Abstract. Luteinizing hormone-releasing hormone (LHRH or GnRH) is not only produced by hypothalamus, but also by other normal and cancer tissues. GnRH peptide agonists and antagonists inhibit the proliferation of breast cancer cells, but their effect on the expression of metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) has not been studied despite the fact that growth and invasiveness of breast cancer cells in adjacent and distant sites is associated with the expression of MMPs. In the present study, the effects of [D-Leu6, desGly10]GnRH-NHEt (commercially available) and [D-Tic3, Deg6, desGly10]GnRH-NHEt on gene expression of MMPs and TIMPs in the breast cancer cell line MCF-7 were examined with semi-quantitative RT-PCR. Results showed that incubation of MCF-7 cells with 30 µM of the synthetic GnRH analogues for 48 h in serum-containing medium resulted in a decrease of MMP-9 expression and increase in MT1- and MT2-MMP mRNA levels. Furthermore, both synthetic analogues induced a significant decrease in TIMP-1 and TIMP-3 mRNA levels and increase in TIMP-2 mRNA levels. The impact of the observed changes on the expression of MMPs and TIMPs warrants further investigation on the effects of GnRH analogues on the invasiveness and metastatic potential of breast cancer cells.

Abbreviations: GnRH, gonadotropin-releasing hormone; LHRH, luteinizing hormone-releasing hormone; MMP, matrix metalloproteinase; TIMP, tissue inhibitor of metalloproteinase; Deg, diethylglycine; D-Tic, D-1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid.

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The hypothalamic decapptide gonadotropin-releasing hormone (GnRH type I or GnRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH2) also known as luteinizing hormone-releasing hormone (LHRH) controls the anterior pituitary secretion of luteinizing hormone and follicle stimulating hormone, which in turn stimulate sex steroid hormone synthesis and gametogenesis in the gonads to ensure normal reproductive function (1). Since the isolation and characterization of GnRH in the late 1960s (2, 3), thousands of GnRH analogues have been synthesized and some have already been used in clinical practice.

The ultimate endocrine effect for both agonists and antagonists of GnRH is reversible chemical castration and, thus, synthetic analogues of GnRH, including leuprolide ([D-Leu6, desPro9]GnRH-NHEt), are used in gynecology and oncology for the treatment of hormone-dependent diseases, such as endometriosis, uterine fibroids, benign prostate hyperplasia, fertility disorders, precocious puberty, and prostate and breast cancer (4). Treatment with GnRH agonists of pre- and peri-menopausal women with advanced metastatic breast cancer has been shown to be effective and safe (5-7). In 1989, Fekete et al. showed the presence of GnRH binding sites in 260 out of 500 biopsy samples of human breast cancers (8). Since then it has consistently been shown that GnRH agonists and antagonists inhibit the proliferation of human cancer cells in a dose- and time-dependent manner (9, 10). Therefore, in addition to its classic hypophysiotropic action, an autocrine/paracrine role of GnRH in normal and malignant peripheral extrapituitary tissues, including the mammary gland, has been suggested (4, 9, 11). Normal and malignant human breast tissues, as well as breast cell lines (including MCF-7 cells), secrete both GnRH-I and GnRH-II (a second form of GnRH, GnRH-II: His5, Trp5, and Tyr8-GnRH-I) and have GnRH binding sites (12).

In human cancers, including that of the mammary gland, cancer invasion/metastasis has correlated with the
overproduction of certain matrix metalloproteinases (MMPs) (13, 14). MMPs are synthesized as zymogens and are collectively capable of degrading almost all extracellular matrix and basement membrane components, thereby allowing cancer cells to penetrate and infiltrate the subjacent stromal matrix. The MMPs are encoded by at least 20 genes and categorized into 4 subclasses based on structural organization and substrate specificity: collagenases (MMP-1, -8 and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -7, -10 and -11) and membrane-type MMPs (MT1- to MT6-MMP) (15). Furthermore, MMPs degrade growth-factor receptors, cell adhesion molecules, chemokines, cytokines, apoptotic ligands and angiogenic factors, and, thus, are also implicated in the host immune response and in the early steps of tumor evolution, including stimulation of cell proliferation and modulation of angiogenesis (16). MMP levels depend on gene transcription and/or proenzyme activation. Moreover, the catalytic activity of the MMPs is regulated by endogenous tissue inhibitors of metalloproteinases (TIMPs); four homologous TIMPs (TIMP-1, -2, -3 and -4) have been identified to date (17).

In a recent thorough study of MMP and TIMP gene expression in a panel of four epithelial breast cancer cell lines, of high and low metastatic potential, and in normal mammary cells it has been shown that MT2-MMP and TIMP-1 are highly expressed in all cancer cells as compared to normal ones, whereas MMP-1 and -7 are overexpressed only in breast cancer cells of high invasive potential, irrespectively of cell culture conditions (18).

The direct effect of synthetic GnRH analogues on the expression of these multipotent MMPs and TIMPs by breast cancer cells has not yet been investigated. The purpose of this study was to study the effects of [D-Leu6, desGly10]GnRH-NHEt (I) and [D-Tic3, Deg6, desGly10] GnRH-NHEt (II) on gene expression of MMPs and TIMPs in the breast cancer cell line MCF-7. The MCF-7 cell line was selected due to the presence of estrogen receptors since it is representative of breast cancer epithelial cells of premenopausal women, which are the most probable recipients of endocrine therapy with GnRH agonists. The synthetic analogue I with Gly6 substituted by D-leucine is commercially available (leuprolide or lupron®). The analogue II with Gly6 substituted by diethylglycine (Deg) and Trp3 substituted by D-1,2,3,4-tetrahydro-isooquinoline-3-carboxylic acid (D-Tic) was synthesized in our laboratories and has shown high antiproliferative activity on breast cancer cells (10). Both synthetic analogues have the common deletion of carboxy- terminal Gly– amide in position 10 of GnRH with the addition of an ethylamidic residue to Pro6. This is the first report that GnRH analogues alter MMP and TIMP mRNA expression in MCF-7 cells.

Materials and Methods

Chemicals and cell line. Eagle’s minimal essential medium (EMEM), fetal bovine serum (FBS), L-glutamine, sodium pyruvate, sodium bicarbonate, non-essential amino acids, penicillin, streptomycin, amphotericin B, gentamycin and HEPEs were all obtained from Biochrom KG (Berlin, Germany). Insulin and glucose were obtained from Sigma Chemicals (Steinheim, Germany). All other chemicals used were of the best available grade. The breast cancer cell line MCF-7 (HTB 22; human breast adenocarcinoma, estrogen receptor-positive, low invasive potential) was obtained from the American Type Culture Collection (ATCC, Midlesex, UK).

Synthesis of GnRH analogues. Analogues I and II were assembled on a [3-((ethyl-Fmoc-amino)-methyl)-1-indol-1-yl]-acetyl AM resin containing 0.85 meq. of amino group/g, as described elsewhere (10, 19). In brief, chain extension was carried out using standard Fmoc (9-fluorenylmethoxycarbonyl) protocols. Completion of the coupling reactions was monitored by the Kaiser test except for Pro and Tic residues, in which completion of the reaction was monitored by the Chloranil test (20). Treatment of the peptide resin with trifluoroacetic acid/ methylene chloride/1,2-ethanediol/anisole/ water (90:5:1:2:2, v/v) (15 ml/g peptide resin) for 4 h afforded the desired products. The peptides were precipitated upon evaporation in vacuo and with the addition of ether. The crude peptides were purified by gel filtration chromatography on Sephadex G-15 (Pharmacia, Uppsala, Sweden) using 15% acetic acid as the eluent. Final purification was achieved by semipreparative HPLC (Mod.10 ÄKTA, Amersham Pharmacia Biotech, Uppsala, Sweden) on reversed-phase C-18 with a linear gradient from 20 to 60% acetonitrile (0.1% TFA) for 30 min at a flow rate of 2.0 ml/min and UV detection at 214, 230 and 254 nm. The appropriate fractions were pooled and lyophilized. Analytical HPLC on Nucleosil 100 C-18 column (5 μm particle size; 250x4.6 mm, Macherey Nagel, Düren, Germany) produced single peaks with at least 98% of the total peptide peak integrals. Electrospray Ionisation-Mass Spectrometry (ESI-MS, Micromass-Platform LC instrument, Milford-Massachusetts, USA) was in agreement with the expected results.

Cell culture. The cells were cultured as monolayers at 37 °C in a humidified atmosphere of 5% (v/v) CO2 and 95% air. Cells were seeded in 75-cm2 plastic tissue-culture flasks. Cancer cells were cultured in EEMEM supplemented with 10% FBS, 2 mM L-glutamine, 1.0 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 0.01 mg/mL of insulin and a cocktail of antimicrobial agents (100 IU/mL penicillin, 100 μg/mL streptomycin, 10 μg/mL gentamicin sulphate and 2.5 μg/mL amphotericin B). According to pilot experiments in respect to growth rate and doubling time, the medium was changed every three days (21). The cells were harvested after treatment with 0.25% (w/v) trypsin in PBS, containing 0.1% (w/v) Na2EDTA.

The effect of GnRH analogues on the expression of MMPs and TIMPs in breast cancer cells was studied following cell culture in serum-containing culture media in the presence and absence of the GnRH analogues. Cells were first grown in 75-cm2 plastic tissue-culture flasks in serum-containing media, as described above, until they reached approximately 60-65% confluence. For studying the effect of the GnRH analogues on the expression of MMPs and TIMPs in serum-containing media, the medium was removed and...
the cells were further grown for 48 h in their respective serum-containing media containing 30% of each GnRH analogue, and total RNA was isolated (see below).

Expression of MMPs and TIMPs using RT-PCR. Total cellular RNA was isolated after cell lysis with guanidium isothiocyanate using the SV total RNA isolation system (Promega GmbH, Mannheim, Germany). The amount of isolated RNA was quantified by measuring its absorbance at 260 nm. All total RNA preparations were free of DNA contamination, as assessed by PCR analysis and the absorbance ratio A<sub>260</sub>/A<sub>280</sub>.

The expression of mRNAs encoded for MMPs [MMP-1, -2, -3, -7, -9, -11, MT1-MMP (MMP-14), and MT2-MMP (MMP-15)] and TIMPs (TIMP-1 to -4) was examined by RT-PCR. Reverse transcription of RNA was performed using the Qiagen® OneStep RT-PCR kit (Qiagen GmbH, Hilden, Germany), on a 2400 GeneAmp PCR System (Perkin-Elmer Corp., Norwalk, Connecticut, USA). The sequences of the primers and the respective conditions used are presented in Table I. Primers were designed as earlier described (18) and were supplied from Operon Biotechnologies GmbH (Cologne, Germany). The amplification was performed through 35 PCR cycles for MMPs and 30 PCR cycles for TIMPs. The RT-PCR conditions were: annealing for 30 sec at annealing temperature, primer extension for 1 min at 72°C and denaturation for 30 sec at 94°C; at the end of all cycles, an additional extension cycle was performed at 72°C for 10 min, before the reaction mixture was cooled to 4°C. Glyceraldehyde phosphate dehydrogenase (GAPDH) was amplified as an internal control under the same conditions as with those for MMPs and TIMPs. The amplification products were separated by electrophoresis in a 2% agarose gel, containing Gel Star® stain (BioWhittaker, Rockland, ME, USA). Bands were visualized on a UV lamp and gels were photographed with a CCD camera. PCR vials initially contained 1 μg RNA of all cells studied. For semi-quantitative analysis, the PCR products of MMPs and TIMPs were expressed as the relative density of each band of MMP/TIMP compared with the band of GAPDH. Image analysis was performed using the UNIDocMv version 99.03 for Windows program (UVI Tech, Cambridge, UK).

Statistical analysis. All values are given as mean±standard deviation of three separate experiments in triplicate. Differences between control and GnRH-treated cells were evaluated using the Student’s t-test (GraphPad InStat version 3.0 Software, GraphPad Software, San Diego, USA) and considered statistically significant at the level of p≤0.05.

Results

To examine the effects of the two GnRH analogues on the expression of MMPs and TIMPs, MCF-7 cells were grown in the presence or the absence of those peptides in serum-containing media, where a mixture of growth factors was

<table>
<thead>
<tr>
<th>MMPs-TIMPs</th>
<th>Primers</th>
<th>Product (bp)</th>
<th>Anneal. T (°C)</th>
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<td>MMP-1 (R)</td>
<td>TTCCAGGTATTTTCTGGGATGAAG 828 57</td>
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<tr>
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<td>GCTAGAGCAACATAGAGCT 515 57</td>
<td></td>
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</tr>
<tr>
<td>MMP-3 (R)</td>
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</tr>
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present and resembled more clinically relevant conditions. Previous studies in our laboratories have shown that MCF-7 cells express mRNAs for MMP-2, MMP-9, MT1-MMP, MT2-MMP and MT3-MMP, as well as TIMP-1, TIMP-2 and TIMP-3 (18, 21). Differences in the expression pattern of these MMPs and TIMPs induced by the GnRH analogues were studied by semi-quantitative RT-PCR analysis. Expression was determined as relative fluorescence obtained for each MMP and TIMP compared to the reference gene (GAPDH).

MCF-7 cells grown in serum-containing media (control) contained high mRNA levels of MMP-9, MT2-MMP and MT1-MMP (Figure 1). Incubation of MCF-7 cells with 30 ìM of GnRH analogue I for 48 h resulted in a 5-fold decrease in MMP-9 expression and almost a 2-fold increase in MT1- and MT2-MMPs (Figure 1). Similarly, GnRH analogue II decreased MMP-9 mRNA levels by 50% and increased MT1- and MT2-MMP mRNAs by 50% (Figure 1). The concentration of 30 ìM was selected since according to pilot experiments both peptide analogues at this concentration significantly inhibited MCF-7 cell proliferation; analogue I inhibited cell proliferation by 25% and analogue II by nearly 40% (10). Furthermore, previous reports (10, 22) have shown that peptide concentrations lower than 10–6 M had no statistically significant effect on cell proliferation within 48 h.

High levels of mRNAs encoding TIMP-1, TIMP-2 and TIMP-3 were detected in control MCF-7 cells (Figure 2); TIMP-4 mRNAs were not detected. After incubation of MCF-7 cells with analogue I, TIMP-1 and TIMP-3 mRNA levels were significantly lower (by 30% and 75%, respectively) and TIMP-2 expression was increased 2-fold in comparison to control values (Figure 2). Analogue II induced similar changes in the expression pattern of TIMPs; TIMP-1 and TIMP-3 mRNA levels were decreased by nearly 60% and TIMP-2 expression was increased 2-fold in comparison to control values (Figure 2).

Discussion

The expression of MMP and TIMP genes and activation of MMPs has been associated with breast cancer progression; the overexpression of some MMPs and TIMPs is associated with
a poor patient prognosis (14, 23). In this study, RT-PCR analysis showed that MCF-7 cells express MMP-9, MT1- and MT2-MMP; these results confirm earlier studies (18, 21) and this may be correlated with the low metastatic potential of this line. However, MMP-2 mRNA was not detected, despite the fact that MMP-2 is often found in malignant breast tissue. Our present finding may be explained by the fact that at the time of mRNA isolation, cell cultures must have reached 90% confluency, a factor of crucial importance since it has been shown that MMP-2 which is expressed in sparsely plated cells is progressively lost as the cells approach confluence (24). Furthermore, in serum-containing media a down-regulation of MMP-2 in cancer cell lines as compared to normal cells has been recorded, indicating that this MMP takes part in the physiological activities of epithelial breast cells (18, 25).

Treatment of cells with GnRH analogues for 48 h resulted in lower levels of MMP-9 mRNA. It has been shown that in contrast to MMP-2, MMP-9 expression is highly inducible and under the control of growth factors, chemokines and other stimulatory signals (14). In this context, overexpression of MMP-9 in serum-containing media is not retained when breast cancer cells are grown in serum-free media, suggesting that some type(s) of growth factors present in serum stimulate the expression of certain MMPs in normal and cancer cells. In particular, it has been reported that, among others, epidermal growth factor (EGF) activates the expression of MMP-9 by cancer cells (14). As far as GnRH cancer biology is concerned, it has been shown that binding of GnRH agonists and antagonists to their receptors inhibits the mitogenic signal transduction pathway of the EGF receptor in MCF-7 cells (26), thus, providing a possible explanation for the observed downregulation of MMP-9 expression. MT1- (MMP-14) and MT2-MMPs (MMP-15), which have approximately the same substrates, are controlled in the same pattern and are upregulated by GnRH analogues. In a previous study on the effect of genistein, a soy isoflavone, on MMP production by MCF-7 cells, the levels of MT1- and MT2-MMP were significantly increased, despite the inhibitory effect on MMP-2 and -9, but did not confer any increase in the invasive capacity; in fact the invasive capacity was significantly lower in the presence of genistein (21). MT1-MMP and TIMP-2 take part in the
main activation route of MMP-2 on the cell surface by the formation of a trimerolecular complex containing MMP-2, MT1-MMP and TIMP-2 (27, 28). In this study, TIMP-2 mRNA levels were also upregulated. Thus, the increased MT1-MMP and TIMP-2 mRNA levels may represent the cells’ attempt to activate proMMP-2.

Our results show that the TIMP-1 and TIMP-3 genes are down-regulated in the presence of GnRH analogues. Upregulation of TIMPs by cancer cells in order to counterbalance the higher proteolytic action of MMPs has been suggested (29). This is probably a defensive mechanism of the human organism so as to be able to deal with the irregular increase in MMPs. Thus, the lower levels of TIMP-1 mRNA may be associated with the lower levels of MMP-9 induced by GnRH analogues, since TIMP-1 inhibits MMP-9 with a high affinity. However, high tumor-tissue TIMP-1 mRNA and protein levels are associated with poor prognosis for breast cancer patients despite the inhibitory action on MMPs. The recently discovered tumor growth stimulating function and the anti-apoptotic and pro-angiogenetic effect of TIMP-1 may in part account for this paradox (23). In this context, the lower TIMP-1 mRNA levels observed might not simply be a result of co-up-regulation and may have beneficial role for cancer patients. In general, the differential expression of TIMPs in breast cancer is a very important issue which requires further investigation.

In conclusion, our results show that two different GnRH analogues significantly alter MMP/TIMP gene expression by MCF-7 cells. [D-Leu\(^6\), desGly\(^10\)]GnRH-NH\(_2\) (analogue I) is a commercially available GnRH agonist which binds to the pituitary GnRH receptor with a high affinity. [D-Tic\(^3\), Deg\(^5\), desGly\(^10\)]GnRH-NH\(_2\) (analogue II) is a synthetic analogue with high antiproliferative activity but a low affinity to the pituitary GnRH receptor (10). Despite their differences, both alter gene expression of MMPs and TIMPs in a similar manner. MMPs and TIMPs are multipotent molecules affecting cancer invasion and metastasis potential and angiogenesis. The impact of the observed changes warrants further investigation, since GnRH analogues are part of the endocrine therapy of pre- and peri-menopausal patients with breast cancer.

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References


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