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The Effects of Estrogen and Antiestrogen on Chromatin in Human Breast Cancer Cell Line, MCF-7. I

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The effects of estrogen and antiestrogen on chromatin in the human breast cancer line, MCF-7 were studied in cells grown in media containing 10^{-8} M estradiol or 10^{-6} M tamoxifen for 1, 3 and 8 d. The nuclear binding sites of the estradiol- and tamoxifen-receptor complexes were determined using intact cells. The nuclear content of the estradiol-receptor complex reached a plateau level on day 1. Nuclear tamoxifen binding sites were still increasing on day 3. The binding capacity of the chromatin for the estradiol-receptor complex was determined in a cell-free system. It was increased by incubation for 1—3 d and then decreased by incubation for 8 d. In addition, the sodium dodecyl sulfate-gel electrophoretic patterns of chromosomal proteins from MCF-7 cells grown in media containing 10^{-8} M estradiol, 10^{-6} M tamoxifen, or vehicle alone for 8 d were compared. The patterns were quite similar, but differences in some bands could be detected.

Keywords——antiestrogen; estrogen; chromatin binding site; MCF-7; electrophoresis

Antiestrogens, which are nonsteroidal triphenylethylenes, are useful for treatment of hormone-dependent breast cancers. After estrogens or antiestrogens bind to cytosol estrogen receptors in the target cell, they translocate into the nucleus and interact with chromatin. The interactions of estrogen- and antiestrogen-receptor complexes with chromatin are thought to differ, although the mechanism of antiestrogen action is not fully understood. Differences in physical properties between the nuclear estrogen- and antiestrogen-receptor complexes suggest that antiestrogens may antagonize estrogen action by promoting a change in the receptor that leads to its association with another cellular component.[1] Based on the striking difference in extractability of 1,3,5(10)-estratriene-3,17β-diol (estradiol)- and *trans*-(*p*-dimethylaminoethoxyphenyl)-1,2-diphenyl-but-1-ene (tamoxifen)-receptor complexes in the nuclei with a non-ionic detergent, Nonidet P-40, the present author and his colleagues[2] suggested in a previous paper that the binding sites of these complexes in the nucleus might be different.

In the present study, the effects of estradiol and tamoxifen on chromatin in MCF-7 cells were studied in order to elucidate the action mechanism of estrogens and antiestrogens. To do this, the nuclear receptor content in intact cells and the receptor binding capacity in a cell-free system to chromatin from cells cultured in the presence or absence of estrogen or antiestrogen for 1, 3 and 8 d were determined. Then the sodium dodecyl sulfate (SDS)-gel electrophoretic patterns of the chromosomal proteins were compared.

Materials and Methods

Cell and Tissue Culture——MCF-7 cells were obtained from the National Institutes of Health (Bethesda, U.S.A.) through Dr. Y. Nomura of the National Kyushu Cancer Center Hospital (Japan) and maintained in closed flasks at 37 °C. Autoclaved Eagle's minimum essential medium (MEM) supplemented with 1% non-essential amino acids, 2 mM L-glutamine, 8% newborn calf serum (Irvine Scientific) and 1% $NaHCO_3$ was used as the growth medium. At

least one week prior to use, cells were grown in medium in which calf serum was replaced by charcoal-dextran stripped calf serum prepared by incubation twice with 0.1% (w/v) dextran-coated 1% (w/v) charcoal (final concentration) (DCC) at 40 °C for 30 min.[2,3] The cells used in the present experiments were in log phase growth.

Nuclear Isolation and Nuclear Exchange Assay——Nuclear isolation was performed following a method described previously.[2,4] MCF-7 cells were suspended in medium S (10 mM Tris–HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol (DTT), 0.25 M sucrose) and homogenized at 0 °C. The homogenate was centrifuged at $1000 \times g$ for 10 min at 4 °C. The crude nuclear pellet was suspended in medium S and then washed with medium S at 0 °C. Deoxyribonucleic acid (DNA) content in the nuclear fraction was determined by the method of Burton[5] with calf thymus DNA as a standard.

Nuclear exchange assays were performed as reported by Anderson *et al.*[6] Triplicate 0.5 ml aliquots of the nuclear fraction were incubated with 10 μl medium S containing [2,4,6,7-^{3}H]estradiol ([^{3}H]estradiol; New England Nuclear, 93 Ci/mmol) at a final concentration of 10 nM in the presence or absence of a 100-fold excess of unlabelled estradiol. The bound [^{3}H]estradiol was extracted twice with 0.5 ml ethanol at room temperature, and counted.

Preparation of Cytosol and the [^{3}H]Estradiol–Receptor Complex——The MCF-7 cells were homogenized in binding buffer [10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM DTT, 0.15 M KCl] at 0 °C using a Polytron PT-10 homogenizer. The homogenate was centrifuged for 60 min at $100000 \times g$ to obtain a clear supernatant (cytosol). The [^{3}H]estradiol–receptor complex was prepared by incubating the cytosol with 10 nM [^{3}H] estradiol (final concentration) with or without a 100-fold excess of diethylstilbestrol (Sigma Chemical Co.) for 2 h at 0 °C and with DCC for 30 min at 0 °C.

Preparation of Chromatin——Chromatin was prepared by a slight modification of the method of Bonner *et al.*[7] MCF-7 cells were homogenized in TES buffer [20 mM Tris–HCl (pH 7.4), 2 mM EDTA, 1 mM DTT, 75 mM NaCl] as previously described.[2] The chromatin pellet was successively washed by centrifugation at $1500 \times g$ for 15 min with TES buffer and TE buffer [10 mM Tris–HCl (pH 7.6), 1 mM EDTA] at 4 °C. Then it was homogenized in a Potter-Elvehjem homogenizer in TE buffer with 10 strokes. The above suspension was layered on 1.7 M sucrose-containing TE buffer and centrifuged at 22000 rpm for 3 h in a Spinco SW 25 rotor at 4 °C. Next, the pellet was suspended in 10 mM Tris–HCl buffer (pH 8.0) and dialyzed against the same buffer overnigh at 4 °C. Absorption of the isolated chromatin at 320 nm was not more than 1/10 of that at 260 nm.

The Cell-free Binding of the [^{3}H]Estradiol–Receptor Complex to Chromatin——The binding of the [^{3}H]estradiol–receptor complex to chromatin was performed in binding buffer at 25 °C for 1 h.[8,9] The bound [^{3}H]estradiol was extracted twice with 0.5 ml of ethanol at room temperature, and the radioactivity was counted. DNA content was measured by Burton's method[5] using calf thymus DNA as a standard before incubation for binding.

SDS-Slab Gel Electrophoresis——The sample (140 μg of DNA) was reduced by boiling for 2 min in TE buffer containing β-mercaptoethanol (10%) and SDS (4%). Electrophoresis was carried out in 10% polyacrylamide gel (140 × 140 × 2 mm) containing 0.1% SDS and 4 M urea in 0.37 M Tris–HCl (pH 8.8) with 3% stacking gel containing 0.1% SDS and 4 M urea in 0.125 M Tris–HCl (pH 6.8) following a modification of the reported method.[10] Bands were visualized by silver staining.[11]

Radioactivity Determination——One ml of ethanol extract was added to 5 ml of scintillation fluid (toluene containing 33.3% Triton X-100 and 0.55% 2,5-diphenyloxazole). Quantitation of radioactivity was carried out with an Aloka scintillation spectrometer (model LSC-900) (30—35% efficiency for tritium).

Results and Discussion

Estradiol at a physiological concentration (10^{-8} M) stimulated cell division, while the antiestrogen, tamoxifen (10^{-6} M), strongly inhibited the growth of MCF-7 cells.[12]

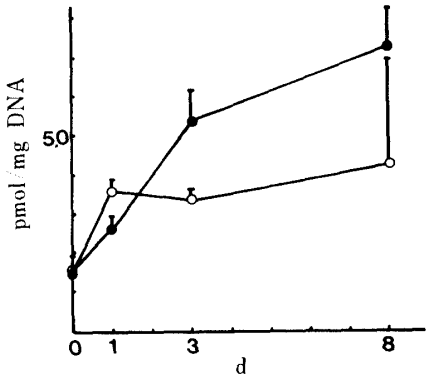

Fig. 1. Nuclear Binding Capacity for Estradiol and Tamoxifen

MCF-7 cells were incubated for the indicated time at 37 °C with 10^{-8} M estradiol (○) or 10^{-6} M tamoxifen citrate (●). The nuclear bindings of estradiol and tamoxifen were measured by exchange assay. All values are means of triplicate determinations ±S.D.

Accordingly, the effects of estradiol and tamoxifen on the chromatin of MCF-7 cells were studied at these concentrations.

The nuclear binding capacities of estradiol- and tamoxifen-receptor complexes in MCF-7 cells grown in media containing 10^{-8} M estradiol or 10^{-6} M tamoxifen were estimated by using the nuclear exchange assay[6] (Fig. 1). The values of nuclear retention of the estradiol-receptor complex were 3.60 ± 0.26, 3.30 ± 0.36 and 4.29 ± 2.67 pmol/mg DNA at 1, 3 and 8 d, respectively, after hormone addition, showing a plateau level after incubation for 1 d. This result suggests that the dynamics of the estradiol-receptor complex were equilibrated on day 1. Nuclear tamoxifen-binding sites were still increasing on day 3 and a progressive increase was noted until the 8 d. It has been reported that the processing step which removes the tamoxifen-receptor complex from the nucleus is somehow defective.[13] Therefore, the increase may have been due to a defect in processing. It is also possible that the tamoxifen-antiestrogen binding site (ABS) complex was translocated to the nucleus.[14] However, since ABS can not bind [^{3}H]estradiol in the exchange assay, the estimated values do not inclued the tamoxifen-ABS complex.

To detect changes in chromatin in MCF-7 cells grown as mentioned above, the binding capacities of chromatins from those cells and KB cells (non-target cells for estrogen) were determined in a cell-free system using the estradiol-receptor complex in the cytosol from MCF-7 cells (Fig. 2A). MCF-7 cell and KB cell chromatins were incubated (25 °C, 1 h) with the [^{3}H]estradiol-receptor complex in the medium containing 0.15 M KCl. The receptor binding capacities of chromatin from cells grown in medium containing 10^{-8} M estradiol for 1, 3 and 8 d were 1.38, 1.61 and 0.85 pmol/mg DNA, respectively. Those of chromatin from cells grown in the medium containing 10^{-6} M tamoxifen for 1, 3 and 8 d were 1.44, 0.84 and 1.06 pmol/mg DNA, respectively. The binding capacity of chromatin from MCF-7 cells grown in the estradiol and tamoxifen-free medium was 0.58 pmol/mg DNA (control value). The receptor binding capacity of chromatin from KB cells was 0.76 pmol/mg DNA. The cytosol-[^{3}H]estradiol mixture was treated with DCC to remove free [^{3}H]estradiol. There was 1.9 pmol/mg DNA of free [^{3}H]estradiol remaining in the cytosol after the DCC treatment. Therefore, the capacity for binding of [^{3}H]estradiol to chromatin was also determined under the above conditions (Fig. 2B). Consequently, the [^{3}H]estradiol binding capacity of the

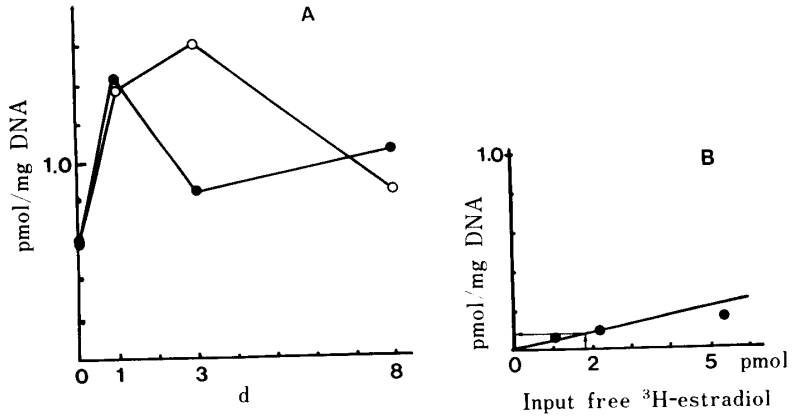

Fig. 2. Cell-free Chromatin Binding of the [^{3}H]Estradiol-Receptor Complex (A), and the Binding of Free [^{3}H]Estradiol to Chromatin (B)

(A) MCF-7 cells were incubated for the indicated time at 37 °C with 10^{-8} M estradiol (○) or 10^{-6} M tamoxifen citrate (●). The chromatin was prepared as described in Materials and Methods. Each tube contained chromatin (20—80 μg of DNA) and cytosol containing 48 fmol of [^{3}H]estradiol-receptor complex and 41 fmol of [^{3}H]estradiol. All values (A) are means of duplicate determinations, which showed no significant variation.

(B) The tubes contained chromatin (22 μg of DNA) and the indicated amount (changed into pmol/mg DNA in each tube) of [^{3}H]estradiol.

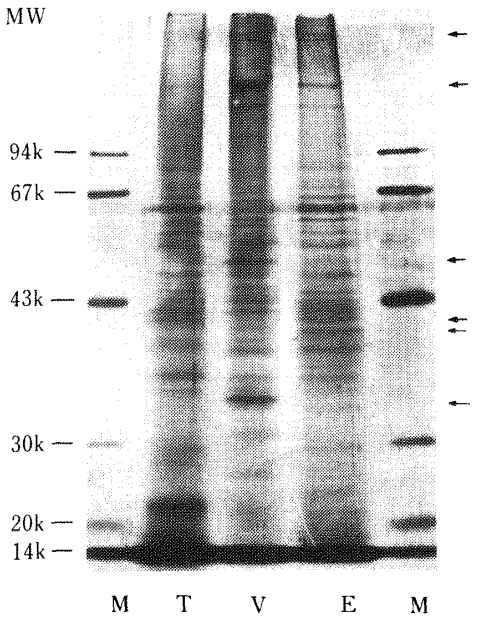

Fig. 3. SDS-Gel Electrophoretic Patterns of Chromosomal Proteins from MCF-7 Cells

The cells were incubated for 8 d at 37 °C in the absence (V) or presence of 10^{-8} M estradiol (E) or 10^{-6} M tamoxifen citrate (T). The chromatins were prepared and the electrophoresis was carried out as described in Materials and Methods. Phosphorylase b, albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and α-lactoalbumin were used as molecular weight marker proteins (M). The arrows indicate major different bands among samples.

chromatin was found to be 0.08 pmol/mg DNA. It has been reported that the steroid–receptor complex binds to acceptor sites in isolated nuclei that are chemically similar to, but topographically different from, those in the intact cell.[15] Accordingly, the binding capacity of the estradiol–receptor complex to chromatin was determined without removing estradiol or tamoxifen from cells grown in medium containing estradiol or tamoxifen. The value in chromatin from MCF-7 cells grown in the absence of estradiol or tamoxifen was similar to that in KB cells. After administration of estradiol or tamoxifen, the binding capacity increased initially and then decreased, until it reached the level of non-target cell chromatin (Fig. 2A). It has also been reported that the acceptor sites in non-target tissues are masked by protein and that the extent of masking in target tissues varies under biological influences.[16] The chromatin binding sites of the estradiol–receptor complex in a cell-free system may be masked before exposure and after prolonged exposure of the cell to estradiol or tamoxifen. Antiestrogen may be more effective in the masking of acceptors to which the estradiol–receptor complex binds. The change in the receptor binding capacity of chromatin in the cell-free system may reflect the conformational change in chromatin that is thought to be the result of translocation of the receptor to the nucleus.[17] Thymidine incorporation into DNA in cells grown under the same conditions was more effective in the presence of estradiol than of tamoxifen after 1—3 d and decreased in the presence of either estradiol and tamoxifen after 3—8 d.[18] These are favorable results for receptor binding to chromatin in a cell-free system.

In order to study whether the composition of chromosomal protein was changed, the SDS-gel electrophoretic patterns of chromatins from MCF-7 cells grown in media containing 10^{-8} M estradiol, 10^{-6} M tamoxifen or vehicle alone for 8 d were examined. The patterns were quite similar to each other, as shown in Fig. 3, but differences in some main bands could be detected visually, as shown by the arrows. Thus, it seems possible that the proteins in which the changes were observed are related to the chromatin binding sites for the respective complex or/and the masking protein(s).

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