Abstract. Background: Although progestins have been used for the treatment of endometrial neoplasias, the mechanisms of progestin-induced growth suppression remain undetermined. Materials and Methods: Immunostaining for steroid receptor coactivators (SRC-1, p300/CBP), corepressors (NCoR, SMRT) and Ki-67 in 15 neoplastic endometria before and after the treatment with medroxyprogesterone acetate (MPA) was performed. The effect of progestin on cell proliferation and cofactors expression were examined using T47D cells. Results: Of the 15 cases, 10 showed good histological responses to MPA (Responder) and 5 poor responses (Non-responder). In Responders, MPA treatment resulted in reduced expression of Ki-67 by 78% \((p=0.0076)\) along with increased NCoR expression by 158 \%(p=0.0077). Progestin treatment for T47D cells resulted in up-regulation of NCoR mRNA and protein with the suppression of cell proliferation. Immunoprecipitation revealed that NCoR was bound to estrogen receptor \(\alpha\), but not to progesterone receptor in T47D cells. Conclusion: The up-regulation of NCoR by progestins is associated with the suppression of estrogen-induced growth of neoplastic cells.

A subset of endometrial hyperplasia and carcinoma expresses estrogen receptors (ER) and progesterone receptors (PR), and their proliferation is known to be stimulated by estrogen and suppressed by progesterone (1). Progesterone-derivatives such as medroxyprogesterone acetate (MPA) also suppress the growth of endometrial hyperplasia and carcinoma expressing PR, and have been used for the treatment of patients with these diseases (2). The mechanisms of progestin-induced growth suppression of endometrial glandular cells have been investigated in several aspects such as down-regulation of ERs (3, 4), altered levels of steroid metabolizing enzymes (5), growth factors or cytokines (6), and up-regulation of p27Kip1 (7). These steroid hormone-related growth mechanisms have been generally considered to be mediated via their receptors, however, the molecular pathways downstream of the receptors that promote the transcription of target genes have not been fully elucidated.

Recent research has identified a group of molecules termed as steroid receptor cofactors (8). These factors bind to steroid receptors in a ligand-dependent fashion, and the receptor-bound cofactors then bind to the basal transcriptional machineries of the target genes, resulting in transcription. Thus, the steroid receptor cofactors are important molecules intervening between the receptors and target genes. These cofactors are now functionally divided into two subclasses, i.e., coactivators and corepressors. The former stimulates, and the latter suppresses the transcription of target genes. The cyclic changes in the expression of the two coactivators, steroid receptor coactivator-1 (SRC-1) (9) and cyclic AMP-response element-binding protein, a substantial homologue of p300 (p300/CBP) (10) in normal human endometrium (11) have been previously reported. The reduced expression of SRC-1 and p300/CBP in endometrial carcinoma, as well as increased expression of a corepressor, nuclear receptor corepressor (NCoR) (12), in endometrial hyperplasia (13) have also been reported. However, the involvement of these cofactors in endometrial hyperplasia treated with progestins has not been elucidated. In the present study, the expression of two representative coactivators (SRC-1 and p300/CBP), and two corepressors (NCoR and silencing mediator for retinoid and thyroid-hormone receptors, SMRT (14) were immunohistochemically examined in neoplastic endometrial tissues treated with progestin. Furthermore, the functional
involvement of cofactors in progestin-induced growth suppression was investigated in vitro using PR-positive carcinoma cell lines.

Materials and Methods

Immunostaining

Histological materials. The age of the 15 patients ranged from 20 to 49 (median 34), who visited the hospital during 1996 to 2003. The initial histological diagnosis consisted of 2 cases of complex endometrial hyperplasia without atypia, 8 cases of atypical endometrial hyperplasia and 5 cases of well-differentiated (grade 1) endometrial carcinoma. The patients orally received MPA 400-600 mg/day for 4-6 months. One month after the oral MPA treatment, all the patients underwent final endometrial curettage for the evaluation of treatment effectiveness. The effect of MPA was histologically evaluated with regard to the disappearance of nuclear atypia, or marked epithelial atrophy with nuclear condensation (15). The cases that showed these findings after the treatment were termed as “Responders” and that without these findings as “Non-responders”. Each tissue was used with the approval of the Ethics Committee at Shinshu University School of Medicine after written consent from the patient was obtained.

Staining procedures. Immunohistochemical staining was performed on paraffin-embedded sections by the streptavidin-biotin-peroxidase complex method using a Histofine SAB-PO kit (Nichirei, Tokyo, Japan). Antibodies for SRC-1 and NCoR were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY, USA). An antibody for p300/CBP, which recognizes both p300 and CBP, was obtained from NeoMarkers (Fremont, CA, USA) and an anti-SMRT antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Ki-67, anti-ER that specifically recognizes ERα, and anti-PR antibody that recognizes both PRA and PRB were obtained from Immunotech (Marseille, France). The staining procedures are as described previously (11). Briefly, after routine deparaffinization and rehydration, sections were treated with microwaves in a 0.01-M citrate buffer (pH 6.0) for 15 min. After blocking of endogenous peroxidase activity, the sections were then incubated with specific primary antibodies (diluted 1:100 with PBS/BSA) or control nonimmunized mouse or rabbit serum at 4˚C overnight. After washing with PBS, biotinylated antitoxine or rabbit IgG was applied for 30 min at room temperature. For negative control of the secondary antibody, biotinylated antirabbit or mouse IgG was applied for mouse or rabbit primary antibodies, respectively. After washing with PBS, peroxidase-conjugated streptavidin solution was applied for 30 min and visualized by 0.05% 3'-3' diaminobenzidine with PBS, peroxidase-conjugated streptavidin solution was applied for 30 min and visualized by 0.05% 3'-3' diaminobenzidine. Counterstaining was performed lightly with hematoxylin.

Interpretation of immunohistochemical staining. The specific staining of each antibody was identified by brown-colored products mainly in the nucleus and partially in the cytoplasm. All the control slides yielded negative staining. In the present study, only nuclear staining was evaluated, because cofactors are generally considered to function in the nucleus (8). The positivity of each staining was also described as a positivity index (PI), which indicates the percentage of positive cells in the nucleus in 200 cells from 3 high-powered fields in each section. Statistical analysis for the PI comparison between before and after MPA treatment was performed with Wilcoxon’s signed-ranks test, and that between Responder and Non-responder was done with the Mann-Whitney U-test. A tied p-value less than 0.05 was considered significant.

Cell culture and reagents. An ER- and PR-positive breast carcinoma cell line, T47D, was purchased from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium (Invitrogen, CA, USA) supplemented with 10% FCS (Biomeda, CA, USA) and 1% antibiotic-antimycotic solution (Invitrogen). An ER- and PR-positive endometrial carcinoma cell line, Ishikawa, was kindly provided by Dr. Nishida at Kasumigaura Medical Center (Tsuchiura, Japan). Cells were cultured in DMEM (Invitrogen) with 15% FCS and 1% antibiotic-antimycotic solution. Incubation was carried out at 37˚C under 5% CO2 in air. Estradiol (E2), MPA and progesterone (P4) were purchased from Sigma (St. Louis, MO, USA).

Cell proliferation assay. T47D or Ishikawa cells were seeded in a 96 well multiplate (10,000 cells/well) with 10% FCS. Twenty four hours after seeding, E2, MPA, or P4 dissolved in ethanol was added every 48 hours at a final concentration of 10–8 M or 10–6 M to 1% FCS-supplemented phenol red-free medium. Viable cells were measured after 120 hours of treatment using the WST-1 assay. After the cell culture, WST-1 agent (Roche, Indianapolis, IN, USA) was added according to the manufacturer’s instructions, and then measured at a wavelength of 450 nm with a Multiscan JX microplate reader (Thermo Labsystems, Vantee, Finland).

Western blotting. After T47D and Ishikawa cells were cultured with MPA or P4 at a concentration of 10–8 M or 10–6 M for 120 hours, cells were harvested and subjected to western blotting. Antibodies for NCoR and SMRT used in western blotting were the same as used in immunostaining as mentioned in the previous section. Experimental procedures were performed as previously described (7). Briefly, extracts equivalent to 50 μg of total protein were separated by SDS-polyacrylamide gels (10% acrylamide). The proteins were then transferred to supported nitrocellulose membranes (Amersham Biosciences Corp., Piscataway, NJ, USA) with a plate electrode apparatus (Semi Dry Blotter II, Ken En Tec, Copenhagen, Denmark) for 90 min. Filters were incubated with antibodies against NCoR and SMRT. An antibody for β-actin (Biomakor, Rehovot, Israel) was used as the internal standard. Then, the filters were incubated in peroxidase-conjugated antimouse or rabbit IgG and the bound antibody was detected with an enhanced chemiluminescence system (Amersham Biosciences Corp.).

RT-PCR. To examine the expression of NCoR mRNA in T47D cells treated with progesterone, a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) assay was performed. After T47D cells were cultured with MPA at a concentration of 10–8 M or 10–6 M for 120 hours with or without 10–6 M RU486, total RNA was extracted using TRIzol reagent (Invitrogen, CA, USA), and RT-PCR was performed using a TaKaRa RNA PCR kit (TAKARA BIO. INC., Otsu, Japan), as previously described (16). The primers for NCoR were designed to encompass a specific segment of the cDNA sequence (sense, 5’-TAAGTCTGCTCCAGCTCCT-3’ and antisense, 5’-GGAGTCTGGCAGGAAAACTTG-3’, 292 bp). Those for GAPDH as a loading control (sense, 5’-ACGACACATGGTCAAGCTC-3’ and antisense 5’-GGTGCTACAGGGCAACTG-3’, 224 bp) were prepared according to a previous study (21). The corresponding cDNA fragments were denatured at 94˚C for 30 seconds, annealed at 55˚C for 1 minute, and extended at 72˚C for 1 minute with 30 amplification cycles.
Immunoprecipitation. After T47D cells were cultured in DMEM containing 10% charcoal-filtered FBS with 10\(^{-6}\) M of MPA or P4 for 24 hours, 200 µg of pre-cleared cell lysate was incubated with anti-NCoR (Upstate Biotechnology), anti-ERα (Santa Cruz) rabbit polyclonal antibody or anti-PR rabbit monoclonal antibody (Eiptomics, Burlingame, CA, USA) overnight at 4°C. The following procedures were performed as previously described (11).

Results

Responders and non-responders to progestin in endometrial neoplasia. Of the 15 cases, 10 showed good histological responses (Responder) and 5 showed poor responses (Non-responder) (Figure 1). The initial diagnosis of the 10 Responders consisted of 2 cases of endometrial hyperplasia without atypia, 6 cases of atypical endometrial hyperplasia, and 2 cases of endometrial carcinoma, and that of the 5 Non-responders consisted of 2 cases of atypical endometrial hyperplasia and 3 cases of endometrial carcinoma. The histological diagnosis of the 5 Non-responders after the treatment was same as the initial diagnosis, and none of the 5 Non-responders showed disease progression. The mean age of Responders and Non-responders was 35.2 and 33.2 years of age, respectively.

Progestin-induced up-regulation of immunohistochemical expression of NCoR in endometrial neoplasia along with its growth suppression. The results of immunostaining for each cofactor, ER, PR and Ki-67 in endometrial hyperplasia and carcinoma before or after MPA treatment are shown in Figure 2 and Table I. Before MPA treatment, there was no significant difference in the expression of any of the factors examined between 10 Responders and 5 Non-responders. In the 10 Responders, PI of Ki-67 before the treatment (8.3±5.3, mean±standard deviation) was markedly decreased after MPA treatment (1.8±2.1) with a significant difference (p=0.0076, Figure 2a, b, c). There were no remarkable changes in the expression of ER (Figure 2d, e, f), PR, SRC-1 (Figure 2g, h, i), p300/CBP (Figure 2j, k, l) or SMRT (Figure 2p, q, r) between before and after MPA treatment. Interestingly, the PI of NCoR before the treatment was 21.1±23.4, and was markedly increased (54.4±17.3) after MPA treatment with a significant difference (p=0.0077, Figure 2m, n, o). In addition, the NCoR-positive tumor cells (Figure 2m, n) were also positive for ER (Figure 2d, e). In the 5 Non-responders, the expression of those factors including NCoR and Ki-67 did not show significant changes after MPA treatment (Table I).

Steroid hormone-induced growth regulation in Ishikawa and T47D cells. The addition of E2 at a concentration of 10\(^{-8}\) M for 120 hours significantly increased the cell proliferation of Ishikawa cells by 16% and of T47D cells by 38% (Figure 3a, b). Treatment with 10\(^{-6}\) M MPA for 120 hours significantly decreased the cell proliferation of Ishikawa cells by 18%, but addition of P4 did not show significant growth suppression. In T47D cells, treatment with 10\(^{-8}\) and 10\(^{-6}\) M MPA or 10\(^{-6}\) M P4 significantly suppressed the cell proliferation by 5%, 7% and 10%, respectively.

Progestin-induced expression of NCoR protein and mRNA in T47D cells. Western blot analysis revealed that MPA and P4 markedly induced NCoR protein expression in a dose-dependent manner (Figure 4a) in T47D cells. However, progestin-induced up-regulation of SMRT was negligible in T47D cells. In contrast, neither P4 nor MPA induced the expression of NCoR protein in Ishikawa cells (Figure 4b). The results of PCR indicated that MPA increased NCoR mRNA level in a dose-dependent manner and a progesterone receptor antagonist, RU486, suppressed MPA-induced NCoR expression (Figure 4c).

Binding of progestin-induced NCoR protein to estrogen receptors in T47D cells. When the cell lysates were immunoprecipitated with the anti-NCoR antibody and the precipitates were incubated with anti-ERα or anti-PR antibody, a positive band was observed only with the anti-ERα antibody but not with the anti-PR antibody (Figure 4d). In addition, a positive band was detected when the immunoprecipitates by anti-ERα antibody were examined using an anti-NCoR antibody.

Discussion

The present study revealed a marked up-regulation of NCoR in tissues showing good histological responses to MPA treatment. In addition, the progestin-induced up-regulation of NCoR was associated with progestin-induced growth suppression of T47D cells. These results strongly suggest that the increased expression of NCoR is involved in progestin-induced growth suppression of ER/PR-positive tumor cells. To our knowledge, this is the first report to elucidate the involvement of NCoR in progestin-induced growth suppression of neoplastic endometrial epithelial cells. In the present study, progestin transcriptionally induced NCoR expression in T47D cells. The mechanism of NCoR transcription is largely unknown, partly because the promoter structure and related functions of NCoR are not fully understood. The present study also demonstrated that the RU-486 treatment suppresses the expression of NCoR mRNA, suggesting that the progestin-induced up-regulation of NCoR was mediated with PR. Contrary to expectations, the progestin-induced up-regulation of NCoR was not observed in Ishikawa cells. Although the reason for the difference between the two PR-positive cell lines is not understood, progestin-induced NCoR expression may occur...
only in highly differentiated neoplastic cells including endometrial hyperplasia, which in general show strong ER/PR expression, since T47D cells originated from well differentiated breast cancer cells (17), whereas Ishikawa cells are derived from moderately differentiated cells (18).

Corepressors like NCoR and SMRT were shown to correlate with nuclear receptors such as thyroid hormone receptors and retinoid receptors (12, 14). These studies proposed a working model of corepressors, i.e., corepressors can bind to these receptors in absence of respective ligands resulting in blocking of the transcription of target genes, whereas the corepressors dissociate from the receptors, leading the transcription activation when the respective ligands bind to the receptors. Thus, NCoR or SMRT had been thought to suppress the transcription of target genes when the ligands are not bound to these receptors. In accordance with this model, the present study indicated the binding of ERα to NCoR in T47D cells in the absence of E2. This result resembled a report demonstrating a weak binding between ER and NCoR in E2-stripped MCP-7 cells (19).

The functional involvement of increased expression NCoR in hyperplastic and cancerous tissues after MPA treatment remains unclear. However, recent studies have identified more direct roles of NCoR as a suppressor of gene transcription regardless of ligand binding status. For example, the ER-NCoR fusion proteins suppress the ER-

| Table 1. Result of immunostaining for ER/PR, cofactors, and Ki-67 in patients with endometrial hyperplasia and carcinoma before and after MPA treatment. |
|-----------------------------------|-----------|-----------|-----------|-----------|
|                                   | Responder | Non-responder |
|                                   | Before*   | After      | Before     | After      |
| ER                                 | 61.8±15.7 | 65.5±13.5  | 49.6±29.5  | 33.6±14.3  |
| PR                                 | 68.0±15.5 | 68.2±16.1  | 48.4±27.3  | 27.8±19.5  |
| SRC-1                              | 50.0±17.2 | 60.3±14.9  | 39.0±24.3  | 33.0±22.4  |
| p300/CBP                           | 87.8±6.8  | 78.9±14.4  | 88.6±6.1   | 83.0±2.7   |
| NCoR                               | 21.1±23.4 | 54.4±17.3**| 12.8±10.8  | 13.2±4.6   |
| SMRT                               | 1.4±2.6   | 3.9±10.6   | 0.4±0.9    | 0.4±0.6    |
| Ki-67                              | 8.3±5.3   | 1.8±2.1**  | 7.6±4.5    | 3.7±6.4    |

Each number indicates mean±standard deviation. *There were no significant differences in the expression of any factors between Responders and Non-responders before MPA treatment. **Significantly different from the expression level before MPA treatment (p<0.01).
mediated transcription of breast carcinoma cells (20). In addition, forced expression of NCoR suppresses the induction of RAR (retinoic acid receptor) β gene regardless of the ligand binding in colorectal carcinoma (21). These studies suggest active roles of NCoR in the inhibition of basal signals from the receptors irrespective of ligand binding. Most endometrial tissue samples used in this study are positive for ER, and the growth of these tissues can be stimulated by serum estrogen and possibly by intrinsic estrogen which potentially lead to the consistent growth of

Figure 2. Result of immunostaining for Ki-67 and cofactors before and after the treatment with MPA in Responders. The expression of Ki-67 was significantly reduced after MPA treatment (a-c), and the expression of NCoR was significantly increased after MPA treatment (m-o). The expression of ER (d-f), SRC-1 (g-i), p300/CBP (j-l), and SMRT (p-r) did not show significant changes between before and after the MPA treatment. a, d, g, j, m, and p, as well as b, e, h, k, n, and q are serial sections. *Significantly different (p<0.05).
tumor cells even in low serum E2 levels at postmenopause (22). Therefore, it is likely that the progestin-induced NCoR can block these basal estrogenic growth signals.

Although the present study showed difficulty in the prediction of MPA responsiveness before treatment, cases which were able to express NCoR by MPA exhibited favorable responses to MPA. This result suggests that the capability of NCoR expression may imply a benign biological phenotype. In accordance with this anticipation, a recent study reported favorable responses to tamoxifen in NCoR-positive breast carcinomas (23). In addition, the increased expression of NCoR in breast carcinoma was reported to be associated with favorable disease-free survival (24).

In conclusion, an increased expression of NCoR after MPA therapy in Responders of endometrial hyperplasia and carcinoma has been demonstrated, and progestin-induced growth suppression was associated with increased expression of NCoR in ER/PR-positive carcinoma cells, suggesting that NCoR may be deeply involved in the progesterone-induced growth suppression of neoplastic cells of the endometrium.

Figure 3. Effect of estrogen and progestin on the proliferation of Ishikawa (a) and T47D (b) cells. Addition of estradiol (E2, \(10^{-8}\) M) for 120 hours stimulated the proliferation of both cells with significant differences. Progesterone (P4) or MPA suppressed the proliferation of both cells. *Significantly different from the control (p<0.05).

Figure 4. Progesterin-induced expression of NCoR and its receptor binding in T47D cells. a: The addition of P4 or MPA increased the expression of NCoR protein in T47D cells dose-dependently, but not for that of SMRT. b: Progesterin treatment did not induce the expression of NCoR protein in Ishikawa cells. c: MPA treatment induced the expression of NCoR mRNA dose-dependently (upper panel), and was suppressed by addition of a progesterone receptor antagonist, RU468 (lower panel). d: Immunoprecipitation showed the binding of NCoR to ERα, but not to PR. IP: anti-PR with IB:PR was performed as a positive control. IP: immunoprecipitation, IB: immunoblotting.
References


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