Steroid responsiveness of the human cell line NHIK 3025

Kjetill Østgaard1,3, Einar Wibe2 and Kristen B. Eik-Nes1

Institute of Biophysics1, NTH, University of Trondheim, N-7034 Trondheim-NTH, Norway
Department of Tissue Culture, Norsk Hydro's Institute for Cancer Research2,
The Norwegian Radium Hospital, Montebello, N-Oslo 3

Abstract. The human cell line NHIK 3025, derived from a carcinoma of the uterine cervix, contains a glucocorticoid and an androgen receptor. The effect of various natural and synthetic steroid hormones and antihormones on growth rate of these cells was therefore investigated.

Cells grown in Eagle's MEM with 10% foetal calf serum exhibited reduced growth when cultured with dexamethasone due to prolongation of the cell cycle. Glucocorticoid anti-inducers like progesterone had no significant effect on cell growth.

Methyltrienolone (R 1881) or 5α-dihydrotestosterone did not affect cell proliferation. The reported shortening of the cell cycle by testosterone is probably not directly connected with activation of the androgen receptor present, but possibly dependent on metabolic conversion of testosterone to the more potent growth stimulator 4-androstene-3β,17β-diol.

The effect of several anti-androgens was also studied. The non-steroidal anti-androgens flutamide and SCH 16483 had no significant effect on cell proliferation. It was, however, found that a number of steroid anti-androgens, including R 2956, stimulated cell growth. A significant stimulatory effect by R 2956 was seen within the first cell generation, 4-androstene-3β,17β-diol had to be present during 2 days, and testosteron for even longer times before a similar effect on cell growth could be obtained.

The following abbreviations and trivial names are used:
- Dexamethasone = 9α-fluoro-16α-methyl-11β,17,21-trihydroxy-1,4-pregnadiene-3,20-dione;
- Cortisol = 11β,17,21-trihydroxy-4-pregnen-3,20-dione;
- Progesterone = 4-pregnene-3,20-dione;
- T = testosterone = 17β-hydroxy-5α-androstan-3-one;
- DHT = 5α-dihydrotestosterone = 17β-hydroxy-5α-androstan-3-one;
- 3β,17β = 4-androstene-3β,17β-diol;
- 3α,17β = 4-androstene-3α,17β-diol;
- R 1881 = methyltrienolone = 17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one;
- R 2956 = 17β-hydroxy-2,2,17α-trimethyl-estra-4,9,11-trien-3-one;
- CPA = cyproterone acetate = 6α-chloro-17α-hydroxy-1α,2α-methylene-4,6-pregnadiene-3,20-dion-17-acetate;
- Cyanoketone = 2α-cyano-17β-hydroxy-4,17α-trimethyl-5-androsten-3-one;
- SCH 13521 = Flutamide = α,α,α-trifluoro-2-methyl-4'-nitro-m-propiono-toluidide;
- SCH 16483 = α,α,α-trifluoro-2-methyl-4'-nitro-m-lactotoluidide.

The human cell line NHIK 3025 was established in 1967 from an early stage of a carcinoma of the uterine cervix (Nordbye & Oftebro 1969; Oftebro & Nordbye 1969). Despite their origin, NHIK 3025 cells do not contain detectable receptors for oestradiol or progesterone (Mulder et al. 1978). They do,
however, have a corticoid and an androgen receptor system, in concentrations of 190 and 8 fmol/mg protein, respectively (Mulder et al. 1978).

Testosterone (T) stimulates growth of NHIK 3025, while 5α-dihydrotestosterone (DHT) has no measurable effect (Wibe et al. 1976). This is surprising since DHT has generally a higher receptor affinity than T in these cells (Mulder et al. 1978). Effects of T have, however, been found in several other and widely different DHT-insensitive systems (Powers & Florini 1975; Haug & Gautvik 1978). Of interest in this respect is androgen action observed in normal uterine and vaginal tissue which can not be attributed to DHT (Liao 1976).

Androgen metabolism in NHIK 3025 cells has been studied by Stenstad et al. (1977). DHT was extensively reduced to 5α-androstane-3α,17β-diol, and conversion from T to 4-androstene-3β,17β-diol (3β,17β), the most potent growth stimulator for these cells (Wibe et al. 1976), could be observed. Moreover, the cells also showed high capacity for oxidizing 3β,17β to T.

The subject of our current investigation was to examine the effect of various steroids on proliferation rate of NHIK 3025 cells, with emphasis on specificity and kinetics of the androgen growth stimulation observed.

Materials and Methods

**Materials**

Flutamide and SCH 16483 were donated by the Schering Corp. R 1881 and R 2955 were gifts from the Roussel UCLAF company. Other steroids were supplied by Steraloids Inc. It was necessary to purify 3β,17β by crystallization before use.

The cell culture medium was Eagle’s MEM (Gibco/Biocult) as described by Stenstad et al. (1977), supplied with 10% foetal calf serum (Gibco/Biocult).

**Methods**

Stock cultures of cells were grown as monolayers in Jena G-20 glass flasks, and all experiments performed with cells in the log phase, 2–4 days after replating (Wibe et al. 1976). Electron microscope examination showed no mycoplasma contamination (White 1978).

Cell growth was measured as published (Wibe et al. 1976). Approximately 250 cells were plated/Petri dish (Nunc) and incubated for 4 h at 37°C in order to let the cells attach. Damaged/non-attached cells were then washed off before addition of fresh medium with or without steroids. Further incubation lasted for 4 days.

After incubation, the cells were fixed and stained in situ with Böhmers haematoxylin. Mitotic cells, which are less adhesive, could possibly be lost during this procedure. However, even when this loss is optimized during mitotic selection, it does not exceed 1% of the total cell population (Østgaard et al. 1981). Microscopic inspection verified that cell detachment during staining was negligible, and a possible steroid effect on cell adhesivity thus not detectable.

Cells were found grouped in colonies, each colony originating from one of the ancestor cells originally plated. By counting in an inverted microscope the number of cells per colony in 40 randomly chosen colonies per Petri dish (5 parallel dishes in each group), the mean colony size could be estimated. This permitted even small changes in the cell cycle to be determined with significance. In addition, the total number of colonies per dish was also counted, and the total number of cells estimated as the product of mean colony size and mean number of colonies.

Under these conditions of experiment the mean generation time of control cultures varied from 24 to 26 h, with a corresponding mean colony size of 12–16 cells after 4 days of growth. In order to avoid counting errors any colony with less than 5 cells was neglected (Wibe et al. 1976). It should be noted that spontaneous mitotic ‘accidents’ leading to giant cell formation is generally observed in established cell lines of this type (White et al. 1980), giving a small fraction of colonies below this counting limit even after prolonged incubation periods.

Cells were synchronized by the method of mitotic selection (Pettersen et al. 1977). Cells growing exponentially in plastic culture flasks (Falcon, 75 cm²) were exposed to fresh medium with or without steroids and further incubated an appropriate number of days before mitotic selection. The medium containing the newly selected mitotic cells was transferred to new culture flasks (Falcon, 25 cm²). In each flask a group of approximately 100 cells was selected at random, and the time when each cell in this group reached metaphase was observed in an inverted phase contrast microscope. The median cell cycle time was calculated from the time needed for 50% of the cell population to reach metaphase (Østgaard et al. 1981). All operations were performed in a 37°C room.

**Results**

**Glucocorticoid inducers and anti-inducers**

Effects on cell growth with 10⁻⁷M steroids are summarized in Table 1. Dexamethasone inhibited cell growth significantly. This reduction was due to a prolongation of the cell cycle (Østgaard et al. 1981), resulting in smaller colonies. Fig. 1 shows that dexamethasone had to be present in concentrations above 10⁻⁸M before a significant inhibitory effect was observed.
Cell growth relative to control values after incubation for 4 days in the presence of steroids at a concentration of $10^{-7}$ M. Deviations given are based on standard error of the mean of steroid-treated and control cultures ($n = 5$). Mean generation time is indicated where colony size is significantly different from the control value according to Student’s t-test ($P < 0.05$).

The results of 2 independent experiments are given for each hormone.

<table>
<thead>
<tr>
<th>Steroid added</th>
<th>Colony size</th>
<th>Total No. of cells</th>
<th>Mean generation time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone</td>
<td>0.84 ± 0.02</td>
<td>0.81 ± 0.07</td>
<td>1.08</td>
</tr>
<tr>
<td></td>
<td>0.84 ± 0.03</td>
<td>0.62 ± 0.07</td>
<td>1.08</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.93 ± 0.04</td>
<td>0.95 ± 0.09</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td>0.90 ± 0.01</td>
<td>0.89 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.98 ± 0.03</td>
<td>1.01 ± 0.13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.98 ± 0.03</td>
<td>0.96 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>1.18 ± 0.04*</td>
<td>1.29 ± 0.09</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>1.19 ± 0.03</td>
<td>1.20 ± 0.13</td>
<td>0.95</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>1.02 ± 0.03*</td>
<td>1.00 ± 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.04 ± 0.04</td>
<td>1.03 ± 0.16</td>
<td></td>
</tr>
<tr>
<td>Methyltrienolone (R 1881)</td>
<td>1.05 ± 0.04</td>
<td>1.07 ± 0.12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.03 ± 0.03</td>
<td>1.06 ± 0.10</td>
<td></td>
</tr>
<tr>
<td>CPA</td>
<td>1.15 ± 0.03</td>
<td>1.30 ± 0.11</td>
<td>0.94</td>
</tr>
<tr>
<td></td>
<td>1.12 ± 0.04</td>
<td>1.28 ± 0.16</td>
<td>0.95</td>
</tr>
<tr>
<td>R 2956</td>
<td>1.13 ± 0.05</td>
<td>1.29 ± 0.10</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>1.18 ± 0.03</td>
<td>1.46 ± 0.15</td>
<td>0.93</td>
</tr>
<tr>
<td>Cyanoketone</td>
<td>1.21 ± 0.04</td>
<td>1.29 ± 0.08</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>1.12 ± 0.05</td>
<td>1.20 ± 0.14</td>
<td>0.96</td>
</tr>
<tr>
<td>Flutamide</td>
<td>1.00 ± 0.04</td>
<td>0.97 ± 0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00 ± 0.02</td>
<td>1.00 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>SCH 16483</td>
<td>1.05 ± 0.02</td>
<td>1.05 ± 0.07</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.00 ± 0.02</td>
<td>1.00 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

* Results published earlier (Wibe et al. 1976).

Cortisol also reduced cell growth (Table 1). No significant effect of progesterone was found in the concentration range $10^{-9}$ M to $10^{-7}$ M (Table 1, Fig. 1).

Table 1 indicates that both steroid inhibition and stimulation leads to greater changes in total cell number than in mean colony size. This is at least partly due to changes in the number of colonies too small to be counted (see Methods section) when the total distribution of colony sizes is changed. Steroid hormones does not seem to affect giant cell formation itself (Østgaard et al. 1981). The neglect of the smallest colonies leads thus possibly to a small overestimation of the steroid effect on the number of colonies, and a slight underestimation of the effect on mean colony size. As a precaution, calculation of statistical significance has been based on the latter parameter only.

Androgens and anti-androgens

The lack of growth response by the androgen methyltrienolone (R 1881) (Bonne & Raynaud 1975) is striking (Table 1), since R 1881 binds strongly to androgen receptors in the nuclear extract from these cells (Mulder et al. 1978). Specific association between this receptor and an androgen does not per se affect cell growth. This has also been observed with DHT with respect to no growth promotion (Wibe et al. 1976) and nuclear association (Mulder et al. 1978) in these cells.
Changes in cell growth by different anti-androgens are shown in Table 1. The surprising result is that all steroidal anti-androgens tried stimulated cell growth. This is in contrast to the highly specific effect found earlier where only T and 3β,17β were able to stimulate growth of the NHIK 3025 cells (Wibe et al. 1976; Stenstad et al. 1977). The potent non-steroidal anti-androgen flutamide, or its active metabolite SCH 16483 (Neri 1977), had, however, no significant growth effect on these cells (Table 1). The androgen R1881 has no measurable effect (Fig. 1) on cell growth in the 'physiological' range $10^{-9}M - 10^{-7}M$. However, its 2α,2β-dimethyl-derivative, the potent anti-androgen R 2956 (Azadian-Boulanger et al. 1974), stimulated cell growth at $10^{-8}M$ or higher concentrations.

**Time course of androgen reduction of cell cycle**
Different pre-incubation periods with steroids before selection and cell cycle estimation were tried in order to study the kinetics of growth stimulation. The optimal responses with 3β,17β and T are depicted in Fig. 2. When asynchronous cells are incubated with steroids for 4 days, 3β,17β leads to a greater augmentation of cell number than T (Wibe et al. 1976). It is seen in Fig. 3 that this is not due to a more pronounced reduction of the cell cycle by 3β,17β than by testosterone, but to a later response.
by the latter. In contrast to this very slow response by T and 3β,17β the effect of R 2956 on cell growth could be seen within the first generation after exposure (Fig. 3). On the other hand, the stimulatory effect of R 2956 is lost after 4 days of incubation (Fig. 3). This might be due to some kind of feedback inhibition regulation, since synthetic compounds like R 2956 are generally resistant to metabolism (Azadian-Boulanger et al. 1974).

Discussion

Specific steroid receptors have been found in non target cells (Jung-Testas et al. 1976) and it has been postulated that such receptors are ubiquitously expressed. The 3025 cells, originating from a tumour of the uterine cervix (Oftebro & Nordby 1969), have receptors for dexamethasone, T and DHT but not oestradiol-17β or progesterone (Mulder et al. 1978). Whether these cells ever had receptors for the latter two hormones is difficult to state, after 12 years growth in culture only the glucocorticosteroid and the androgen receptors have survived in measurable concentrations.

Like in the L 929 (Jung-Testas & Baulieu 1979) and MCF-7 cells (Lippman et al. 1976) dexamethasone inhibits cell growth (Table 1, Fig. 1). Progesterone is known as a potent anti-inducer on the glucocorticoid response mediated by binding to the glucocorticoid receptor (King & Mainwaring 1974). Since NHK 3025 lacks a specific progesterone receptor (Mulder et al. 1978), only the possible anti-inducer effect should be evident in our experiments. No significant effect was observed (Table 1, Fig. 1) at concentrations up to $10^{-7}$M. Considering the limited affinity of progesterone for the glucocorticoid receptor compared to dexamethasone (Lippman et al. 1976) this concentration is possibly too low for an optimal effect. The inhibitory effect

![Percentage of synchronized cells having entered metaphase, plotted as a function of time after mitotic selection; ○: control; ●: $10^{-7}$M testosterone present for 4 days before selection and throughout the experiment; ■: $10^{-7}$M 4-androstene-3β,17β-diol present for 4 days before selection and throughout the experiment.](image-url)
of dexamethasone on the 3025 cells (Østgaard 1979) is caused by a change of the cell cycle (Østgaard et al. 1981). With the relatively high concentration of dexamethasone receptor material in these cells (Mulder et al. 1978) and the lack of anti-inducer effect of progesterone, it is likely that the observed growth inhibition by dexamethasone is promoted by the dexamethasone receptor.

Since receptor association with DHT or R1881 exists in the 3025 cells (Mulder et al. 1978) and these compounds have no measurable effects on cell proliferation (Table 1), the question must be raised whether the recorded T or 3β,17β stimulated growth response (Wibe et al. 1976; Stenstad et al. 1977) is mediated by the same system as the anti-androgen stimulation seen in current work (Table 1). 3β,17β had androgen activity in vivo (Verjans & Eik-Nes 1977) possibly via conversion to T. However, DHT-stimulated growth in vitro of Shionogi 115 had been observed by Smith & King (1972), while 3β,17β was inhibitory for this function. If it is assumed that the transformation of 3β,17β to T is not predominant in that system, one possible explanation is that 3β,17β per se or via some unknown metabolite has anti-androgen activity. Of interest in this respect is the observation that 3β,17β will not depress circulating LH in castrated rats at doses where such depression was achieved with T (Verjans & Eik-Nes 1977). Stenstad et al. (1977) recorded that 3β,17β was active in lower concentrations than T with respect to growth of the 3025 cells. Further the isomer 4-androstene-3α,17β-diol had no significant effect on this function although it was more effectively converted to T by these cells. It was therefore suggested that the recorded effect of T on the growth of this cell line was mediated by a small conversion to 3β,17β. The slow effect of T on cell proliferation (Fig. 3) could therefore be due to intracellular accumulation of 3β,17β formed from T.

Steroid action mediated by activation of a steroid-receptor complex has been observed within an hour after hormonal exposure (King & Mainwar- ing 1974). Although growth stimulation of cells is a late type of hormonal response, an effect of T on proliferation of asynchronous Shionogi 115 was seen within 12 h reaching a maximum at 20 h (King et al. 1976). Compared to these data, the effect of 3β,17β on proliferation of NH1K 3025 cells is slow with a lag period of at least 24 h (Fig. 3).
This raises the question whether steroid metabolism of slow rates is obligatory for the processes leading to growth stimulation by $3\beta, 17\beta$

The recorded effect of T or of $3\beta, 17\beta$ in physiological concentrations ($10^{-10}$M to $10^{-7}$M) on growth of the 3025 cells is of borderline nature (Wibe et al. 1976; Stenstad et al. 1977) but highly dependent on the structure of these compounds. The recorded anti-androgen growth stimulation (Table 1) is, on the other hand, apparently not steroid structure specific. The culture medium contains endogenous steroids, including approximately $0.7 \cdot 10^{-9}$M testosterone (Østgaard et al. 1981), that could be of importance for this function. Experiments employing dextran charcoal extracted serum (Østgaard 1979) do indicate that the anti-androgen response is lost when endogenous steroids are removed. Also, with such media a small growth stimulation from exogenous T could be observed. Finally in such media the NHK 3025 cells will continue growing for more than 30 generations with augmented doubling time, indicating that these cells are not dependent on serum androgens for survival and proliferation. The reported growth stimulation by exogenous T or $3\beta, 17\beta$ should therefore be considered as only additive.

The association between specific steroid receptors and changes in cell proliferation may be argued in light of published data from experiments on L 929 cells (Jung-Testas & Baulieu 1979) and cells from the RBA or the NMU lines (Vignon et al. 1979). In spite of a high concentration of androgen receptors in the latter cells, it was difficult to measure androgen effects on cell proliferation. A modest growth response by DHT could be determined in L 929 (Jung-Testas & Baulieu 1979). These cells, as the NHK 3025 cells, showed receptor association with R 1881 but the compound was ineffective with regard to cell proliferation. Such information raises the question whether the receptors in question are vestigial and can no longer convey steroid function or the technique of growing cells in culture is inadequate for determining borderline effects.

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