Presence of Estrogen Binding Sites and Growth-stimulating Effect of Estradiol in the Human Myelogenous Cell Line HL60

Laurence Danel, Geneviève Cordier, Jean-Pierre Revillard, and Simone Saez

Centre de Lutte contre le Cancer, Centre Leon Berard, 28, rue Laennec, 69008 Lyon [L. D., S. S.], and UER-Faculté de Biologie Humaine, Institut National de la Santé et de la Recherche Médicale, U80, CNRS-ERA 782, Pavillon P, Hôpital Edouard Herriot, 69003 Lyon [G. C., J-P. R.], France

ABSTRACT

In the present study, we investigated the effects of estrogens on the growth of the HL60 line in vitro and the presence of estrogen binding sites in the same cells.

Cell proliferation was estimated by cell counts, [3H]thymidine incorporation, and determination of the percentage of cells in the S phase by flow cytometry.

Cells maintained in a medium containing physiological concentrations of estradiol (10<sup>-9</sup> M, 10<sup>-8</sup> M, 10<sup>-7</sup> M) exhibited a growth stimulation, shown by an increase in the percentage of cells in the S phase, whereas a pharmacological concentration (10<sup>-6</sup> M) produced a growth inhibition. Furthermore, the addition of the specific antihormone, tamoxifen, inhibited the stimulating effect of the estrogens.

Receptor analysis showed the presence of specific estrogen-binding sites with an apparent dissociation constant of about 5.3 x 10<sup>-10</sup> M. The effect of estrogen was therefore associated with the presence of estrogen receptors in the human leukemic cell line HL60.

INTRODUCTION

In addition to being a responsive target to glucocorticoids, leukemic cells have also been reported to respond to sex steroids (9, 24). Previous studies on blast cells from patients with acute leukemia have shown the presence of specific proteins which bind androgen, estrogen, and progesterin with a high affinity (6).

Our aim was to investigate the role of these receptors. Since studies on fresh leukemic cells have been limited in part by the restricted survival of leukemic cells in vitro, it appeared that the action of sex steroids would be best studied by using a cultured human cell line. A human myelogenous cell line (HL60) was recently established from the peripheral blood of a woman with an acute promyelocytic leukemia (3, 4, 13). This cell line would provide a suitable tool to investigate the regulation of cell growth and differentiation in acute myeloid leukemia. In a first attempt, we examined the in vitro effects of estrogens on the growth of the HL60 cell line in order to determine whether these effects were mediated through estrogen receptors.

MATERIALS AND METHODS

Cells

HL60 cells were kindly provided by Dr. R. Gallo (National Cancer Institute, Bethesda, Md.). Cells were cultured at 37° in an atmosphere of 5% CO<sub>2</sub> in air, in slightly sealed 25-cm<sup>2</sup> tissue culture flasks (Corning Plastic, Corning, N. Y.), in Roswell Park Memorial Institute Tissue Culture Medium 1640 (Gibco-Biocult, Glasgow, N. Y.), supplemented with 10% heat-inactivated (56°, 30 min) fetal calf serum (Gibco-Biocult), 1% L-glutamine (Bio-Merieux, Lyon, France), penicillin (40 IU/ml), and gentamicin (40 μg/ml).

All experiments were done with the same batch of fetal calf serum. Estradiol concentration in the serum-containing media in which the cells were grown was less than 10<sup>-11</sup> M. The cells were seeded at 2.5 x 10<sup>5</sup> cells/ml. Every 6 to 7 days, they were split at a ratio 1:10.

Steroid-binding Assay

Five x 10<sup>6</sup> cells were obtained by growing cells in mass culture. Cells were harvested by centrifugation at 300 x g and washed 3 times in PBS. Binding assay on cytoplasmic extracts was performed as described previously (6) but with a slight modification. Following homogenization, 2 ml buffered sucrose was added to produce a final concentration of 0.25 M, and leupeptin (10 nm) or aprotinin (17 IU/ml) was added to protect soluble proteins against proteolysis (24). Aliquots of cytosol (100 μl) were incubated at various concentrations (2.5 x 10<sup>-11</sup> to 6 x 10<sup>-9</sup> M) of the specific synthetic estrogen [3H]R2858 (specific activity, 88 Ci/mmol; New England Nuclear, Boston, Mass.) in the presence or absence of an excess of unlabeled R2858 (Roussel Uclaf, Romainville, France) to correct nonspecific binding (21). Radioactivity was counted in 5 ml of emulsifier-scintillator, and the counts were corrected for quenching. Results were plotted according to Scatchard (25). The dissociation constant was calculated and the bound radioactivity was expressed as fmol/mg protein evaluated by the method of Lowry et al. (18).

Nuclear Acceptor Sites

Samples containing 5 x 10<sup>6</sup> cells were incubated in 0.3 ml of Roswell Park Memorial Institute Tissue Culture Medium 1640 without serum with 5 nm [3H]R2858 with or without 100-fold excess of non-radioactive steroid, for 30 to 180 min at 37°. After incubation, each sample was chilled and washed 3 times in cold PBS. Cells were diluted in hypotonic buffer (10 nm Tris and 3 mm MgCl<sub>2</sub>) and homogenized in 1 ml TED buffer. All assays were done in duplicate.

The homogenate was centrifuged at 800 x g for 15 min at 4°. The supernatant was centrifuged at 100,000 x g for 60 min at 4° and then treated with charcoal to absorb free steroids, and the radioactivity was counted.

The crude nuclear pellet was washed in TED buffer. Bound [3H]-R2858 was extracted in TED buffer containing 0.6 M KCl. Following centrifugation of KCl extract, the pellet was treated by 1 ml of ethanol at room temperature for 15 min to complete the extraction. The radioactive activity was counted in both KCl and ethanol extracts, and the total nuclear associated [3H]R2858 was measured. The results are expressed in number of sites per cell (6).

In some experiments, isolation of purified nuclei was performed according to Stevens et al. (26, 27). The crude nuclear pellet obtained...
from disrupted cells as described previously was purified through a sucrose barrier. Specifically bound \(^{3}H\)R2858 was extracted with KCl and ethanol as above. The results were expressed in fmol/mg DNA. Aliquots of nuclei were studied by electron microscopy to confirm the absence of cytoplasmic contamination.

### Measurement of Cell Growth

**Cell Counts.** Cells were seeded in complete medium for 24 hr before the addition of various concentrations of steroids. In the absence of hormone, cells grew with a doubling time of approximately 60 hr. Growth saturation was obtained with 2.5 to 3 \(\times\) 10^6 cells/ml in 6 to 7 days. Nonradioactive steroids were provided by Roussel Uclaf. The antiestrogen tamoxifen was kindly donated by ICI-Pharmaceuticals, Engehein, France.

Steroid solutions were prepared in absolute ethanol and added to the culture system at final concentrations never exceeding 0.1%. Control cultures contained the same concentration of ethanol. Each experiment was done in quadruplicate. Cell growth was evaluated by daily counts, using a Couter Counter. Viability was checked by the trypan blue exclusion test. Changes in the morphology of cells studied on cytocentrifuge-prepared slides stained with May-Grunwald-Giemsa.

**Steroid Effect on Nucleic Acid Precursor Incorporation.** The incorporation of \(^{3}H\)thymidine (specific activity, 28 Ci/mmol; CEA, Saddle, France) was measured during growth in the absence (control) or presence of nonradioactive estradiol; 5 \(\mu\)Ci of \(^{3}H\)thymidine per ml were added for 2-hr pulse labeling (10). The reaction was terminated by the addition of ice-cold PBS. The cells were washed 3 times and then disrupted by sonication in 5 mM NaCl and 1 mM EDTA. DNA measurement of the homogenized sample was carried out by the fluorescence technique of Karsten (12). The remaining part was precipitated by the addition of 10% trichloroacetic acid. Acid-insoluble material was collected, washed extensively with 10% trichloroacetic acid, and counted after hydrolysis for its radioactivity content. The rates of incorporation were calculated as cpm/µg DNA.

**Cell Cycle Analysis.** Cell cycle analysis was performed at the time of the \(^{3}H\)thymidine addition. Aliquots of cells were washed with PBS, fixed in absolute ethanol (2 volumes/volume of pelleted cells), and stored at 4°C before analysis. The content of DNA per cell was evaluated by ethidium bromide staining according to the method of Gray (7). Briefly, aliquots of cells were treated for 5 min at room temperature with RNase (1 mg/ml in Tris-KCl buffer, pH 7.2) and then for 10 min with pepsin (2800 ID/ml) after which ethidium bromide solution (50 µg/ml in PBS) was added. Measurements were carried out within 10 min after staining. Cells were analyzed on the Cytofluorograf 50 H (Ortho Instruments, Westwood, Mass.).

With this staining procedure, the intensity of red fluorescence is proportional to the DNA content of the cell. Doublets were distinguished from cells in G2 and M phase by measuring the peak and the area of the red fluorescence pulses. The relative percentage of cells in the G1, S, and G2 + M phases were evaluated from the fluorescence histograms by using the estimation of Baisch et al. (1).

### RESULTS

**Characterization of Estrogen Receptors in HL60 Cells.** A representative curve of \(^{3}H\)R2858 binding on cytosol after 3 hr of incubation at 0-4°C is shown in Chart 1. Since the specificity of R2858 binding for estrogen receptors and the absence of cross-reaction with other steroid receptors has been demonstrated (21), this result suggests that cytosol preparations from HL60 cells possess high-affinity estrogen-binding proteins. The Scatchard plot was fitted by a single straight line suggesting one class of binding sites of uniform high affinity and low capacity: 14 to 16 fmol/mg protein on 2 experiments.
Table 1

<table>
<thead>
<tr>
<th>Concentration of estradiol (M)</th>
<th>24^a</th>
<th>48</th>
<th>72</th>
<th>96</th>
<th>120</th>
<th>144</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24 ± 3</td>
<td>373 ± 57.6</td>
<td>481 ± 78.4</td>
<td>615 ± 78.4</td>
<td>607 ± 179.8</td>
<td></td>
</tr>
<tr>
<td>10^-9</td>
<td>148 ± 21.7</td>
<td>388 ± 90.5</td>
<td>626 ± 54.3</td>
<td>632 ± 121.4</td>
<td>645 ± 168.1</td>
<td></td>
</tr>
<tr>
<td>10^-8</td>
<td>151 ± 28.8</td>
<td>385 ± 49.9</td>
<td>541 ± 93.9</td>
<td>616 ± 159.2</td>
<td>657 ± 240.7</td>
<td></td>
</tr>
<tr>
<td>10^-7</td>
<td>158 ± 24.1</td>
<td>375 ± 89.7</td>
<td>514 ± 72.6</td>
<td>552 ± 78.4</td>
<td>515 ± 64.0</td>
<td></td>
</tr>
<tr>
<td>10^-6</td>
<td>147 ± 21.4</td>
<td>358 ± 74.7</td>
<td>445 ± 78.0</td>
<td>552 ± 78.4</td>
<td>500 ± 114.1</td>
<td></td>
</tr>
</tbody>
</table>

*^a^ Culture time (hr).

Results are from one representative experiment. Determination of cell numbers and cell viability were made as described in “Materials and Methods.” Values are expressed as in Table 1.

from the control curve until the third day, at which point an inhibitory effect is seen. At 10^-7 M and 10^-6 M concentrations the stimulatory effect was largest on the fourth day of culture, while at 10^-5 M estradiol the maximum stimulatory effect occurred on the third day of culture. Hence, the kinetics of the stimulatory effect depended on the dose of estradiol added to the culture.

The specificity of the effect was studied by adding 10^-8 M estradiol at the beginning of the culture simultaneously with 10^-7 M to 10^-6 M of the antiestrogen, tamoxifen. Cell growth was unaffected by 10^-7 M tamoxifen (Chart 4A), but markedly inhibited cell growth was observed after 5 days at 10^-6 M. Addition of both 10^-8 M estradiol and 10^-7 M tamoxifen resulted in a marked inhibition of the stimulatory effect seen with estradiol alone (Chart 4B).

In attempts to understand the biological effect of estradiol on HL60 cells, we measured the thymidine incorporation and the number of cells in S phase, both in controls and after estradiol addition. There were no apparent differences between treated and control populations for the first 24 hr, after which estradiol-stimulated cells began to incorporate thymidine at a rate greater than did control cells (Chart 5A). The marked rise in thymidine incorporation occurred at 96 hr with 10^-6 M and at 72 hr with 10^-5 M. At 10^-7 M concentration, the increase was less marked. All of these increases were correlated to cell counts.

As shown in chart 5B, physiological concentrations of estradiol clearly stimulated cell division and increased the percentage of cells in S phase. This phenomenon would be expected

---

**Chart 3.** Effects of steroids on growing HL60 cells. HL60 (2.5 to 3 × 10^5 cells/ml) were cultivated in Roswell Park Memorial Institute Tissue Culture Medium 1640, supplemented with 10% heat-inactivated fetal calf serum and 1% L-glutamine, in the presence of 10^-9 M, 10^-8 M, 10^-7 M, 10^-6 M, or no (control) estradiol (E2). Results are from one representative experiment. Determination of cell numbers and cell viability were made as described in ‘Materials and Methods.’ Values are expressed as in Table 1.

**Chart 4.** Effect of antiestrogen (tamoxifen), alone (A) or associated with a stimulatory concentration of estradiol (B), on growing HL60 cells. TAM, tamoxifen; E2, estradiol.
to precede the increase in cell counts (Chart 3), which is indeed the case at 10^{-9} M, 10^{-8} M, and 10^{-7} M.

At 10^{-6} M estradiol, cell counts are below control levels after 72 hr, but low nucleotide incorporation was still observed until 96 hr. A slight increase in the percentage of cells in the S phase was observed during the first 48 hr.

**DISCUSSION**

Many investigators have shown that the biological sensitivity of acute lymphoid leukemia cells to glucocorticoids in vitro and in vivo is not always related to their content of steroid receptors (10, 17). However, human lymphocytic cell lines that possess high-affinity glucocorticoid receptors are sensitive to glucocorticoids. Sensitive clones have been isolated (20) whose growth is completely blocked by dexamethasone. Cell cycle analysis has also shown that dexamethasone-treated cells are killed upon entry to G1 (8). These techniques have provided an understanding of the mechanism of action of glucocorticoids on cell metabolism and have also been exploited to investigate the effects of drugs used in chemotherapy of acute lymphoid leukemia (11, 19, 23). Nevertheless, glucocorticoids appear to have only a marginal activity in human myelogenous leukemia (15).

Although HL60 cells contain glucocorticoid receptors, they are apparently resistant to the cytolytic effects of glucocorticoids (5, 14). A number of other steroids such as progesterone and androgen have been tested on this cell line (14); no significant effect on colony formation in vitro was detected. In this paper, we have presented data characterizing the response of the cell line to estrogen and antiestrogen treatment.

The HL60 cell line possesses specific estradiol-binding sites characterized in the cytosol and in the nuclei. The dissociation constant for estrogen receptors obtained from Scatchard analysis of the binding data is about 5.2 × 10^{-10} M, a value in agreement with results obtained previously in estrogen target tissue (16). Physiological concentrations of estradiol, which are adequate to saturate estradiol receptors, show reproducible stimulation of the cell line growth.

We observed a dose-dependent, time-dependent response curve, expressed in cell counts, and correlated to nucleotide incorporation and to the percentage of cells in the S phase. However, slight differences in the kinetics of the stimulatory effect were observed depending on whether thymidine incorporation or flow cytometry was used. These differences may be accounted for by the differences in the number of cells recorded by each technique. Flow cytometry measures the number of cells in the S phase at a precise time, while thymidine incorporation reflects the number of cells entering the S phase during the pulse period. The lack of synchronization of growing cells minimizes the estrogen effect.

The early increase of the S-phase proportion and the slight decrease of thymidine incorporation observed for cells cultivated with 10^{-6} M estradiol may indicate that this large con-
centration has an effect distinct from low doses. It does not prevent DNA synthesis but may act at later stages of the cell cycle or interfere with the viability of dividing cells. In our experimental conditions, antiestrogen blocked the estrogen cycle or interfere with the viability of dividing cells. In our work has suggested that the mechanism of this action might be more complex. Further investigations are needed to understand this mechanism.

In conclusion, our results show that the use of the HL60 cell line as an in vitro model for the study of estrogen receptors was also helpful in demonstrating a biological effect of hormones on cell growth at physiological concentrations (22). In this respect, the use of a serum-free culture could be of particular interest (2).

ACKNOWLEDGMENTS

We are grateful to Dr. R. Gallo of National Cancer Institute, Bethesda, Md., for providing line HL60. We thank Dr. G. A. Pangalis for helpful review of the manuscript and Ray Lefebvre for technical editing.

REFERENCES


Presence of Estrogen Binding Sites and Growth-stimulating Effect of Estradiol in the Human Myelogenous Cell Line HL60

Laurence Danel, Geneviève Cordier, Jean-Pierre Revillard, et al.