Norethindrone Scatters Silver-stained Nucleolar Organizer Regions of Ishikawa Cells

Yasuhiro Yokoyama,¹ Kenji Niwa, and Teruhiko Tamaya

Department of Obstetrics and Gynecology, Gifu University School of Medicine, Gifu University, Tsukasa-machi 40, Gifu City, Gifu Prefecture 500, Japan

ABSTRACT

We investigated the effects of sex steroids on silver-stained nucleolar organizer regions (Ag-NORs) and DNA/RNA kinetics in Ishikawa cells. Norethindrone and its isomer norethynodrel exclusively caused Ag-NORs to scatter in the nuclear matrix, from the nucleolus. No such effect occurred with the other sex steroids tested, including progesterational, androgenic, and estrogenic compounds. Nuclear argyrophilic substances induced by norethindrone, as well as nucleolar ones, were neither DNA nor RNA but protein. Electron microscopy showed that norethindrone caused nucleolar segregation, in which the fibrillar components disappeared, and it produced islets, consisting of dense fibrillar materials, in the nucleoplasm. Ag-NORs observed on the fibrillar components in control nucleoli were translocated onto the dense fibrillar materials in the nucleoplasm. Although scattering was preferentially found in the cells synthesizing DNA, the scintillation assay of DNA/RNA kinetics suggested that scattering was related to the inhibition of RNA synthesis. These results imply that norethindrone preferentially interacts with intranucleolar DNA when its duplication is occurring and then interferes with rRNA synthesis. Scattering of Ag-NORs might not be caused by the hormonal activity of these agents but by a pharmacological effect derived from their molecular structures.

INTRODUCTION

NORs² are located on the secondary constrictions of five acrocentric chromosomes and can be selectively stained by silver staining (1–3). This technique demonstrates nonhistone proteins associated with the NORs, rather than the rDNA itself (1, 4). In interphase cells, Ag-NORs can be observed on the fibrillar centers and peripheral dense fibrillar components of the nucleoli (4–7). The exact localization of rDNA genes during interphase has never been elucidated, but recent investigations have suggested that fibrillar centers might be the sites where they are located (8–10). Ag-NOR proteins exist in close proximity to rDNA genes and so may play an essential role in the transcription of rRNA in interphase nuclei. However, the exact biological role of these Ag-NOR proteins has not yet been elucidated.

The characteristics of Ag-NORs have been investigated in many types of cells, and differences in the number of Ag-NOR dots between benign and malignant cells have been demonstrated in some tumors (11–16). Moreover, the staining intensity of Ag-NORs has been reported to be related to the activity of RNA synthesis in fibroblasts (17) and HeLa cell (18) treated with actinomycin D. Thus, the staining of Ag-NORs seems to indicate a transcriptional potential of rDNA.

Estrogen is associated with a risk of endometrial carcinogenesis (19), while some progestogens have been used for the treatment of advanced endometrial carcinoma. Sex steroids are closely associated with the development of endometrial carcinoma, but the mechanism of their effects remains to be elucidated. Progesterone and estrogen have effects on the nucleolus and Ag-NORs in the endometrial epithelial cell (20–22). If endometrial carcinoma shows the same behavior in response to sex steroids as does its benign counterpart, sex steroids will also have an effect on its Ag-NORs.

Therefore, we investigated the effects of progestogens, as well as the other kinds of sex steroids, on Ag-NORs of endometrial carcinoma cells.

MATERIALS AND METHODS

Ishikawa cells are a continuously cultured endometrial carcinoma cell line, established by Nishida et al. (23). These cells have undergone 50–70 passages and possess both estrogen and progesterone receptors. The cells used in this study were grown in Eagle's minimum essential medium supplemented with glutamine (292 mg/liter) and 10% fetal bovine serum treated with charcoal. Cultures were maintained at 37°C, in a humidified atmosphere of 5% CO₂/95% air. The cells were cultured in Lab-tek chamber slides or 24-well culture plates, for 2 days, and were then incubated with various sex steroids at 10⁻⁴ M.

The sources of steroid hormones were as follows. Progesterone, norethindrone, norethynodrel, norethindrone acetate, ethisterone, testosterone, 17α-methyl-testosterone, 19-nor-testosterone, 17α-ethyl-estradiol-3-methyl-ether (mestranol), and ethynyl estradiol were purchased from Sigma Chemical Co. (St. Louis, MO). Norgestrel, danazol, gestrinone, chlormadinone acetate, and medroxyprogesterone acetate were kindly provided by Wyeth-Ayerst Research (Princeton, NJ), Tokyo Tanabe Pharmaceutical Inc. (Tokyo, Japan), Luel Medicina Inc. (Tokyo, Japan), Shionogi Pharmaceutical Inc. (Osaka, Japan), and Kyowa Hakko Inc. (Tokyo, Japan), respectively. Allylestrenol and lynestrenol were kindly provided by Organon International B.V. (Bhf Oss, Netherlands). These sex steroids were dissolved in dimethylsulfoxide, and aliquots were added to the culture medium at the given concentrations. The concentration of dimethylsulfoxide in the culture medium did not exceed 0.1%. The concentration of dimethylsulfoxide in controls was adjusted to 0.1%, which had no effect on Ishikawa cells.

Light Microscopy. After incubation for specified time intervals, the cells were fixed with 6% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), at 4°C for 1 h. All sex steroids inhibited cell growth at the concentration of 10⁻⁴ M.

Ag-NOR staining was performed by the method of Howell and Black (2). More than 500 cells in three different fields were examined for each culture.

Digestion tests of argyrophilic substance associated with scattering of the Ag-NORs by norethindrone were conducted using the following treatments: (a) DNase (1 mg/ml) in 0.1 M Tris buffer (pH 7.3) containing MgCl₂ and CaCl₂, at 37°C for 2 h, (b) 0.1% RNase in 0.1 M phosphate buffer (pH 6.0), at 37°C for 1 h, and (c) 0.01% trypsin in phosphate-buffered saline, at 37°C for 10 min. The successful extraction of DNA or RNA was checked by acridine orange fluorescence.

Evaluation of DNA and RNA Synthesis. The percentage of inhibition of RNA or DNA synthesis by the sex steroids was investigated in the following manner. Approximately 50,000 cells were grown in the culture wells. Forty-eight h later, the cells were reacted with sex steroids at 10⁻⁴ M and various concentrations of norethindrone. After 12 h of incubation, RNA or DNA synthesis was monitored by pulse-labeling cells, for 30 min, with 1.0 μCi/ml [³H]uridine (New England Nuclear, Boston, MA) or 1.0 μCi/ml [³H]thymidine (New England Nuclear), respectively. In the former assay, 10 μmol of thymidine was simultaneously added to the medium.

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¹To whom requests for reprints should be addressed.

²The abbreviations used are: NOR, nucleolar organizer region; Ag-NOR, silver-stained nucleolar organizer region.

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Norethindrone or norethynodrel decreased to 44 ± 10% (mean exposure to the various sex steroids is shown in Fig. 4. The elimination by trypsin treatment, but not by either RNase or DNase treatment.

The scattered Ag-NORs observed with norethindrone treatment, as well as the nucleolar Ag-NORs, were completely eliminated by trypsin treatment. The relationship between labeling with bromodeoxyuridine and scattering of Ag-NORs was investigated in >500 cells from each culture.

Electron Microscopy. Cells were fixed with 2.5% glutaraldehyde in 0.1 m phosphate buffer (pH 7.2) for 1 h at 4°C and with 0.1% osmic acid in 0.1 m phosphate buffer (pH 7.2) for an additional 1 h at room temperature. The cells were dehydrated, detached with propylene oxide, and embedded in Epon. Ultrathin sections were cut and then counterstained with uranyl acetate and lead citrate.

For the histochemical study of Ag-NOR localization, the cells were fixed with 2% paraformaldehyde in 0.1 m phosphate buffer (pH 7.2), at 4°C for 1 h, and were stained for NORs by the same method described above. Cells were then fixed with 1% osmic acid for 1 h at room temperature, dehydrated, detached with propylene oxide, and embedded in Epon. Ultrathin sections were cut and counterstained with uranyl acetate and lead citrate. The sections were viewed with a Hitachi HU12 electron microscope (75 kV; Hitachi Instruments Inc., Ibaragi, Japan).

Statistics. The data obtained were evaluated by the x² test.

RESULTS

Light Microscopy. Ag-NORs were observed in the nucleoli of Ishikawa cells during interphase. Among the sex steroids tested, only norethindrone and its isomer norethynodrel caused morphological changes in the Ag-NORs. Treatment with these agents caused the emergence of Ag-NORs into the nuclei. The nuclear Ag-NORs showed stronger NOR staining than did nucleolar ones. We designated this morphological feature "scattering" (Fig. 1). The nucleolar Ag-NORs in cells with the scattering of Ag-NORs induced by treatment with 20 µg/ml norethindrone for 12 h became obscure. At 60 µg/ml norethindrone, the nucleoli of the cells with the scattering dwindled in size (Fig. 2).

None of the other sex steroids caused any changes in the distribution of the Ag-NORs or nucleolar morphology. Fig. 3 shows the percentage of cells showing scattered Ag-NORs after 12 h of incubation with 10⁻⁴ m sex steroids. After 12 h of treatment with norethindrone and norethynodrel, 74% and 25% of the cells, respectively, showed scattering of the Ag-NORs, while the cells incubated with the other sex steroids did not show scattering. The other sex steroids could not cause the scattering at any concentrations.

In order to identify the argyrophilic substance related to the scattering, digestions with various enzymes were performed. The scattered Ag-NORs observed with norethindrone treatment, as well as the nucleolar Ag-NORs, were completely eliminated by trypsin treatment, but not by either RNase or DNase treatment.

DNA and RNA Kinetics. DNA/RNA synthesis after 12 h of exposure to the various sex steroids is shown in Fig. 4. The percentage of radioactive uridine uptake in the presence of norethindrone or norethynodrel decreased to 44 ± 10% (mean ± SD) or 59 ± 18% of the control level, respectively, whereas the thymidine uptake did not decrease. Fig. 5 shows the relationship between scattering of Ag-NORs and DNA/RNA kinetics at different concentrations of norethindrone. Since the medium was fully saturated at approximately 70 µg/ml norethindrone, the percentage of inhibition and percentage of scattering showed limited changes at higher concentrations. RNA synthesis was inhibited in a concentration-dependent manner, while DNA synthesis was not inhibited up to a concentration of 40 µg/ml.

Relationship of Ag-NOR Scattering to the S Phase. The relationship between the phase of the cell cycle and Ag-NOR scattering is shown in Fig. 6. After 6 h of exposure to norethindrone, scattering occurred dose dependently, and the percentage of cells in the S phase decreased. Cells labeled with bromodeoxyuridine were significantly more likely to show scattering of Ag-NORs at any concentration of norethindrone (Fig. 7).

Electron Microscopy. The nucleolus consisted mostly of granular and fibrillar components. Fibrillar centers were not obvious in Ishikawa cells. Nucleoplasm consisted mostly of euchromatin (Fig. 8). After treatment with norethindrone (60 µg/ml) for 12 h, nucleolar segregation occurred in some nucleoli. Loose fi-
Norethindrone affects nucleolar organizer regions

% of cells with scattered Ag NORs

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Fig. 3. Percentage of cells showing scattering of Ag-NORs after 12 h of exposure to 10^{-4} M sex steroids. Ag-NORs were evaluated in >500 cells in three different fields for each culture. *MPA, medroxyprogesterone acetate.

Fig. 4. Percentage of inhibition of DNA and RNA synthesis after 12 h of exposure to 10^{-4} M sex steroids. Synthetic activity was evaluated by 30 min of pulse labeling with 1 μCi/ml [3H]thymidine (DNA) or 1 μCi/ml [3H]uridine (RNA). The experiments were performed in quadruplicate. MPA, medroxyprogesterone acetate.

brillar materials were observed to migrate from the nucleolus toward the nucleus, and patches of dense fibrillar materials emerged in the nucleoplasm (Fig. 9). An additional 6 h later, the fibrillar components disappeared out of the nucleoli, and only condensed granular components remained. Many islets consisting of dense fibrillar materials emerged in the nucleoplasm (Fig. 10).

Ag-NORs were localized on the dark staining area of the nucleolar body (Fig. 11). Most granular components and nucleolar interstices were negative for staining. After treatment with norethindrone (60 μg/ml) for 12 h, Ag-NORs were stained irregularly in such nucleoli and spottily in the nucleoplasm (Fig. 12). The Ag-NORs in the nucleoplasm corresponded to dense fibrillar materials. The loose fibrillar materials migrating from the nucleolus were free from staining. The staining intensity of nucleolar Ag-NORs was heterogeneous.

DISCUSSION

With transmission electron microscopy, three morphologically distinct components are usually observed in interphase...
NORETHINDRONE AFFECTS NUCLEOLAR ORGANIZER REGIONS

During the past several years, numerous investigations have validated the usefulness of the enumeration of Ag-NORs for cancer diagnosis in certain tumors (11–15). In addition, relations of the number of Ag-NOR dots in tumor cells to their proliferative activity (16, 26–28) and prognosis (29, 30) have been reported. However, it is not entirely certain whether Ag-NORs are an index for the transcriptional activity of rDNA. As shown in this study, norethindrone appeared to increase Ag-NOR dots in Ishikawa cells, but the total RNA synthesis and the proliferative activity clearly decreased. Under this experimental condition, Ag-NOR dots merely indicated possible locations of rDNA in Ishikawa cells, and the number was related neither to RNA synthetic activity nor to proliferative activity.

Cells labeled by bromodeoxyuridine were liable to show scat-

nucleoli (24, 25). The fibrillar center is a possible area where rDNA exists, the fibrillar component usually exists at the periphery of the fibrillar centers and is the site of pre-rRNA synthesis, and the granular component is the main body of the nucleolus, which is composed of ribonucleoprotein particles, the terminal products of rRNA synthesis in the nucleolus. The fibrillar center is a conspicuous structure in many kinds of cells but in some cells is not obvious and may be absent (25). In Ishikawa cells, the fibrillar center was not obvious and the Ag-NORs were observed mostly on the fibrillar components. Thus, in Ishikawa cells, the fibrillar components will be the sites where rDNA exists and pre-rRNA is synthesized. Norethindrone exclusively affected this specific site, causing inhibition of RNA synthesis.

Norethindrone segregated the nucleolar structure of Ishikawa cells and caused the following changes: (a) disappearance of fibrillar components from the nucleolus, (b) condensation of the granular components, and (c) appearance of dense fibrillar materials in the nucleoplasm. The scattering of Ag-NORs observed in Ishikawa cells appears to be due to the argyrophilic nature of dense fibrillar materials emerging in the nucleoplasm. Because the dense fibrillar materials structurally resemble the fibrillar components of the nucleolus and are also argyrophilic, both may be relevant substances. Therefore, the scattering of Ag-NORs may be associated with the translocation of rDNA.

Fig. 7. Cells double-stained with Ag and immunohistochemistry for bromodeoxyuridine. Dark cells (arrows) were positive for bromodeoxyuridine. × 1330.

Fig. 8. Control cell fixed with 2.5% glutaraldehyde and 1% osmic acid and contrasted with uranyl acetate and lead citrate. × 17,000. FC, fibrillar components; GC, granular components.

Fig. 9. Cell treated with norethindrone at 60 μg/ml for 12 h, fixed with 2.5% glutaraldehyde and 1% osmic acid, and contrasted with uranyl acetate and lead citrate. Note the nucleolar segregation. × 17,000. GC, granular component; LFM, loose fibrillar materials; DFM, dense fibrillar materials.

Fig. 10. Cell treated with norethindrone at 60 μg/ml for 18 h, fixed with 2.5% glutaraldehyde and 1% osmic acid, and contrasted with uranyl acetate and lead citrate. × 9,000. NO, nucleolus. Arrows, dense fibrillar materials. Nucleolus consists only of the granular components.
corresponded to fibrillar components in the nucleolus. × 8,000. NO, nucleolus. After counterstaining with uranyl acetate and lead citrate, silver deposits occurred irregularly on the granular component of Ishikawa cells. All the progestational compounds we investigated would bind to the progesterone receptor of Ishikawa cells. If the scattering was an event subsequent to progesterone receptor binding of progestational agents, all progestogens would cause scattering. This result suggests that scattering is not related to pure progestational effects. Furthermore, norethindrone and nor the estrogenic agents used in this study cause such changes, suggesting that scattering was not related to the pure hormonal effects of those agents. The other 19-nortestosterone progestogens used in this study, including norethindrone acetate, norgestrel, lynestrenol, and allylestrenol, have the same multipotentiality as norethindrone but did not cause scattering. These results indicate that scattering of Ag-NORs is a pharmacological effect derived from the molecular structure of these two compounds.

The facts that only a few kinds of progestational agents, which contain an acyl group at the 17α-position, are used clinically against endometrial carcinoma, that the progestrone receptor content of the tumor is not always a key factor for the efficacy of progestogen therapy, and that a high loading dose of progestational agents is necessary for their efficacy suggest that the mechanism of action of progestogens against endometrial carcinoma is not due to their hormonal effects but to a pharmacological effect derived from their molecular structures.

There are no available reports on the efficacy of norethindrone against endometrial carcinoma. As shown in this study, the site of action of norethindrone is quite different from that of the other progestogens. This specificity suggests that norethindrone might be effective for tumors that are resistant to current progestational agents.

REFERENCES

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