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Progesterone Regulates Human Telomerase Reverse Transcriptase Gene Expression via Activation of Mitogen-activated Protein Kinase Signaling Pathway

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Abstract

Emerging evidence indicates that sex steroid hormones regulate telomerase in target tissues. We have reported that estrogen activates telomerase through transactivation of the telomerase catalytic subunit, human telomerase reverse transcriptase (hTERT). Progesterone usually antagonizes estrogen action in reproductive organs, but the effect on telomerase remains unclear. In this study, we examine the effects of progesterone on the gene expression of hTERT in breast and endometrial cancer cell lines expressing progesterone receptor. Progesterone significantly induced hTERT mRNA expression within 3 h after exposure. This transient effect peaked at 12 h and then decreased. In contrast, exposure to progesterone for >48 h antagonized estrogen effects and inhibited the estrogen-induced activation of hTERT expression; the cyclin-dependent kinase inhibitor p21/Waf1/Cip1 plays an integral role in this inhibition. Thus, progesterone exerts diverse effects on hTERT mRNA expression in a time-dependent manner. We also found that the mitogen-activated protein kinase signaling pathway mediates both the short-term and long-term effects of progesterone on hTERT gene expression. These findings support the notion that hTERT gene is a target of both estrogen and progesterone.

Introduction

Telomeres are essential elements that protect chromosome ends from degradation and ligation, contributing to chromosomal stability (1, 2). Telomeres undergo progressive shortening with cell division because of the inability of DNA polymerase to completely replicate the ends of chromosome DNA (3). The critical shortening of telomeres with cell division induces replicative senescence. Further dividing of cells beyond senescence results in a serious loss of telomeres, causing chromosomal instability. This includes end-to-end fusions and dicentric or multicentric chromosomes, and leads to cellular crisis (4). Telomerase is a specialized ribonucleoprotein polymerase that directs the de novo synthesis of telomeric repeats at chromosome ends (1). Telomerase is not active in most somatic tissues but is widely activated in cancer cells (5, 6). Telomerase activation is thought to be required for cells to continuously divide beyond replicative senescence and may, therefore, be a critical step in cellular immortality and carcinogenesis (7, 8).

Three major subunits comprising the human telomerase complex have been identified. The RNA component of human telomerase (hTR) provides the template for telomere repeat synthesis (9). Telomerase-associated protein (TP1) is a cloned telomerase component, the function of which remains unclear (10, 11). The most important component responsible for the enzymatic activity of telomerase is hTERT (12, 13). Some studies have found that hTERT is expressed in most malignant tumors but not in normal tissues and that hTERT expression is closely associated with telomerase activity, whereas two other factors are constitutively expressed in both tumors and normal tissues (14–18). In addition, introducing the TERT gene into telomerase-negative cells lead to telomerase expression and telomere elongation and to extended cellular life spans (19–22). These findings suggest that hTERT is a rate-limiting determinant of telomerase enzymatic activity. We have cloned the hTERT promoter, which enables the molecular mechanisms of hTERT regulation to be investigated.

Evidence indicates that telomerase activity is critically regulated by steroid hormones in their target tissues (23–26). We found that the human endometrium expresses telomerase activity despite its somatic origin, and that this activity is tightly regulated in a menstrual phase-dependent manner, which suggests that sex steroids are involved in this regulation (23, 24). We also demonstrated that estrogen activates telomerase via the direct interaction of ligand-activated ER with the hTERT promoter (27). Progesterone usually antagonizes estrogen action and inhibits estrogen-induced cell proliferation in reproductive tissue such as the endometrium. Consequently, progesterone is therapeutically applied to inhibit the growth of estrogen-dependent cancers (28). However, little is known about the role of progesterone in modulating telomerase. The present study examines the effects of progesterone on hTERT gene expression. The study found that progesterone regulates hTERT transcription via the MAP kinase cascade.

Materials and Methods

Cell Cultures. T47-D cells obtained from the American Type Culture Collection were incubated in RPMI 1640 containing 10% FCS and 10 μg/ml insulin under a 5% CO2 atmosphere at 37°C. To assay estrogen- or progesterone-induction, the cells were cultured in phenol red-free medium containing 10% dextran-coated charcoal-treated FCS for 24–48 h prior to hormone exposure.

RNA PCR Analysis. The expression of hTERT mRNA, c-myc mRNA and p21(Waf1/Cip1/Md16) mRNA was analyzed by semiquantitative RT-PCR amplification as described previously (12, 29, 30). Briefly, hTERT, c-myc and p21 mRNAs were amplified using the primer pairs: 5′-CGGAAAGGTGCTCTGGAGCA-3′ (LT4) and 5′-GGATAGACGAGAGTCGTTTTGGA-3′ (LT5) for hTERT; 5′-AAGCTCTGAGGTCCCGAA-3′ and 5′-GCTGGGCTCCCAGCACGAGC-3′ for c-myc; and 5′-CCTTCTGCCGCACTGGAGAC-3′ and 5′-CCTTCTGCCGCACTGGAGAC-3′ for p21. Total RNA was isolated from the cells using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer’s protocol, and cDNA was synthesized from 1 μg of RNA using the RNA PCR Analysis.

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3 The abbreviations used are: hTERT, human telomerase reverse transcriptase; ER, estrogen receptor; MAP, mitogen-activated protein; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MPA, 6a-methyl-17α-hydroxyprogesterone acetate; PR, progesterone receptor; E2, 17β-estradiol; ERK, extracellular signal-regulated kinase; MEK, MAP/ERK kinase; MBP, myelin basic protein.

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PCR kit version 2 (TaKaRa, Ohtsu, Japan) with random primers. Serially diluted cDNA was reverse transcribed from 1 μg of RNA (corresponding to 50 ng to 1 μg) was first amplified by RT-PCR to generate standard curves. Band intensity was determined using NIH Image picture analyzing software. The correlation between band intensity and dose of cDNA templates was linear under the conditions described below. Typically, 2-μl aliquots of the reverse-transcribed cDNA were amplified by 28 cycles of PCR in 50 μl of 1× buffer (10 mM Tris-HCl [pH 8.3], 2.5 mM MgCl₂, and 50 mM KCl) containing 1 mM each of dATP, dCTP, dGTP, and dTTP, 2.5 units of Taq DNA polymerase (TaKaRa), and 0.2 μM each, specific primers. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 45 s. PCR products were resolved by electrophoresis in 7% polyacrylamide gels and stained with SYBR green I (FMC BioProducts, Rockland, ME). The efficiency of cDNA synthesis from each sample was estimated by PCR with GAPDH-specific primers as described previously (21).

**Transient Expression Assays.** T47-D cells cultured in phenol red-free medium containing 10% dextran-coated charcoal-treated FCS for 24 h in 24-well dishes were transfected with 1 μg of hTERT-promoter ligated into the luciferase reporter plasmid pGL3–3328, using Lipofectamine (Life Technologies, Inc., Gaithersburg, MD) according to the protocols recommended by the manufacturer, and were incubated in the absence or presence of 1 nM MPA or progesterone (MPA). After 24 h or 72 h, the cells were harvested and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI), in which Renilla luciferase plasmids were cotransfected as controls to standardize transcription efficiency. All of the experiments were performed at least three times for each plasmid, and the results represent average relative luciferase activity.

**In Vitro Kinase Assay.** T47-D cells were cultured in 150-mm dishes. The medium was replaced with phenol red-free RPMI 1640 containing 10% dextran-coated charcoal-treated FCS for 48 h. Various concentrations of MPA were added to the wells and incubated for 5 or 15 min at 37°C. The cells were then washed with PBS and lysed in ice-cold HNTG buffer [50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 10 mM sodium diphosphate, 100 μM sodium orthovanadate, 100 mM NaF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethanesulfonyl fluoride]. The extracts were separated by centrifugation to recover supernatants. The protein content of the supernatants was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Richmond, CA). The rabbit polyclonal antibody ERK1 was bound to protein A-Sepharose beads, and 300 μg of proteins from the lysate samples were immunoprecipitated at 4°C for 1 h. The protein complexes were washed once in HNTG buffer, twice in 0.5 M LiCl, 0.1 M Tris (pH 8.0), and once in kinase assay buffer [25 mM HEPES (pH 7.2–7.4), 10 mM MgCl₂, 10 mM MnCl₂, 1 mM DTT, and 0.5 mM diethylpyrocarbonate] and were resuspended in 30 μl of kinase assay buffer containing 10 μg of MBP and 40 μM [γ-32P]ATP (1 Ci) as described previously (31). The kinase reaction that proceeded at room temperature for 5 min was stopped by adding Laemmli SDS sample buffer (31). Reaction products were resolved by SDS-PAGE.

**Introduction of p21** (Waf1/Cip1/Mda6) Gene and Antisense p21 Gene into T47-D Cells. The recombinant p21 adenoviral vector Ad5CMV-p21 contains human p21 cDNA driven by the cytomegalovirus promoter as well as the SV40 polyadenylation signal in a minigene cassette inserted into the E1-deletion region of modified Ad5 (32). The control viral vector contains a β-galactosidase cDNA insert instead of p21 cDNA (Ad5CMV-β-gal). Virus stocks were quantified by a plaque-forming assay using 293 cells, separated into portions, and stored at −80°C. To overexpress p21, T47-D cells were infected at a multiplicity of infection of 30 or 100 with either Ad5CMV-p21 or Ad5CMV-β-gal, collected 24 h and 48 h after infection and RT-PCR-assayed for hTERT mRNA. To block the induction of p21 by progesterone, the pCEP-WAF1-AS antisense p21 expression plasmid or pCEP4 empty vector, provided by Dr. Bert Vogelstein (33) was transfected into T47-D cells using the LipofectAmine Plus system (Life Technologies, Inc.) and was cultured in the presence of MPA for 48 h. Thereafter, the cells were collected, and hTERT mRNA was assayed by RT-PCR.

**Results**

To examine the effects of progesterone on hTERT gene expression, PR-positive T47-D human breast cancer cells were cultured in the absence or presence of MPA at various concentrations, and hTERT gene expression was semiquantitated using RT-PCR (Fig. 1). MPA at 1 nM or more induced a significant increase in hTERT mRNA after 3 h. Induction of hTERT mRNA expression peaked at 12 h after exposure (more than 5-fold compared with control), then decreased at 24 h. Thus, progesterone rapidly and transiently induced hTERT mRNA expression. In PR-positive endometrial cancer cells, Ishikawa (data not shown), hTERT mRNA was similarly induced, which suggests that this regulation is not specific to breast cancer cells. We found that E2 up-regulates hTERT mRNA (27). To analyze the effect of progesterone on E2-induced hTERT mRNA, we incubated T47-D cells with E2 alone or E2 plus MPA. Estrogen alone up-regulated hTERT mRNA in T47-D cells as in MCF-7 cells, and this activation continued for >48 h (27). However, MPA exhibited biphasic effects on hTERT mRNA expression in the presence of E2. For up to 24 h, MPA synergized with E2 to activate hTERT expression (Fig. 2), but over a longer period, it antagonized estrogen and inhibited E2-induced hTERT expression. Thus, progesterone appeared to exert biphasic effects on hTERT mRNA expression in a time-dependent manner.

We examined whether or not the effects of progesterone are attrib-
were not affected by MPA (data not shown). Several tumor suppressor
expression. However, RT-PCR assays revealed that c-myc mRNA levels
examined the notion that progesterone down-regulates c-myc expres-
sion, p21(Waf1/Cip1/Mda6) expression is up-regulated by
progesterone (41), which suggests p21 as a candidate modulator for
this regulation. We, therefore, examined whether or not progesterone
induces p21 expression in T47-D cells. The RT-PCR assay results
showed that MPA alone or together with E2 significantly activates
p21 mRNA expression (Fig. 5A). This late effect of progesterone
occurred after 48–72 h. In contrast, E2 alone did not induce p21
expression. To examine the effects of p21 on hTERT mRNA expres-
sion, p21 expression vectors were introduced into T47-D cells by
adenovirus-mediated gene transfer. Fig. 5B shows that p21 overex-
pression causes the significant inhibition of hTERT mRNA expres-
ion. To further confirm the role of p21 in progesterone-induced
hTERT repression, T47-D cells were transfected with the antisense
p21 expression vector pCEP-WAF1-AS and were cultured in the
absence or presence of MPA (Fig. 5C). Preliminary studies confirmed
that pCEP-WAF1-AS effectively blocked p21 induction by MPA
(data not shown). In addition, RT-PCR assays revealed that the
inhibitory effects of progesterone on hTERT expression were largely
eliminated in cells transfected with pCEP-WAF1-AS but not in those
transfected with the empty vector, pCEP4. These findings suggest that
p21 plays an integral role in the long-term effect of progesterone. The
induction of p21 by progesterone was completely blocked by expo-
sure to PD98059 for >48 h (Fig. 5A), which suggests that the MAP
kinase pathway also mediates this induction as a late effect. This
blockade consequently abrogated progesterone-induced hTERT-
repression (Fig. 5A). Thus, the MAP kinase cascade involves short-
and long-term effects of progesterone on the regulation of the hTERT
promoter.

**Discussion**

To our knowledge, this is the first attempt to examine the effect of
progesterone on hTERT gene expression. The present results showed
that progesterone rapidly induces hTERT transcription in breast and
donometrial cancer cells. We previously reported that estrogen activ-
vates hTERT transcription via the direct interaction of ligand-acti-
ated ER with the estrogen-responsive element that is located in the
hTERT promoter (27). Whereas the effects of estrogen on hTERT
activation lasted for >48 h, progesterone action was transient and
although to transcriptional control of hTERT gene. Transient expression
assays were performed using luciferase reporter constructs containing
cloned 3.3-kb hTERT promoter sequences (pGL3–3328; Ref. 34; Fig.
3). Exposing T47D cells to either E2 or MPA for 24 h stimulated the
transcriptional activity of pGL3–3328. The effects of MPA were
transient and diminished after 72 h, whereas E2 continued to activate
hTERT mRNA expression. However, long-term exposure to MPA
together with E2 (72 h) completely inhibited the E2-induced tran-
scriptional activation of pGL3–3328, which was consistent with the
results of RT-PCR assays. Thus, hTERT expression appears to be
regulated by progesterone at the transcriptional level. Progesterone-induced hTERT activation is extremely rapid; it is
initiated within 3 h, which suggests that it does not depend on de novo
protein synthesis. This was confirmed by the finding that cyclohexi-
mide did not affect progesterone action in T47-D cells (Fig. 4A).
Progesterone stimulates oocyte maturation through activation of the
MAP kinase pathway (35). Moreover, progesterone activates the
Src/Ras/ERK pathway in T47-D cells (36). We, therefore, examined
whether or not the MAP kinase signal transduction pathway mediates
progesterone-induced hTERT expression. T47-D cells were exposed
to MPA in the absence or presence of PD 98059, a highly selective
inhibitor of MEK (37). PD98059 completely blocked MPA-induced
hTERT mRNA expression (Fig. 4B). We also used an in vitro kinase
assay to examine whether or not ERK, a key factor in the MAP kinase
signaling pathway, is activated in response to progesterone. T47-D
cells were incubated with MPA at various concentrations, then cell
lysates were extracted and immunoprecipitated with anti-ERK anti-
body. The activity of ERK kinase was evaluated by measuring its
ability to phosphorylate its substrate protein, MBP. ERK kinase was
significantly activated within 15 min after exposure to progesterone in
a dose-dependent manner (Fig. 4C). These findings suggest that
progesterone activates the MAP kinase cascade to rapidly induce
hTERT mRNA expression.

We next investigated the mechanisms through which progesterone
inhibits the effects of E2-induced hTERT transcription. We showed
that estrogen up-regulates c-myc expression, which contributes to
indirect mechanisms of estrogen activation of hTERT (27). We, thus,
examined the notion that progesterone down-regulates c-myc expres-
sion. However, RT-PCR assays revealed that c-myc mRNA levels
were not affected by MPA (data not shown). Several tumor suppressor
gene products function as negative regulators of telomerase (38–40).
Among them, p21(Waf1/Cip1/Mda6) expression is up-regulated by
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ated ER with the estrogen-responsive element that is located in the
hTERT promoter (27). Whereas the effects of estrogen on hTERT
activation lasted for >48 h, progesterone action was transient and

decreased within 24 h. The classical pathway of progesterone action is mediated via the interaction of ligand-activated PR with progesterone-responsive element in the promoters of target genes (42, 43). However, a computer-assisted homology search revealed that the hTERT promoter lacks a canonical progesterone-responsive element. Our results indicate that the MAP kinase cascade mediates the progesterone-induced transcriptional regulation of hTERT.

A variety of growth factors or hormones are transduced into adaptor molecules attached to the intracellular domains of membrane receptors, in which they activate a series of serine-threonine kinases (Ras, Raf, and MEK) as well as ERK kinases by sequential phosphorylation reactions. Activated ERKs translocate into the nucleus, in which they phosphorylate specific transcription factors to activate promoters of various growth-regulating genes (44–46). The present study found that a MEK inhibitor completely blocked progesterone-induced hTERT activation. In vitro kinase assays also revealed that ERK is activated in response to progesterone. These findings suggest that the MAP kinase pathway is involved in progesterone-induced hTERT activation.

FIG. 4. Activation of MAP kinase cascade by progesterone. A, T47-D cells were incubated with 1 nM MPA in the absence or presence of cycloheximide at 10 mg/ml. Twelve h later, RNA was extracted, and RT-PCR assays were performed to detect hTERT mRNA. B, T47-D cells were incubated with 1 nM MPA in the absence or presence of MEK inhibitor PD98059 at 50 μM. Twelve h later, RNA was extracted, and RT-PCR assays were performed to detect hTERT mRNA. C, ERK activation by progesterone. T47-D cells were incubated with MPA at various concentrations. Five or 15 min later, cell lysates were recovered and immunoprecipitated with anti-ERK1 antibody. Immunoprecipitates were incubated with MBP in the presence of [γ-32P]ATP. Reaction products were resolved by SDS-PAGE and visualized by autoradiography.

FIG. 5. Involvement of p21 in progesterone-induced repression of hTERT. A, T47-D cells were incubated with MPA and/or E2 at 1 nM in the absence or presence of MEK inhibitor PD98059 at 50 μM. Seventy-two h later, RNA was extracted, and RT-PCR assays were performed to detect p21 and hTERT mRNA. B, T47-D cells were infected with adenoviral expression vectors Ad5CMV-p21 for p21 overexpression or Ad5CMV-βgal as a control. Forty-eight h later, RNA was extracted, and RT-PCR assays were performed to detect hTERT mRNA. C, T47-D cells were transfected with p21 antisense expression vector pCEP-WAF-AS or control empty vector pCEP4. Twenty-four h later, cells were incubated with or without E2 in the absence or presence of MPA at 1 nM for 72 h. RNA was then extracted, and RT-PCR assays were performed to detect hTERT mRNA.
T47D cells to the MEK inhibitor blocked the induction of p21 expression. This blockade abrogated hTERT-repression by progesterone as shown in Fig. 5A. Thus, the MAP kinase cascade may play a key role in both short- and long-term regulation of the hTERT promoter. At present, it remains unclear how p21 inhibits the hTERT promoter.

Although the present study did not address the effects of progesterone on cell cycle regulation, studies have demonstrated that progesterone induces cell cycle arrest in T-47D cells through up-regulation of p21 (41). It is well known that telomerase activity is critically regulated depending on the status of cell proliferation, and that cell cycle arrest leads to down-regulation of telomerase activity (50, 51). Therefore, hTERT inhibition may in part be attributable to secondary effects of cell cycle arrest, other than the primary effects of p21 on hTERT promoter.

Our findings may have important implications concerning hormone-dependent telomerase regulation. In particular, the present results can explain the molecular mechanisms of menstrual cycle-dependent telomerase regulation in the human endometrium. Telomerase activity increases as of the proliferative phase progresses, then peaks at the late-proliferative phase. In contrast, the activity significantly decreases as the secretory phase progresses and activity is either absent or minimal at the late-secretory phase. Local and circulating levels of estrogen that increase during the proliferative phase probably cause telomerase activation. In contrast, high levels of progesterone secreted during the secretory phase may contribute to the suppression of telomerase activity. The expression of p21 is induced during the secretory phase (52), a fact with which the in vitro findings of this study agree. Furthermore, telomerase activity remain high even at the beginning of the secretory phase (23, 24), which may also be explained by the short-term effects of progesterone.

In summary, we demonstrated that the hTERT gene is a target of progesterone. The actions of progesterone were biphasic depending on the duration of exposure, which was mediated by MAP kinase signaling pathways. These findings may provide insight into the molecular mechanisms of telomerase regulation by sex steroids in their target tissues as well as the therapeutic applications of progesterone against estrogen-dependent tumors.

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