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Insulin-like Growth Factor 2 and its Receptors (IGF 1R and IGF 2R/ Mannose 6-Phosphate) in Endometrial Adenocarcinoma

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Summary

Objective. To investigate the consequences of IGF proteins dysfunction in development of endometrial adenocarcinomas.

Methods. The expression of IGF 2 and IGF 1R was correlated with the expression of IGF 2R and apoptosis rate in 59 human endometrial adenocarcinomas, 10 endometrial hyperplasias and 7 normal tissues. The presence of mutations in the IGF 2R gene was followed in 46 adenocarcinomas. We also examined the effect of IGF 1 receptor blockage on cancer cell proliferation. In groups of either IGF 2 positive or IGF 2 negative tumors (stages III and IV) the expression of IGF 1, IGF 1R was correlated with cell proliferation index and telomerase activity.

Results. The expression of IGF 2 and IGF 1R was much higher in malignant tissue of stages III and IV than in tumors of stages I and II and normal or hyperplastic endometrium. This correlated with a decreased apoptosis rate and IGF 2R expression. Eight adenocarcinomas expressed biallelic mutation of the IGF 2R gene. The specific inhibition of IGF 1R and IGF 2 decreased tumor cell proliferation in IGF 2/IGF 1R-positive tumors. Also, the positive correlation between increased expression of IGF 1 and IGF 1R proteins and increased telomerase activity and cell proliferation index was found in both, IGF2 negative and IGF 2 positive tumors.

Conclusion. Our data suggest that IGF 1, IGF 2 and their receptors, are involved in the progression of endometrial adenocarcinomas. As cancer cell proliferation can be abrogated by blocking mRNA or protein products of these genes, tumors with extensive involvement of the IGF 2 pathway would be candidates for the therapeutics strategies aimed at interference with this pathway.

Introduction

Under normal circumstances, proteins of the IGF family (IGF 1 and IGF 2, their receptors and binding proteins) regulate cell proliferation, metabolism and maintain differentiated cell function. In addition, in endometrium, the IGF protein system is thought to function as a mediator of steroid hormone actions through autocrine/paracrine mechanisms. Namely, endometrial stroma cells produce IGF 1 and IGF 2 as well as high affinity IGF-binding proteins (IGF BPs), the most abundant being IGF BP-1, whereas epithelial cells and, to a lesser extend, stromal cells contain cell membrane receptors for IGF's. On the other hand, estrogen stimulates IGF 1/IGF 1R gene expression, associated with endometrial carcinoma development [1].

The IGF 1 and IGF 2 signaling pathway involve two specific receptors. IGF 1R (which is virtually ubiquitous) mediates IGF 1 and IGF 2 action on prenatal growth and IGF 1 action on postnatal growth. On the other hand, as far as IGF ligands are concerned, IGF 2R/M6-P is involved in the clearance of IGF 2 from the circulation. Furthermore, at least six IGF-binding proteins (IGF BP 1-6) also regulate the function of both, IGF 1 and IGF 2. It is obvious from the above mentioned, that the inappropriate expression of any IGF peptide could be involved in abnormal cell function. It is known that not only IGF 2, but also IGF 1R, are commonly overexpressed in many tumors. As their appropriate level and/or interaction regulate cell growth, it is obvious that overexpression of either of them (or both) may be involved in enhanced tumor growth [2]. The proposed mechanism of autocrine IGF 2 action on malignant proliferation came from its elevated secretion by tumors and tumor derived cell lines [3, 4].

On the other hand, the physiological significance of IGF 1R activation by either IGF 1 or IGF 2 was brought into focus by gene targeting experiments in mice [5]. Mice with homozygous targeted mutations of the IGF 1 receptor were severely growth retarded and died immediately after birth, indicating these receptors are important for cell proliferation and

survival. Later it was also shown that signaling through these tyrosine kinase growth factor receptors is one of the key regulators of cell motility and adhesion. However, functional IGF 1R is also required for cell transformation, which appears to involve autocrine stimulation through overexpressed IGF 1R, at least in tumors of epithelial origin [6]. Moreover, it has been shown that a blockade of the IGF 1R function reverses the transformed phenotype of tumors derived from both experimental animals and humans [7, 8].

Contrary to the already mentioned IGF 2/IGF 1R oncogenic action, IGF 2R/M6-P protects the cells from neoplastic impulses, at least those caused by IGF 2. Namely, the binding of IGF 2 to IGF 2R/M6-P results in the degradation of ligand, making it unable to activate IGF 1R. However, IGF 2R/M6-P has been shown to be mutated in a number of human tumors, identifying it as a tumor suppressor.

In developed countries, adenocarcinoma of the endometrium is the most common gynecological cancer. Histopathologically, it is characterized by the proliferation of abnormal glands in an abnormal relationship to one another. Evidence has been accumulated that links IGF family peptides with the development of this tumor type. However, their exact role in endometrial adenocarcinoma development is still unclear.

The aim of this study was to investigate the role of IGF 2 and its receptors (IGF 1R and IGF 2R/M6-P) in endometrial adenocarcinoma. We measured expression of IGF 2 and its receptors mRNA in order to analyze the possible correlation among these genes activity and cell proliferation, as well as the tumor stage. As disruption of the IGF 2/IGF 1R pathway can be accomplished through the binding of growth factors, blockage of receptors or inhibition of secondary targets, we also examined the effect of IGF 1 receptor blockage by αIR3 monoclonal antibodies or antisense oligodeoxynucleotides on cell proliferation. An additional purpose of our investigation was to determine if IGF 2R/M6-P is a target for mutation. Our data suggest that increased expression of IGF 1, IGF 2 and IGF 1R may be an important

contributor to the risk of endometrial cancer. Together with concomitant down regulation of IGF 2R they are involved in the progression of endometrial adenocarcinoma. In addition, oncogenic expression of IGF 1, IGF 2 and IGF 2R strongly correlated with the late stage disease compared to with early stage. Therefore evaluation of these IGF's expression may have clinical implication in endometrial cancer prognosis and treatment. Our results also indicate that IGF 2/IGF 1R antagonists can reduce the growth of human endometrial cancer and could be used as an alternative adjuvant therapy for management of endometrial cancer.

Materials and Methods

Tumor tissues

Ten endometrial hyperplasias and 91 human primary endometrial adenocarcinomas (34 of stages I and II, and 57 of stages III and IV) were obtained from the Croatian Human Tumor Bank. These were obtained from patients treated surgically (females, aged 45 to 78, mean 62 year). Endometrial hyperplasias (obtained from post-menopausal women) were proliferative lesions that showed minimal glandular complexity and crowding and had no cytological atypia. The epithelial lining was one cell layer thick, and the stroma between the glands was abundant. Endometrial carcinomas were pure or endometrial adenocarcinomas, composed entirely of glandular cells. All tumors were tested immunohistochemically – Alcian-PAS, CEA, variety of keratins. The histopathology was confirmed by a pathologist. Immediately after surgical removal, the one part of tissue was snap frozen in liquid nitrogen and stored at -80^0C, and another was brought to the laboratory for subsequent cultivation of tumor cells. Sections of frozen samples (10 hyperplasia, 34 adenocarcinomas of stages I and II and 25 adenocarcinomas of stages III and IV) adjacent to those used for histopathology were used for RNA extraction. Forty six samples were used for mutational analysis. For each tumor sample, normal tissue, not immediately adjacent to cancerous tissue, was used as a

control for LOH/mutation analysis. Part of each tumor sample was also embedded in paraffin for immunohistochemical analysis of IGF protein expression. Sections of each paraffin block were stained with hematoxylin and eosin to confirm the exact tissue analyzed (Table 1).

Seven normal post-menopausal endometrial tissue specimens were used as a control for mRNA analysis.

For the proliferation assay, [^{3}H]-thymidine labeling, apoptosis and telomerase assay, fresh resected samples were collected into sterile vessels containing RPMI 1640 medium with 10% heat-inactivated fetal calf serum and brought to the laboratory for subsequent cultivation of tumor cells.

All persons gave their informed consent prior to inclusion in the study. The local ethics committee approved the study.

Immunohistochemistry

This was performed on formalin-fixed, paraffin-embedded tissue using the avidin-biotin-peroxidase method. Sections, cut at 4 µm, were subjected to a heat-induced epitope retrieval technique in 10 mM citrate buffer (pH 6.0) in an 850 W microwave oven for 10 minutes. Anti-IGF1 (goat; 50 µg/ml; R&D Systems), anti-IGF 2 (mouse, Upstate Biotechnologies, Lake Placid, N.Y) and anti-Ki-67 (mouse, 10 µg/ml; DAKO Cytometrics, Denmark) monoclonal antibodies were diluted 1:50, and incubated for 15 to 18 hours. Anti IGF 1R (Santa Cruz Biotechnologies, Santa Cruz, CA) was diluted 1:100 and incubated for 15-18 hours. Detection was achieved using the DAKO LSAB 2 kit (Carpinteria, CA). Anti IGF 2R/M6P rabbit polyclonal antibody was a generous gift from Zeneco, USA (http://www.geneimprint.com). Detection was achieved using the DAKO LSAB 2 kit, in accordance with the manufacturer's instructions. Negative controls were stained by substitution of the primary antibodies with non-immune mouse or rabbit immunoglobulins. Appropriate positive controls (thyroid gland tissue for IGF 1R, Wilms' tumor for IGF 2 and

normal colon for IGF 2R/M6P) were stained positively. The tumor cells showed strong diffuse cytoplasmic immunopositivity for IGF 2 and IGF 2R/M6P, and cytoplasmic and focal membranous reactivity for IGF 1R. The intensity of staining was arbitrarily judged as: negative (0), weak (1), moderate (2) and strong (3).

RNA extraction and RNase protection assay

Total RNA extraction was performed as described previously [9]. Tissue mRNA for IGF 2 and IGF 1R was analyzed by an RNase protection assay. A sample of total RNA (20 μg) was hybridized in a buffer containing 80% formamide, molar excess of [^{32}P]UTP-labeled antisense RNA probe ($1x10^6$ dpm; 603 and 411 bases long for IGF 2 and IGF 1R, respectively), a control human β-actin probe (247 bp), and tRNA as a negative control [10]. The hybridization was carried out at 45^0C for 16 hours. After hybridization, the RNA samples were digested with RNases A and T1. Protected hybrids were extracted with phenol/chloroform, ethanol precipitated, and electrophoresed on 8% polyacrylamide/8 M urea denaturing gel. The autoradiograms were scanned by densitometer (Pharmacia, LKB, Uppsala, Sweden) [10].

Loss of Heterozygosity Analysis and Mutation Detection in IGF 2R/M6P

We utilized two polymorphisms, dinucleotide (GT) repeat sequence and a tetranucleotide insertion/deletion (ACAA) site, present in the 3'-untranslated region of the human IGF 2R/M6P gene, to determine the frequency of LOH [11]. The reaction conditions for PCR, primer sequences and a detailed method description have been published previously [12].

In tumors with LOH at one IGF 2R/M6P locus, the remaining allele was screened for mutations in the ligand binding regions by direct sequencing of PCR products [13]. The regions screened for mutations were exons 8-11, exons 27-29, exon 31, exons 33 and 34 and

exons 37-39. The exon specific forward and reverse PCR primers that were used have been published [14].

Cultivation of tumor cells

Tumor tissue, without necroses, was cut into small pieces and pressed through a nylon sieve. Cells (approximately 4 x 10^5) were put into T75 flasks covered with an extracellular matrix with an RPMI medium supplemented with 10% fetal bovine serum, 5% human serum, 1% glutamine, and 20 mM HEPES. Tumor cell cultures were maintained as monolayers [15].

Cell proliferation and apoptosis after blocking of IGF 2/IGF 1R by monoclonal antibodies or antisense oligodeoxynucleotides.

Tumor cell proliferation was evaluated by [^{3}H]-thymidine incorporation assay. Cancer cells, seeded in 96-well plates ($2x10^3$ cells/well) were grown in DMEM medium for four days with or without the addition of 1000 ng/ml of αIR3 monoclonal antibodies (AMS Biotechnology, Lugano, Switzerland) or with or without the addition of sense and antisense oligodeoxynucleotides applied to $3x10^4$ cells/well. Eighteen hours before the end of incubation [^{3}H]-thymidine (spec. activity 25 mCi/ml) was added to a final concentration of 1 μCi/ml. The cells were harvested on glass wool filters and the incorporated radioactivity was measured in a scintillation counter. Tumor cell proliferation was expressed in cpm/minute [10]. All oligodeoxynucleotides were synthesized as mixed phosphorothiodates. The IGF 1R: antisense 5'TCCTCCGGAGCCAGACTT3' (complementary to codons 21-29 of the signal sequence of the human IGF 1R) and sense 5'AAGTCTGGCTCGGAGGA 3'; IGF 2: antisense 5' TTCCCCATTGGGATTCCCAT 3' (corresponding to the IGF 2 mRNA initiation site) and sense 5' ATGGGAATCCCAATGGGGAA 3'.

For the apoptosis analysis, $2x10^5$ cells/well were treated in the same way as described for the [^{3}H]-thymidine proliferation assay.

Analysis of Apoptosis

A FACScan apoptosis analysis was performed as described previously [16]. Two million cells per sample were fixed with 2% paraformaldehyde in PBS, washed twice with TBS (50 mM Tris HCl in saline solution), and permeabilized for 1 min with ice cold acetone. Staining was performed by incubating cells for 1 hr at 37^0C in 25 μL of TUNEL reaction mixture – *in situ* Cell Death Detection Kit (Boehringer, Mannheim, Germany). Samples were analyzed by FACScan (Becton Dickinson, Erembodegem-Aalst, Belgium).

Telomerase assay

The telomerase assay was performed with a commercial telomerase PCR ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN) in accordance with Bosserhoff et al [17] and the manufacturer's protocol. Absorbance at 450 nm was measured using an ELISA plate reader (Labsystems, Multiscan MS) [18]. Cells were lysed and protein concentrations were determined by Bradford assay. Two μg of protein were used for each telomerase PCR reaction. Telomerase assay was also performed using positive and negative controls as specified in the kit.

Statistical analysis

Data are shown as mean ± standard deviation. A Student's t-test was performed to compare the results between different groups of samples. The normality of the data was tested by Kolmogorov test. The assumption of equal group variances was tested with Levene's test. In the case of violated assumptions for the analysis, the maximum likelihood analysis suggested the optimal transformation data.

Results

Expression of IGF 2 and IGF 1R mRNA and Apoptosis

The expression of the IGF 2 and IGF 1R gene mRNA was much higher in malignant than in normal or hyperplastic endometrial tissue ($p<0.001$). Moreover, it was significantly higher in stages III and IV versus cancers of stages I and II ($p<0.001$, Fig. 1).

The immunohistochemical analysis of IGF 2, IGF 1R, as well as IGF 2R/M6-P protein expression, was carried out on 34 endometrial adenocarcinomas of stages I and II, 25 adenocarcinomas of stages III and IV, as well as 10 hyperplasias. The expression of IGF 2/IGF 1R proteins was negligible (negative or of weak intensity, 32 out of 34 samples for IGF 2 and 31 out of 34 samples for IGF 1R) in tumors of stages I and II (not shown). However, it was much higher, of moderate or strong positivity, in tumors of more advanced stages (III and IV; $p<0.0001$; Chi-square = 27.28, df = 6 for IGF 2 and $p<0.0001$, Chi-square = 37.11; df = 6 for IGF 1R) (Table 2) compared to both normal tissue (4 samples negative and 3 of weak positivity for IGF 2/IGF 1R proteins), as well as to the tumors of stages I and II. The IGF 2 protein was overexpressed in 60% of tumors, of which 86% overexpressed IGF 1R. There was also a strong correlation between extend of IGF 2 protein expression and number of apoptotic cells. When compared to the data for these two parameters in hyperplasia samples statistical significance was at the level of $p<0.001$. The opposite, although to a lesser extend, was observed for the IGF 2R/M6-P protein expression. Most of the tumors of stages I and II (28 out of 34 samples, 82 %) were of strong or moderate IGF 2R/M6-P positivity (not shown). Among tumors of stages III and IV, 10 were either negative or of weak intensity for IGF 2R/M6-P protein ($p<0.001$, Chi-square = 29.32; df = 6 compared to both tumors of stages I and II and hyperplasia. All these tumors also expressed a decreased number of apoptotic cells ($p<0.001$). The remaining samples expressed the IGF 2R/M6-P protein with moderate or strong intensity. However, it is interesting to note that almost all the samples with negligible

IGF 2R/M6-P expression overexpressed at the same time IGF 2 and/or IGF 1R, and vice versa. These findings point to the fact that, in endometrial adenocarcinomas, IGF 2 and IGF 1R act as oncogenes, while IGF 2R/M6-P acts as a tumor suppressor gene.

The opposite results were observed in hyperplasias. Most of the IGF 2/IGF 1R weak positive hyperplasias were strongly or moderately positive for IGF 2R/M6-P having also a higher number of apoptotic cells.

Mutation of IGF 2R/M6-P Gene

Two polymorphisms in the 3'-untranslated region of the IGF 2R/M6-P gene were used to screen 46 endometrial adenocarcinomas for LOH. Of 46 normal tissue specimens, seventy percent (32 samples) were informative (heterozygous) for at least one polymorphic site. Of the corresponding 32 endometrial adenocarcinomas, 16 had LOH at the IGF 2R/M6-P locus. The remaining allele, in 8 out of 16 LOH patients, contained mutation in the mannose 6-phosphate IGF 2 binding domain (Table 3). The following exons were affected: 27, 28, 33 and 34.

Tumor Cell Proliferation After Blocking of IGF 2/IGF 1R Pathway

Eight different endometrial adenocarcinomas, 5 in stage 2 and 3 in stage 3, were grown in culture and used to determine the correlation between IGF 1R mRNA expression and the extend of cell proliferation (^{3}H-thymidine incorporation). Thymidine uptake correlated well with the higher level of IGF 1R mRNA (Table 4).

The effect of the IGF 1 receptor blocking antibody on cell proliferation is shown in Table 5. In IGF 2 positive cells, cell proliferation was strongly diminished. On the contrary, there was no statistically different effect of αIR3 treatment on IGF 2 negative cell culture.

To ascertain the role of IGF 2 and IGF 1R as a potential mediator in growth promotion, 2 endometrial adenocarcinomas positive for IGF 2 mRNA and one negative, grown *in vitro*, were treated with sense and antisense oligodeoxynucleotides to IGF 1R or IGF

2 mRNA (Table 5). Antisense oligodeoxynucleotides to IGF 2 in concentration of 20 μM significantly reduced the growth of both cell lines for 95% and 85%, respectively. At a concentration of 10 μM, antisense oligodeoxynucleotides to IGF 1R also inhibited the growth of both tested cell lines by 46% and as much as 86%. Contrary to this, sense oligodeoxynucleotides to IGF 2 or IGF 1R had no effect on tumor cell proliferation. There was no effect of either antisense or sense deoxyoligonucleotides on either IGF 2 or IGF 1R in IGF 2 negative cells.

The relationship among IGF 1/IGF 2/IGF 1R protein and telomerase activity and proliferation index

The positive correlation between the increased expression of protein for both IGF 1 and IGF 1R and increased telomerase activity ($p<0.0267$ for IGF1 and $p<0.3707$ for IGF 1R) and the cell proliferation index ($p<0.3707$ for IGF 1 and p=05. for IGF 1R) was found in all eleven IGF 2 positive endometrial adenocarcinomas of stages III and IV. Similar results were also observed in a group of 21 IGF 2 negative tumors of stages III and IV (IGF 1/telomerase $p<0.0062$; IGF 1R/telomerase $p<0.0515$; IGF 1/Ki-67 $p<0.0001$; IGF 1R/Ki-67 $p<0.0013$) (Table 6).

Discussion

IGF 1 and 2 secreted by tumor cells play a major role in tumor progression mostly through the binding and activation of IGF 1R. Such an interplay has been shown in a variety of human tumors. Here we provide the evidence on the role of IGF system in endometrial malignancy and provide novel insights into the complex mechanisms that contribute to its oncogenic potential.

In our study, the majority of endometrial adenocarcinomas overexpressed IGF 1/IGF 2 and/or IGF 1R. At the same time, these tumors, especially those of advanced stages, failed to

express IGF 2R. Together with the fact that almost all such samples had a decreased apoptosis rate, the increased proliferation index, as well as increased telomerase activity, indicate the important role of proteins of the IGF family in the pathogenesis of endometrial adenocarcinoma. Similar finding were also shown for colon [3], lung [8], gastric [10] and gynecological cancers [19], as well as hemangiopericytomas [20]. Furthermore, all these data point to the autocrine action between IGF 1 and /or IGF 2 and their receptors, supported by the fact that abrogation of the IGF 2/IGF 1R function inhibited cancer cell proliferation *in vitro*.

Data concerning the level of IGF 1 in plasma or in endometrial adenocarcinoma cells are different. Although there are some reports indicating its decreased or unchanged presence [21], we and some others have shown the opposite. Contrary to these, are the findings concerning IGF 1R, which have been shown continuously to be overexpressed in endometrial adenocarcinoma [22, 23]. Knowing that the action of IGF 1 is mediated by IGF 1R, it is obvious that some other proteins might interfere with IGF 1 action, contributing to the different findings concerning the IGF 1 level in cancer cells. In this respect, it has been shown that in endometrial adenocarcinoma epithelial cells, IGF 1 binding to IGF 1R is also modulated (usually inhibited) by the presence of IGF-binding proteins (mostly IGF BP3) which are secreted into the circulation by IGF 1 target cells [23, 24, 25]. However, the function of cell-surface-associated insulin-like growth factor binding proteins in IGF 1 stimulation is controversial. Bermont et al. [26] showed that in HEC-1A endometrial adenocarcinoma cells responsive to IGF 1, the IGF BP3 is the main binding protein expressed, and both soluble and cell-associated forms act as inhibitors of IGF 1-induced cellular proliferation. In addition to IGF BPs, insulin, as well as nutritional energy balance and physiological activity levels, appears to be an important determinant of IGF 1 bioactivity [27].

When expressed normally, IGF 2 is an indispensable mitogenic peptide for a number of cell types. However, there are now a number of disease situations in which the level of IGF 2 is elevated, leading to increased cell proliferation and inhibition of apoptosis, as seen in many human and animal tumors. In endometrial adenocarcinoma, the IGF 2 protein/mRNA level is also increased [21, 25] Although the exact meaning of these findings and the role of IGF 2 in the etiology of endometrial cancer is still unclear, the accumulated evidence points to its autocrine stimulation through IGF 1R, which is an absolute requirement for at least the maintenance of the transformed phenotype. In support to this possibility, there are also the results presented in this paper. The concomitant elevated expression of IGF 2 (sometimes associated with elevated IGF 1) and IGF 1R resulted in an increased proliferation rate and telomerase activity, and a decreased apoptosis rate of cancer cells, all characteristics of neoplastic growth. Additionally, when the IGF 2 and/or receptor function was blocked by using monoclonal antibodies or antisense oligonucleotides, the cell growth *in vitro* was efficiently abrogated, showing for the first time that these two kinds of targeted therapies can be successfully applied to the treatment of endometrial adenocarcinoma. As far as we know there are only limited data on targeted therapy of endometrial adenocarcinoma. So it was shown that the growth of endometrial adenocarcinoma cells can also be inhibited by the use of the LH-RH antagonist SB-75 (Ac-D-Nal(2)1,D-Phe(4Cl)2,D-Pal(3)3,D-Cit6,D-Ala10)-GnRH [28]. This interferes with the autocrine activity of IGF 2 and also directly inhibits the growth-stimulatory effects of IGFs, probably through effects on a post-receptor mechanism. Of a special clinical interest might also be the finding that overexpressed IGF 1R signaling might provoke resistance to trastuzumab (Herceptin) an anti HER2/neu receptor monoclonal antibody [29], main drug for treatment breast cancer overexpressing HER2/neu receptors. However, amplification of HER2/neu has also been reported for endometrial cancer, and

trastuzumab is currently under Phase II clinical trial for stage III and IV, and recurrent endometrial adenocarcinoma.

All together, ours, as well as other experiments have unequivocally shown that a decrease in the level of IGF 2 and number of IGF IR causes a reversal of the transformed phenotype [7, 8, 10, 12, 30].

The mechanism by which the IGF 2 gene is overexpressed in endometrial adenocarcinomas presented in this paper was not explored. However, it is known that IGF 2 overexpression can be accomplished by multiple mechanisms such as loss of imprinting (LOI), loss of heterozygosity (LOH) with paternal duplication, and alteration in IGF-binding proteins [31]. Which of the mechanisms underlay IGF 2 overexpression in endometrial adenocarcinoma is still in question, although biallelic expression was shown to occur in some (3 out of 12 tested) endometrial tumor samples [32]. However, as LOI did not correlate with IGF 2 mRNA overexpression, the authors of these results suggested «that biallelic expression of IGF 2 may be subject to down regulation, despite loss of imprinting».

Consistent with the IGF's autocrine regulation of endometrial adenocarcinoma growth are also the findings of mutations in IGF 2R gene reported previously to be present in 15% of examines samples [33]. Expressed at the cell surface, IGF 2R is constitutively endocytosed, where its main role is binding and internalization and subsequent degradation of non-glycosylated IGF 2. As shown in this paper, the mutated IGF 2R gene caused decrease in the level of corresponding protein, which was a more common event in advanced stages of endometrial adenocarcinoma. As these changes were associated with the concomitant overexpression of IGF 2/IGF 1R, it is very likely that the IGF 2R gene acts as a tumor suppressor in endometrial adenocarcinoma, similar to what has been shown for some other human tumors. Namely, biallelic mutations, usually point mutation or small deletion in one allele and loss of heterozygosity in the other, have been reported in a variety of human

malignancies, including hepatocarcinoma and adrenocortical tumors [34, 35], aggressive early breast cancer [36], and lung cancers [12, 13]. Furthermore, the IGF2/M6P locus at 6q has been reported to be a hot spot for mutation in tumors, including malignant melanoma [37], ovarian cancer [38] non-Hodgkin lymphoma [39], and renal cell carcinoma [40]. Down regulation of the IGF 2R/M6P promotes the growth of transformed cells by sustaining IGF 2, which binds to and activates IGF 1R and the insulin receptor to increase intracellular growth signals [41]. However, it has also been shown that IGF 2R/M6P is a receptor for granzyme B during immune system mediated apoptosis [42]. Therefore, tumor cell lines deficient in functional IGF 2R/M6P would also have an inherent resistance to the immune system, which could contribute to the easier tumor development. In support of this belief, there is also the observation that IGF 2R/M6P is secreted by breast cancer lines and primary metastatic breast cancer cells [43]. If it is supposed that granzyme could also bind to this soluble form of IGF 2R/M6P (at least its larger form), then again, cytotoxic T-cell induced apoptosis mediated by granzyme would be inhibited, allowing, when such conditions exist, overexpressed IGF 2 to bind without restriction to IGF 1R, promoting increased cell proliferation. Contrary to such deliberation, stand the results showing that the soluble form of IGF 2R/M6P retains its ability to bind IGF 2 and blocks IGF 2-stimulated DNA synthesis in isolated rat hepatocytes, thus blocking tumor growth mediated by IGF 2 [44].

In support of the autocrine loop between IGF 2 and IGF 2R, are the data obtained with mutant mice lacking IGF 2R/M6P. These usually die perinatally, but are completely rescued from lethality in the absence of IGF 2. IGF2R/M6-P deficient mice have also elevated levels of the circulating IGF binding protein (IGF BP-3) and show a strong IGF BP-6 immunoreactivity in all pancreatic islet cells and in secretory granules of different size in acinar cells and interlobular connective tissue of exocrine pancreas. Fibroblasts derived from

double mutant mice missort the lysosomal protease cathepsin D, and are able to degrade endocytosed IGF BP-3 intracellularly, though with lower efficiency [45].

In addition to IGF's, but in close relation to them, estrogens are also strong growth stimulators of endometrial tumors. Paradoxically, tamoxifen, a known antiestrogen, also stimulates their growth. The mode of action of estrogen can be partially explained by the modulation of the insulin-like growth factor autocrine or paracrine action. On the other hand, tamoxifen does not affect the number or affinity of IGF 1R [46]; rather, similar to estradiol, it increases IGF 1-stimulated tyrosine phosphorylation of cellular substrates. In contrast, in MCF-7 mammary cancer cells, tamoxifen reduced IGF 1-induced tyrosine phosphorylation in the presence of estradiol. Tamoxifen caused a 3-fold decrease in IGF BPs. Moreover, a reduction in soluble IGF BPs was also observed, making the IGF peptides more available to the receptors [46].

In conclusion, our results support the hypothesis that the production of IGF 1, IGF 2, IGF 1R, and IGF 2R, different from that in normal tissue, influence endometrial adenocarcinoma growth by autocrine mechanism. Tumor cells overexpress IGF 1R which efficiently binds, also overexpressed, IGF 2, since IGF 2 “clearance”-IGF 2R are at the same time underexpressed and/or nonfunctional. These lead to increased telomerase activity, and the inversion of the balanced proliferation/apoptosis rate usual for normal cells. The already described loop can be abrogated by targeted therapy.

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Tables

Table 1. Number of specimens and type of analysis

Analysis	Number of tissue specimens			
	Normal	Hyperplasia	Endometrial adenocarcinoma stages I, II	stages III, IV
RNase assay				
IGF 2/IGF 1R	7[a]	10[b]	34[c]	25[d]
Immunohistochem.				
IGF 1	7[a]	10[b]		32[e]
IGF 2	7[a]	10[b]	34[c]	25[d] + 32[e]
IGF 1R	7[a]	10[b]	34[c]	25[d] + 32[e]
IGF 2R	7[a]	10[b]	34[c]	25[d]
Ki-67				32[e]
Apoptosis		10[b]	34[c]	25[d]
Telomerase				32[e]
[^{3}H]-thymidine incorporation			5[c]	3[d]
Mutations			21 (12 inf)[c]	25 (20 inf)[d]
Blocking assay			1[c]	2[d]

a, b, c, d, e – the same specimens
Inf – informative samples

Table 2. IGF 2, IGF 1R, IGF 2R/M6P protein expression and apoptosis in endometrial adenocarcinoma and hyperplasia

Samples	IGF 2	IGF 1R	IGF 2R/M6P	Apoptosis
ADENOCARCINOMA*				
1	3	3	0	13
2	3	3	0	12
3	3	3	0	19
4	3	2	0	19
5	3	3	1	26
6	3	3	1	18
7	3	3	1	10
8	3	3	1	15
9	2	3	1	30
10	3	3	2	49
11	3	2	2	17
12	3	2	3	31
13	2	2	2	25
14	2	1	3	51
15	2	1	2	29
16	1	2	3	66
17	1	1	3	58
18	1	2	1	44
19	1	2	3	37
20	1	2	2	41
21	1	1	2	40
22	1	2	3	32
23	0	2	3	63
24	0	2	2	60
25	0	1	3	70
HYPERPLASIA				
1	0	0	2	44
2	0	0	2	38
3	0	1	2	70
4	1	1	2	47
5	1	2	2	39
6	1	2	3	69
7	1	2	3	59
8	2	1	3	50
9	2	2	3	55
10	2	3	1	46

* stages III and IV

Table 3. Mutations in IGF 2R/M6P gene in endometrial adenocarcinoma

Tumor stage	**LOH (allele 1)**	**Mutation (allele 2)**
1	yes	**G**GT to **C**GT in exon 27
1	yes	**G**GG to **C**GG in exon 33
2	yes	G insert in poly-G repeat region in exon 28
2	yes	**G**CC to **A**CC in exon 34
3	yes	**G**GG to **A**GG in exon 34
3	yes	**G**GG to **C**GG in exon 33
3	yes	**G**GG to **A**GG in exon 34
4	yes	G insert in poly-G repeat region in exon 28

Table 4. IGF 1R mRNA expression and [^{3}H]-thymidine incorporation in endometrial adenocarcinomas

Tumor stage	IGF 1R mRNA*	[^{3}H]-thymidine incorporation**
2	310	7649±1207
	210	3950±879
	130	3144±1126
	50	1260±331
	40	860±311
3***	440	9384±2155
	290	4251±977
	130	1592±213

*The values of IGF 1R mRNA represent the relative density of the bands expressed as an percentage of decrease or increase compared to the expression in normal tissue (100%)

** cpm calculated as mean ± standard deviation of three parallel samples in two independent experiments; fourth passage of the cells used for experiments; even after fifth passage the cells retained their initial phenotype

*** moderately differentiated formed by glandular elements; nuclei were vesicular, pleomorphic. Mitotic figures are abundant and abnormal. Cells are moderatly or poorly differentiated growing in solid sheets. Positive for c-myc, p53 and erb B-2.

Table 5. Effect of IGF 1 receptor blocking antibody and antisense deoxyoligonucleotides to IGF 2 or IGF 1R on cell proliferation of IGF 2 positive and negative adenocarcinoma cells[†]

Tumor stage	Treatment	IGF 2 positive/negative tumors	Cell proliferation[††] cpm	% [†††]	P
2[*]	DMEM	+	4124		
	αIR3		1158	72↓	P<0.001
3[**]	DMEM	+	5624		
	αIR3		429	92↓	P<0.001
3[***]	DMEM	-	2520		
	αIR3		1914	24↓	NS
2[*]	sense IGF 2 (20 μM)	+	3115	25↓	NS
	antisense (20 μM)		224	95↓	P<0.001
3[**]	sense IGF 2 (20 μM)	+	4904	13↓	NS
	antisense (20 μM)		819	85↓	P<0.001
2[*]	sense IGF 1R (10 μM)	+	7200	74↑	P<0.001
	antisense (10 μM)		2247	46↓	P<0.001
3[**]	sense IGF 1R (10 μM)	+	6824	21↑	NS
	antisense (10 μM)		818	86↓	P<0.001
3[***]	sense IGF 2 (20 μM)	-	3405	35↑	NS
	antisense (20 μM)		4041	60↑	NS
3[***]	sense IGF 1R (10 μM)	-	3307	31↑	NS
	antisense (10 μM)		3979	58↑	NS

- [†]the same type of experiments was conducted on all together 8 tumor cell lines, 4 positive and 4 negative for IGF 2. As the results were the same among cell lines of a certain group, and to contribute to the simplicity of the table, only the results for 3 cell lines (2 positive and one negative for IGF 2) were presented. IGF 2 positive (negative) tumors retained their ability to express (or not) IGF 2 in culture.
- [† †]measured by [^{3}H]-thymidine incorporation (cpm); mean of three parallel samples
- [†††] % of inhibition ↓ /stimulation ↑
- *, **, *** - the same tumor cell lines
- NS – not significant

Table 6. Expression of IGF 1 and IGF 1R protein and telomerase activity in IGF 2 positive and negative tumors of stages III and IV.

Patient	IGF 1	IGF 1R	Telomerase*	Ki-67
		IGF 2 positive tumors		
1	3	3	250	3
2	3	2	220	3
3	3	3	200	3
4	3	3	170	2
5	3	3	410	1
6	3	3	280	3
7	3	3	400	3
8	2	1	190	1
9	0	2	170	3
10	0	3	110	1
11	0	2	110	2
		IGF 2 negative tumors		
1	3	3	370	2
2	3	2	240	2
3	3	3	130	2
4	3	3	120	3
5	3	2	380	3
6	3	2	120	3
7	3	2	200	2
8	3	2	150	2
9	3	2	430	3
10	3	3	370	3
11	1	2	120	2
12	1	2	100	1
13	2	0	190	2
14	1	0	120	2
15	1	3	170	2
16	1	0	140	0
17	2	3	200	3
18	2	3	210	2
19	0	1	130	1
20	0	0	100	0
21	0	0	100	1

* the values represent the relative telomerase activity expressed as a percentage of increase when compared to referral values (100%)

Figure legend

Figure 1. Expression of insulin-like growth factor 2 mRNA (A) and insulin-like growth factor receptor type 1 (IGF 1R) mRNA in endometrial hyperplasia (10 specimens) and endometrial adenocarcinoma (stages I and II, 34 specimens; stages III and IV, 25 specimens) compared with normal adjacent tissue (7 specimens). The values represent the relative density of the bands expressed as a percentage of decrease or increase when compared to referral values (100%) in normal tissue. There was a significant difference ($p<0.001$) between normal and cancer tissues and hyperplasia and cancer as well as between endometrial adenocarcinoma of stage I and II versus stages III and IV (⊥ Non-Outlier Max, Non-Outlier Min; ⌈ 75%, 25%; ▫ median).