Inhibition of Cyclic AMP-triggered Aromatase Gene Expression in Human Choriocarcinoma Cells by Antisense Oligodeoxynucleotide

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ABSTRACT

Aromatase, an endomembrane-bound cytochrome P450, is the key enzyme of estrogen biosynthesis. Aromatase inhibitors, therefore, are clinically important tools in the treatment of estrogen-dependent tumor growth. To improve the specificity of these tools, inhibition at the nucleic acid level was examined. An antisense oligodeoxynucleotide complementary to the translation start region of human aromatase transcripts (antisense-arom) was synthesized and used to inhibit cyclic AMP-triggered aromatase gene expression in a human choriocarcinoma cell line (JEG-3), both as occurring in an autocrine fashion by secreted human chorionic gonadotropin or as induced by application of the membrane-permeating dibutyryl cyclic AMP. Significant inhibition was obtained in both cases, reaching 70% and 60%, respectively. In addition, the antisense-arom treatment led to accelerated mRNA degradation. The inhibition at the nucleic acid level was accompanied by a decrease of both the aromatase protein and microsomal aromatase activity. The data appear to indicate the antisense strategy to be a most promising approach for the development of a novel type of specific aromatase inhibitor.

INTRODUCTION

Estrogens are growth factors for a number of tumors, particularly those of ovaries, endometrium, and breast (1). Consequently, inhibitors of estrogen biosynthesis are among the therapeutic tools of clinical oncologists. In order to sharpen these tools, further detailed studies, both of the estrogen generating system at the molecular and cellular level and the applicability of novel strategies of inhibition, are of utmost importance.

Estrogens are generated solely by an enzyme termed aromatase. The term expresses the essence of the catalyzed reaction, the conversion of androgens to estrogens due to aromatization of ring A of the steroid ring system. The aromatization is the consequence of oxidative removal of the angular C-19 methyl group. As a member of the cytochrome P450 superfamily (2), a family of endomembrane-bound monoxygenases (EC 1.14.14.1) inserting oxygen atoms into diverse hydrophobic substrates with the simultaneous consumption of reducing equivalents, aromatase introduces oxygen atoms into this methyl group in three successive steps, eventually resulting in its release as formiate (3).

Tissue aromatase activity and tumor growth have been reported to correlate in cases such as breast cancer (4) or prostate cancer (5). More directly, a correlation has been demonstrated in cell cultures. A human mammary carcinoma cell line devoid of aromatase and non-responsive to androgens as proliferation stimuli developed increased proliferation in the presence of androgens when transfected with an aromatase expressing vector. Addition of an aromatase inhibitor prevented the proliferation increase (6, 7).

Aromatase is located at the endoplasmic reticulum of estrogen-producing cells (8) receiving reducing equivalents (mainly) from NADPH-cytochrome P450 reductase, a ubiquitous FMN- and FAD-requiring protein (9). The clinically applied aromatase inhibitors are exclusively designed for binding to the enzyme protein. This approach may fail short of the mark. Other members of the P450 superfamily may also react, resulting in severe side effects. For example, the P450 arom3 inhibitor aminogluthethimide also acts significantly on P450ccc, the enzyme responsible for cleavage of the cholesterol side chain. As a consequence, generation of pregnenolone, the mother compound of all steroid hormones, is inhibited (10). To avoid such effects, the inhibitor design must be based on structural motifs strictly unique for aromatase; therefore, considerations should include the nucleic acid level. At this level, powerful possibilities such as the antisense strategy may be applied. This strategy is aimed at hybrid formation of antisense molecules with single-stranded nucleic acids. Gene transcripts can specifically be trapped and their biological activity altered; hybridization of mRNAs with antisense molecules blocks translation and, as a consequence, leads to a decrease of the proteins they are coding for. Inhibition of one copy of mRNA will theoretically be much more efficient than inhibiting a protein, since one mRNA gives rise to multiple protein copies (11, 12). The unraveling of the aromatase gene and its transcripts over the past several years enables application of this approach.

The human aromatase gene (CYP 19) has a peculiar structure. It spans more than 76 kilobases and contains 9 coding exons, exon II-X (13-15). Exon I is noncoding and unusually variable. Several exons I, exon I.1 through exon I.4, have been found whose transcripts are alternatively spliced to exon II. As a consequence, the resulting aromatase mRNAs contain the same coding region but variable 5' untranslated regions. Each of the exons I appears to have its own promoter (P I.1 through P I.4) responsible for the tissue-specific expression of the different aromatase mRNA species. In addition, a promoter contiguous to exon II exists, promoter P II (16).

A variety of factors can trigger aromatase gene promoter activation and cause aromatase induction, a typical feature of the cytochrome P450 superfamily (17). These include adenylate cyclase stimulators such as the follicle-stimulating hormone, luteinizing hormone, or hCG and, consequently, membrane-permeating analogues of cAMP such as dibcAMP. Glucocorticoids, phorbol esters, undefined serum factors, etc. These may potentiate or inhibit each other's effects. On the other hand, factors belonging to the growth factor family have been shown to inhibit (18).

For examination of aromatase gene expression and its inhibition at the nucleic acid level, a suitable cell culture model is required, ideally a model that constitutively shows measurable expression of aromatase and, in particular, has preserved the characteristic of aromatase induction. On screening a large number of cell types in culture, it turned out that a human choriocarcinoma cell line, JEG-3, meets the requirements. This cell line originates from a choriocarcinoma fragment
(Erwin-Turner tumor; Ref. 19), i.e., of the mesoderm-covered trophoblast, and is hypertriploid with a mean chromosome number of 71 and a moderate degree of differentiation. The cells have kept many properties of the normal trophoblast such as the synthesis of placental steroid hormones including estrogens. JEG-3 cells have been characterized already in some detail, and aromatase has been found to be regulated at the mRNA level. mRNA generation, when induced via the cAMP signaling pathway, is mediated by cyclheximide-sensitive elements, probably labile protein(s) including RNA stabilizing protein(s) (20, 21). This expression regulation at the mRNA level is a particularly favorable condition for affecting induction of aromatase by the antisense approach.

We show here that aromatase mRNA and, as a result, aromatase protein and aromatase activity are significantly decreased by synthetic antisense oligodeoxynucleotide directed against aromatase transcripts when aromatase gene expression is stimulated via the cAMP signaling pathway. The antisense molecules were designed for an interaction with the translation start region, a strategy already successfully applied in different biological systems (11, 12, 22, 23), including our effort to define a role of a protein kinase, casein kinase II, in the mitogenic signaling of cells (24, 25). Furthermore, this strategy should be applicable to any of the aromatase transcripts independent of tissue-specific use of the various aromatase gene promoters, because the translation start is in exon II, and exon II is present in all of the different aromatase transcripts generated in different tissues. In order to arrive at these results, the expression of aromatase in JEG-3 cells via the cAMP signaling pathway had to be characterized. These data suggest an autocrine expression mechanism mediated by hCG and, on the other hand, provided the necessary chronological details of aromatase induction by dbcAMP to allow for examination of antisense oligodeoxynucleotide effects.

MATERIALS AND METHODS

Cell Culture

JEG-3 cells, obtained from American Type Culture Collection (HTB36), were grown in Eagle's minimum essential medium (GIBCO) supplemented with 10% FCS (GIBCO) at 37°C in a 95% air and 5% CO2 atmosphere. In cases of serum-free propagations, cells were cultured in the presence of serum until reaching subconfluency. Then the medium was changed to serum-free Eagle's minimum essential medium and incubation continued. Cells were harvested by scraping off in phosphate-buffered saline and sedimented by centrifugation at 800 X g. When not analyzed immediately, cell pellets were stored at —80°C until use. Cell numbers were determined using a Coulter Counter (Coulter Electronics, Krefeld, Germany). To induce aromatase, cells were exposed to either dbcAMP or 10 mM EDFA (pH 8.0, 50% formamide, and 15% formaldehyde) in the presence or absence of serum (Amersham, Braunschweig, Germany) by capillary transfer in 10 X SSC [15 mM NaCl-0.15 mM sodium citrate (pH 7.0)]. RNA was fixed to filters by UV cross-linking for 1 min followed by baking at 80°C for 2 h prior to hybridizations. As a probe for aromatase mRNA, a 495-base pair P450arom cdNA BamHI/KpnI fragment (position 125 to 619) comprising part of exon II plus exon III, exon IV, and part of exon V, was used. Following prehybridization, hybridization was carried out (20 h at 42°C) using the 32P-labeled probe (labeling was by random priming with [α-32P]dCTP (Amersham)). Filters were washed under stringent conditions at 60°C in 1 X SSC containing 0.1% SDS for 20 min, followed by 0.1 X SSC and 0.1% SDS for 20 min three times. Autoradiography was performed on X-Omat (Kodak) with intensifying screens at —80°C. For control of applied RNA amounts, the filters were probed with a 1.7-kilobase EcoRI cdNA fragment of GAPDH. Autoradiographic signals were quantified with a video-densitometric scanner (Vilber Lourmat/Fröbel, Lindau, Germany).

Preparation of Microsomes

Cell pellets were resuspended in homogenization buffer, pH 7.4 (10 mM potassium phosphate, 150 mM KCl, and 10 mM EDTA). Cells were disrupted by sonification, and microsomes were obtained in two steps by centrifugation of the homogenate at 10,000 X g for 20 min and of the resulting supernatant at 300,000 X g for 20 min. All operations were performed at 4°C. Human placental microsomes were obtained as described previously (27).

Determination of Aromatase Activity

HPLC Assay. After washing, microsomal pellets were carefully resuspended in reaction buffer, pH 7.4, containing 50 mM potassium phosphate, 2.5 mM glucose-6-phosphate, 0.25 units/ml glucose-6-phosphate dehydrogenase, and 10 µM androstenedione or testosterone as substrate. The mixture was preincubated at 37°C for 5 min, and the reaction was started by adding NADPH to a final concentration of 100 µM. After 5 h, the reaction was stopped by adding dichloromethane in a 1:1 volume, and the steroids were extracted. Then the organic phase was removed under vacuum, and the dried samples were redissolved in 20 µl of 40% acetonitrile. After a short centrifugation, the steroids were reextracted.

Preparation of Oligodeoxynucleotides

Unmodified oligodeoxynucleotides were synthesized with an automatic synthesizer (Applied Biosystems). After precipitation with ethanol and sodium acetate, they were vacuum dried and redissolved in water (for oligodeoxynucleotide sequences and target sequence, see Fig. 4).

Isolation of Cytoplasmic RNA

Cytoplasmic RNA was purified according to the method of Gough (26) with the following modifications. Cells were vigorously vortexed in 400 µl 10 mM Tris-HCl (pH 7.5) containing 0.15 mM NaCl, 1.5 mM MgCl2, and 0.65% Nonidet P-40 and centrifuged at 800 x g for 5 min; supernatant was put into 400 µl 10 mM Tris-HCl (pH 7.5) containing 10 mM EDTA, 0.35 mM NaCl, 1% SDS, and 7 M urea. Extraction of RNA was performed in two steps, with 800 µl phenol:chloroform (1:1) followed by 1 volume of chloroform:isoamylalcohol (24:1).

Western Blot Analysis

Microsomes were resuspended in homogenization buffer and separated by the Laemmli gel electrophoresis using 12% polyacrylamide gels in the presence of 2% sodium dodecyl sulfate (SDS-PAGE). Protein (70—100 µg) was
mixed with SDS sample buffer [50 mM Tris-HCl (pH 6.8) containing 10% glycerol, 2% SDS, 0.2% dithiothreitol, and 0.002% bromophenol blue], separated by SDS-PAGE, and transferred to a PVDF membrane (Immobilon P; Millipore) by semidry blotting (Trans-blot SD; Bio-Rad, Munich, Germany). Transferred proteins were stained with 0.3% Ponceau S in 3% trichloroacetic acid and destained in water. After incubation with blocking solution [10 mM Tris-HCl (pH 7.4), 10% dry milk, 1% bovine serum albumin, 0.3% Tween 20, and 150 mM NaCl], the membrane was incubated with a monospecific rabbit polyclonal antibody (1:5000) raised against human placental aromatase (27). Detection of bound antibody was performed by goat anti-rabbit IgG conjugated to peroxidase (1:2000; Dianova) and visualization with o-dianisidine.

**Protein Assay.** Protein concentrations were measured according to the method of Bradford (29) using bovine serum albumin as a standard.

**Determination of hCG Secretion.** Media of cell cultures were collected, and the concentration of hCG was determined with an enzyme immunoassay kit using two monoclonal antibodies against the β subunit of hCG (DDV Diagnostica, Marburg, Germany).

**RESULTS AND DISCUSSION**

**Aromatase Expression in the JEG-3 Cell Model System.** JEG-3 cells started colonization 1 day after seeding, followed by formation of cell islands after 2 days, subconfluency after 3 days, and, finally, confluency after 4 days, indicating a generation time of roughly one day (Fig. 1A). The generation time varied somewhat with the passage number of cells; roughly 17 and 26 h were determined as generation times for cells of passages 5 and 35, respectively (Fig. 1B). Other choriocarcinoma cell lines such as HM, HCCM-5, or NUC-1 have generation times of up to 48 h (30). The relatively short generation time aside, further experimental advantages of JEG-3 cells are their reasonably effective uptake of foreign DNA, of importance for experiments with antisense oligodeoxynucleotides, and their relatively constant cell physiological behavior when compared to that of primary culture systems such as adipose cells or granulosa cells frequently used in aromatase investigations. With the latter, reproducibility of results in our hands was rather poor because of low cell yields and significant donor-dependent variabilities.

The estrogen biosynthesis of JEG-3 cells is mediated by aromatase gene transcripts 3.4 and 2.9 kilobases in length (Fig. 1C, inset). This is as expected since two alternatively used polyadenylation sites occur in the gene (13). The two mRNA species are also found in human placenta and other aromatase-positive tissues and cells in culture (31). The 3.4-kilobase mRNA represents, by far, the more abundant form. The ratio of the two mRNA species appears to be rather stable and
Fig. 2. Secretion of hCG by JEG-3 cells in comparison to expression and activity of aromatase. After reaching subconfluency in the presence of FCS, incubation of cells was continued under serum-free conditions for the indicated times. A, secretion of hCG (0) was determined by EIA based on antibodies specific for $\beta$-hCG. Microsomal aromatase activity (0) was determined by incubation of resuspended post-nuclear 100,000 × g pellets in the presence of testosterone followed by E2-EIA. Expression of aromatase mRNA (iner) and secretion of E2 (0) were determined as described in the legend to Fig. 1C. Bars, SD. B, Western blot. Microsomal aromatase protein was assessed by SDS-PAGE separation of microsomal proteins (70 μg each), transfer to PVDF membrane, and identification of aromatase protein by monospecific polyclonal aromatase antibody with o-dianisidine/peroxide staining reaction coupled to the second antibody. Microsomes of human placenta (5 μg protein) were used as a positive aromatase control (P1, arrowhead). Bottom line, incubation of JEG-3 cells in the absence (–) or presence (+) of 1 mM dbcAMP and times of cell harvesting after dbcAMP addition. Positions of marker proteins are given on the right.

While aromatase mRNA is constitutively expressed in JEG-3 cells, other members of the cytochrome P450 superfamily are not. Using specific probes for members of family 1 (CYP1A1), family 2 (CYP2B6, 2C9, and 2D6), and family 3 (CYP3A4) in Northern blots, no signals were obtained (data not shown). This is of some interest because a number of cis-acting elements are present in the promoters of these P450 genes, which also occur in the P450 arom promoters (2).

The subconfluent state turned out to be that most suited for a change to serum-free conditions, a prerequisite for reliable studies on aromatase expression and its modulation. This is because serum significantly and uncontrollably affects aromatase expression. When determined 24 h after changing to serum-free medium, the level of aromatase mRNA in JEG-3 cells showed significant differences in dependence on the serum preparation used, and there were differentially strong memory effects on aromatase induction; in the presence of different serum preparations, we observed inhibition of aromatase induction by growth factor addition in one experiment but even stimulation in another (data not shown). Thus, the set of experiments shown here has strictly been based on the use of one and the same...
Table 1  Induction of aromatase by dbcAMP in the absence and presence of serum

Shown are the microsomal aromatase activities in JEG-3 cells 48 h after stimulation by 1 mM dbcAMP. Cells stimulated in the absence of serum were kept serum free for 24 h prior stimulation. The estrogen production was determined by HPLC using 10 μM androstenedione as substrate. Given are the mean values ± standard deviations or maximum deviations of four (absence of serum) or two (presence of serum) separate experiments, respectively.

<table>
<thead>
<tr>
<th>Serum present</th>
<th>dbcAMP stimulated</th>
<th>Aromatase activity a</th>
<th>Stimulation factor b</th>
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<td>13 ± 3</td>
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<tr>
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<td>yes</td>
<td>62 ± 22</td>
<td>7-8</td>
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a pmol estrogen formed per 5 h and mg microsomal protein
b Ratio of microsomal aromatase activity of dbcAMP-stimulated cells and non-stimulated cells.

Aromatase Expression Is Strongly Induced by dbcAMP. The intracellular cAMP increase due to peptide hormones such as secreted hCG was mimicked with the membrane-permeating cAMP derivative dbcAMP. When dbcAMP was added to the medium of JEG-3 cells, aromatase was induced significantly. Addition of dbcAMP to the medium of confluent cells kept under serum-free conditions increased the specific microsomal aromatase activity 2-3-fold above constitutive levels (Table 1). Again, serum had a strong impact. In the presence of serum, dbcAMP addition resulted in a 7-8-fold increase of aromatase.

Fig. 3. Kinetics of aromatase induction by dbcAMP in JEG-3 cells. JEG-3 cells, having reached subconfluence in the presence of serum, were kept serum-free for 24 h before 1 mM dbcAMP was added and incubation continued. At the time of dbcAMP addition or at the indicated times after that, aromatase mRNA (A) and microsomal aromatase activity (B) were determined as described in the legends to Fig. 1C and Table 1, respectively; bars, SD. Values obtained in the presence of dbcAMP were compared to values obtained in parallel experiments in the absence of dbcAMP at each time point (controls). Note that the increase in aromatase mRNA level precedes that of aromatase activity (shaded area). Arrows pointing downwards, times of successive application of antisense oligodeoxynucleotides, bent arrow, time of cell harvesting and determination of antisense effect on aromatase expression (see Figs. 5 and 6).
prior to the acceleration of microsomal aromatase activity increase was chosen (Fig. 3, shaded area). Unmodified antisense oligodeoxynucleotides such as those used here usually have short half-lives (34). However, deoxyribonucleotides have a greater stability than oligoribonucleotides (11). Thus, a repeated application was performed, and the cells were analyzed for aromatase 6 h after the last application (Fig. 3, arrows). The concentration used was in the range of 16 μM antisense-arom, a range that had been calculated to be effective in our previous work with antisense oligodeoxynucleotides (24, 25). A concentration of 5–200 μM is the suggested range for antisense oligodeoxynucleotide experimentation in general (22). No apparent toxicity is observed at this range. Only a small portion appears to become available in the cytoplasm, probably because the majority is degraded in lysosomes (35). This is the reason for the application of such a relatively high concentration of oligodeoxynucleotides.

When antisense-arom was applied at 14, 16, and 18 h post application of dbcAMP and the cells were harvested at 24 h, aromatase induction in JEG-3 cells was significantly inhibited. The level of aromatase mRNA measured at 24 h post stimulation was decreased by roughly 60% (Fig. 5). In addition, an accelerated degradation of aromatase mRNA was noted. Antisense-mediated mRNA destabilization was also observed in other cases and explained by endonuclease activity specifically targeting mRNA in DNA/RNA hybrids (12). Neither decreased aromatase mRNA nor RNA degradation was seen in cells treated with the sense-arom or the mutated antisense-arom or in cells without oligodeoxynucleotide treatment. Furthermore, the antisense-arom effect was neutralized upon simultaneous application of sense-arom (data not shown). Interestingly, not only was the dbcAMP-triggered aromatase mRNA increase inhibited by antisense-arom, but inhibition was also noted for the autocrine aromatase application. One would necessarily expect, therefore, that the detected decrease of the arom mRNA level would also have led to a decreased cellular aromatase. This was, in fact, the case. Significant decreases of both the specific microsomal aromatase activity and the microsomal aromatase protein were observed (Fig. 6). Both decreases matched in extent the decrease in mRNA.

The data appear to verify the hypothesis that expression of the human aromatase gene should be sensitive to inhibition by the antisense strategy. The results suggest that the aromatase antisense oligodeoxynucleotide used is taken up by JEG-3 cells, hybridizes with aromatase mRNAs, and, as a consequence, prevents translation into aromatase protein, resulting in decreased cellular aromatase levels. In addition, the hybridization seems to cause accelerated degradation of aromatase protein, resulting in decreased cellular aromatase. This was, in fact, the case. Significant decreases of both the specific microsomal aromatase activity and the microsomal aromatase protein were observed (Fig. 6). Both decreases matched in extent the decrease in mRNA.

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