the cell into S phase (Sherr and Roberts, 1999). Recent studies have identified S-phase kinase-interacting protein-2 (Skp2) as the specific substrate recognition subunit of the ubiquitin ligase E3 that targets p27 for conjugation and subsequent degradation following its phosphorylation on Thr187 (Carrano et al., 1999). The Skp2, F-box subunit, is part of a larger SCF (Skp1, Cullin, F-box protein)–ligase complex (Deashies, 1999). It has been reported that p27 levels are markedly reduced in several malignancies, such as those of the breast (Tan et al., 1997), prostate (Tsihlias et al., 1998) and endometrium (Shiozawa et al., 1998; Oshita et al., 2002). In some of the tumours studied, a strong correlation has been found between the low level of p27, the aggressiveness of the disease and poor prognosis of the patients (Loda et al., 1997; Tan et al., 1997). Interestingly, the p27 in all these tumours is of the wild-type species, and its deregulation has been attributed to aberrant accelerated ubiquitin-mediated degradation of the protein. The endometrium is a unique tissue in that the level of p27 is hormone dependent. Estrogen exposure has been considered as an important risk factor in developing endometrial cancer (Tong and Pollard, 1999). Up-regulation of p27 by progesterone has been demonstrated in glandular cells (Shiozawa et al., 2001). IVF patients treated with gonadotrophins prior to oocyte retrieval are exposed to extremely high estrogen levels, particularly those at high risk for developing ovarian hyperstimulation syndrome (OHSS), which is one of the major complications of infertility treatment. In this syndrome, the high level of
endometrium has been shown to induce an increase in capillary permeability which in turn results in a shift of fluid from the intravascular space into the abdominal cavity and may lead to circulatory and excretory disorders (Speroff et al., 1994).

Only a few studies have examined the possible relationship between ovulation induction drugs during IVF treatments and cancer. Most of these have been epidemiological in nature. In the present study, levels of p27 and its ubiquitin ligase, Skp2, were examined in endometrium of IVF patients at high risk for developing OHSS, and were compared with normal secretory, proliferative and malignantly transformed endometrium.

Materials and methods

Patient population

We analysed endometrial biopsies from four groups of patients: (1) day 22 endometrium obtained from patients undergoing IVF being at high risk of developing OHSS, from whom all embryos were cryopreserved; (2) day 22 endometrial biopsies from secretory endometrium of non-stimulated normal cycling women; (3) day 10 endometrial biopsies from proliferative endometrium of non-stimulated normal cycling women; (4) endometrial carcinoma samples.

The histology type in patients in group 4 were all endometroid carcinomas grade 1 and 2 (four cases were of grade 1 and nine were of grade 2). In 10 cases the tumour was stage I, two cases were of stage III and one tumour was of stage IV, according to the classification of the International Federation of Gynecology and Obstetrics (FIGO). The age of patients was 28.9 ± 5.0 in group 1, 36.5 ± 5.4 in group 2, 36.1 ± 5.3 in group 3, and 64 ± 10.0 in group 4.

Patients were considered at high risk for the development of OHSS when estradiol (E2) serum level was >12 000 pmol/l, and >20 medium-sized (13–15 mm) follicles were observed on transvaginal ultrasound examination (TVS) on the day of ovulation triggering.

The Local Ethical Committee of the Carmel Medical Center approved all study protocols.

Ovarian hyperstimulation was performed in all women with long down-regulation protocol using GnRH agonist buserelin (Suprefact® nasal spray; Hoechst, Germany) 1000 µg/day, continued up to the day when hCG was administered. When laboratory testing indicated pituitary suppression (estradiol <40 pg/ml), treatment with i.m. hMG (Pergonal; Serono, Israel), 2–3 ampoules per day, was started. Follicular monitoring by serum estradiol, LH, progesterone and serial TVS scan was performed as previously described (Dirnfeld et al., 1993). When three or more follicles of >18 mm in diameter were present on TVS and E2 was not >15 000 pmol/l, patients received 5000 IU of hCG i.m. TVS-guided follicular aspiration was scheduled 35–36 h after hCG administration. When E2 was >12 000 pmol/l, all embryos obtained were cryopreserved.

Immunohistochemistry

Endometrial biopsies, derived from eight IVF patients all of whose embryos were cryopreserved due to high risk for OHSS, 26 normal ovulating patients (13 in the secretory phase and 13 in the proliferative phase) and 13 patients with endometrial carcinoma, were sectioned. The sections were deparaffinized.

For epitope retrieval, slides were heated in a microwave oven at 92 °C for 20 min in a Tris–EDTA buffer, pH 8.0.Slides were then incubated overnight at 4 °C with the primary antibodies (monoclonal anti-human Skp2 (Zymed Laboratories, USA) diluted 1:100, monoclonal anti-human p27 (Transduction Laboratories) diluted 1:2000, and anti-Ki67 clone MIB-1 (Dako, Denmark) diluted 1:90). Staining of Skp2 was completed with EnVision kit (Dako, Denmark), and staining of p27 and Ki67 with Histosave Plus kit (Zymed Laboratories). Colour reaction product was developed with aminoethylcarbazole as the chromogen. All sections were counterstained with haematoxylin. For negative controls, sections were incubated with 1% bovine serum albumin (BSA) instead of the primary antibody. Tissue sections known to be positive for staining for p27, Skp2 and Ki67 served as positive controls. These controls were run in parallel.

Immunofluorescence

For double immunofluorescence staining of the paraffin-embedded sections, antigen retrieval and blocking steps were performed as described above for immunohistochemistry. Sections were incubated (overnight at 4 °C) simultaneously with monoclonal anti-Skp2 antibody (1:100; see above), and a rabbit polyclonal anti-p27 (1:400; Santa Cruz Biotecnology Inc.). After washing with phosphate-buffered saline (pH 7.4), secondary antibodies were applied at room temperature for 1 h. These antibodies were Fluororescein-Cy3-conjugated donkey anti-mouse antibody and Rhodamine-Red-X-conjugated anti-rabbit antibody (Jackson ImmunoResearch Laboratories, USA). The fluorescent probes were photographed using a fluorescence microscope axioskop 2 (Zeiss, Germany) and a CCD camera, and colour processing was performed using the Micromax imaging system. For negative controls, sections were incubated with 1% BSA instead of the primary antibody as was performed for the immunohistochemistry.

Tissue extracts

Tissues were extracted as previously described (Hershko et al., 2001). Briefly, endometrial samples were immediately frozen and stored at −70 °C. Samples were homogenized at 0 °C (using Polytron 1200 homogenizer) with 3 volumes (v/w) of cold Tris–HCl buffer (50 mmol/l, pH 7.2), 10 µg/ml leupeptin, 10 µg/ml chymostatin, 10 µg/ml pepstatin, 5 mmol/l N-ethylmaleimide, and 0.25 mmol/l EDTA. The homogenates were centrifuged at 14 000g for 15 min. The supernatants (100 µg from each sample) were mixed with electrophoresis sample buffer and resolved via sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (12.5% acrylamide).

Immunoblotting

The SDS–PAGE-resolved proteins were blotted onto nitrocellulose membranes (Scheicher & Schuel, Germany). The membranes were blocked with 8% non-fat dry milk in Tris-buffered saline (20 mmol/l, pH 8.0) containing 0.01% Tween-20 (TBS-T) and probed with anti-human Skp2 (1:500), anti-human p27 (1:1000), and anti α-tubulin (Sigma) (1:2000). After washing with TBS-T, membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Jackson ImmunoResearch Laboratories) diluted 1:10 000. Proteins were visualized by enhanced chemiluminescence system (ECL Super-signal; Pierce) and exposed to X-Ray film (Fuji).

Statistical analyses

Quantitative analyses of p27, Skp2 and Ki67 expression (by immunohistochemistry) in tumour and normal cells were performed using the Mann–Whitney U-test. Expression of the proteins was compared in the epithelial cells of all four groups using the Kruskal–Wallis non-parametric analysis of variance, followed by Dunn’s post-test. Two-tailed P ≤ 0.05 was considered to be statistically significant.

Results

Endometrial samples derived from patients at high risk of OHSS, patients with normal secretory endometrium, patients with normal proliferative endometrium or patients with endometrial malignancy, were stained for p27, Skp2 and Ki67. Results are presented in Tables I and II, and in Figures 1 and 2.
endometrium derived from patients at high risk of ovarian hyperstimulation (compare Figure 1D–F with G–I).

In contrast, a significantly lower expression of p27 and a significantly higher expression of Skp2 and Ki67 were observed in endometrial carcinoma cells (Figure 1J–L).

The expression of these proteins was further analysed in stromal cells, which are known to be the main target of progesterone stimulation. Similar expression of p27 and Ki67 was found in stromal cells derived from secretory endometrium and from endometrium following ovarian hyperstimulation. Skp2 expression was higher in stromal cells derived from endometrium of patients with ovarian hyperstimulation compared with stromal cells derived from normal secretory endometrium (4.22 ± 3.32 and 2.14 ± 0.86 respectively), but this difference was not statistically significant (Table II). Stromal cells derived from endometrial carcinoma samples are not presented in Table II, because in malignant tissue, stromal cells cannot be recognized and only desmoplastic stroma can be seen in some cases.

Table I. Percentage (± SD) of epithelial cells that stained positive for p27, Skp2 and Ki67

<table>
<thead>
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<th></th>
<th>p27</th>
<th>Skp2</th>
<th>Ki67</th>
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<tbody>
<tr>
<td>Sec</td>
<td>97.76 ± 2.24</td>
<td>0.46 ± 0.66</td>
<td>0.87 ± 0.86</td>
</tr>
<tr>
<td>HS</td>
<td>94.3 ± 4.4</td>
<td>0</td>
<td>0.33 ± 0.51</td>
</tr>
<tr>
<td>EC</td>
<td>41.12 ± 21.12</td>
<td>25.75 ± 7.26</td>
<td>27.0 ± 9.5</td>
</tr>
<tr>
<td>Prol</td>
<td>38.8 ± 23</td>
<td>16.9 ± 7.3</td>
<td>32.4 ± 12</td>
</tr>
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$^aP < 0.0001$, $^bP < 0.006$, the proliferative phase compared with the endometrial carcinoma.

$^a$Non-significant.

In the present study, p27 staining has been found to be significantly lower in the proliferative phase than in the secretory phase. Ki67, a marker for proliferation, has also been found to be similar in these two groups of patients. In contrast, the levels of p27 and its specific ligase Skp2 have been similarly lower in the proliferative phase than in the secretory phase. Unlike the histochemical method, fluorescence microscopy enables one to detect simultaneously several antigens in the same cell. It clearly emphasized the differential distribution of p27 and Skp2 in different cells of the same section. Thus, we utilized fluorescent microscopy to observe p27 and Skp2 in normal secretory and Skp2 in malignant endometrial tissue; here, an inverse expression of these proteins was observed: Skp2-positively stained cells can be seen clearly, while the level of p27 is lower compared to the benign tissue. In the malignant cells the nuclei are elongated.

The results presented in Table I further corroborate the microscopic observation. The percentage of epithelial cells of secretory endometrium that were stained positively to Skp2 is 0.46 ± 0.66%. This level of expression is similar to the epithelial cells of hyperstimulated samples which were found to be negative for Skp2. In contrast, in epithelial cells from endometrial carcinoma, the staining reached 25.75 ± 7.26%; a 50-fold increase compared to the normal secretory or hyperstimulated endometrium (Table I). In the proliferative phase, 16.9 ± 7.3% of the epithelial cells were stained for Skp2. This level of expression is significantly higher than its level in samples from secretory endometrium (Table I). p27 level in the proliferative phase was significantly lower than its level in the secretory phase (Table I).

Table II. Percentage (± SD) of stromal cells that stained positive for p27, Skp2 and Ki67

<table>
<thead>
<tr>
<th></th>
<th>p27</th>
<th>Skp2</th>
<th>Ki67</th>
</tr>
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<tbody>
<tr>
<td>Sec</td>
<td>69.58 ± 12.25</td>
<td>2.14 ± 0.86</td>
<td>6.35 ± 5.3</td>
</tr>
<tr>
<td>HS</td>
<td>61.34 ± 15.9</td>
<td>4.22 ± 3.32</td>
<td>6.4 ± 4.5</td>
</tr>
<tr>
<td>Prol</td>
<td>38.92 ± 17.0</td>
<td>9.07 ± 6.6</td>
<td>12.0 ± 7.5</td>
</tr>
</tbody>
</table>

$^aP < 0.001$, $^bP < 0.02$.

$^a$Non-significant.

In this study, endometrial samples were taken from the late secretory phase of the cycle when estrogen and progesterone are at their highest level. In this stage, OHSS becomes a risk in these IVF patients.

Similarly, the levels of p27 and its specific ligase Skp2 have been examined in normal proliferative endometrium obtained from IVF patients at high risk for OHSS and in endometrial carcinoma. Although statistically non-significant, Skp2 level has been demonstrated to be elevated in hyperstimulated stromal cells in comparison with normal secretory endometrium. This finding is probably the result of the cells’ response to the hormonal treatment. Ki67, a marker for proliferation, has also been found to be similar in these two groups of patients. In contrast, in the endometrial carcinoma, Ki67 and Skp2 expression were high, whereas p27 level was low.

In the present study, p27 staining has been found to be significantly lower in the proliferative phase than in the secretory phase.
This result is in agreement with previous reports (Shiozawa et al., 1998; Watanabe et al., 2002). In fact, our study shows that p27 expression in the proliferative phase is similar to that observed in endometrial carcinoma. The proliferative phase of the endometrial cycle is marked by increased mitotic activity and extensive cell divisions. However, we unexpectedly observed a 30-fold increase of Skp2 level in the endometrial samples from the proliferative phase compared with the secretory endometrium. Nevertheless, Skp2 level is significantly lower in the proliferative phase compared with its level in the endometrial carcinoma Skp2 expression increases during the S-phase of the cell cycle. It may increase also in certain conditions in normal tissue (Waltregny et al., 2001). However, the reversed high level of Skp2 in the proliferative endometrium raises questions about the role of Skp2 in normal and in malignantly transformed tissues.

The levels of Ki67 in the proliferative phase and in the endometrial carcinoma observed in the present study are similar to those reported in previous studies (Utsunomiya et al., 2001; Illouz et al., 2003).

Decreased level of p27 in endometrial neoplasia and hyperplasia has been demonstrated in previous reports (Bamberger et al., 1999). Surprisingly, in a study performed on p27 expression in endometroid adenocarcinoma of the uterine corpus, positive correlation was found between increased level of p27 and higher histological grade of the tumour. In this study, however, p27 levels in malignant samples were not compared with the normal tissue (Watanabe et al., 2002).

Although for a short period, IVF patients are exposed to a high dose of steroid hormones. The finding that p27 is regulated by progesterone on the one hand (Dai et al., 2002) and on the other hand was found to be correlated with pre-malignancy (Shiozawa et al., 1998) and malignancy (Bamberger et al., 1999) of the endometrium, prompted us to examine its level in the hyperstimulated tissue. One important unknown is: how long before malignant transformation occurs, is p27 down-regulated and Skp2 up-regulated. Since overexpression of Skp2 can be oncogenic (Signoretti et al., 2002), it is reasonable to believe that the aberrations in the relationship and steady state level of these proteins precede the malignant

**Figure 1.** Immunohistochemical staining of p27, Skp2, and Ki67 expression in normal secretory (Sec) and proliferative (Prol), hyperstimulated (HS) and malignant endometrial (EC) tissues. p27 is in A, D, G and J, Skp2 is in B, E, H and K, and Ki67 is in C, F, I and L. Magnification ×200.
transformation. Therefore, it was not unreasonable to assume that these aberrations may occur during the late secretory phase of patients at high risk for OHSS. Bebington et al. (2000) reported that during a natural cycle, but not during a hyperstimulated cycle, the level of ubiquitin in the endometrium is increased between days 4 and 10 post ovulation. However, since ubiquitin is essential for targeting numerous substrates, the significance of this finding, in particular as related to the fate of specific substrate, has yet to be elucidated.

p27 has to be phosphorylated on Thr187 before being recognized by the Skp2 ubiquitin ligase. Nevertheless, the levels of Skp2, as high as they may be, cannot regulate p27 by itself, and it is the kinase that governs its stability. It is not known whether the kinase (or a phosphatase) is affected in malignancies. Thus, the mechanism or mechanisms that underlie the down-regulation of p27 in malignant cells are still obscure, and the finding that Skp2 is up-regulated in all these cases is circumstantial at the moment, though the notion that it plays a causative role is attractive. Experimentally, p27 is rapidly degraded following phosphorylation, even when normal cellular levels of Skp2 are present. Thus, as in other studies of p27 in different tumours, we assumed that the steady state level of phosphorylated p27 could be low and therefore hard to detect. In a recent study on breast and ovarian tumours, the same results were demonstrated using antibody against p27 and against Serine-phosphorylated p27, but the latter showed a weaker signal (Yang et al., 2003).

The parallel high level of progesterone in IVF patients during the late secretory phase may have prevented elevation in Skp2 expression and a decrease in p27 level. Progesterone inhibits estrogen-induced cell proliferation and is currently routinely used to oppose estrogenic effects in post-menopausal women during HRT (Tong and Pollard, 1999). Progesterone has recently been found to inhibit endometrial cancer by inhibiting the cell cycle: Dai et al. (2002) found that the number of cells in G1 is significantly increased following treatment by progesterone.

Levels of p27 and Skp2 may serve in the future as prognostic indicators for malignancy or even pre-malignancy of the endometrium only in the secretory phase, where the level of p27 is expected to be high and the level of Skp2 is expected to be low. In view of our findings, the possible implications of ovarian hyperstimulation treatments and proliferation events in IVF patients should be further analysed using molecular and biochemical methodologies.

References


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