out at a wavelength of 514 nm and maximum power for 50 iterations of a box representing 20% of the nuclear volume. A time-interval mode of Time Series was used with the time interval varied in different groups of experiments according to the mobility of the protein: 500 μsec was set for the Ad4BP/SF-1-H89 group (Fig. 8A), 2 sec was for the DAX-1-Ad4BP/SF-1-H89 group (Fig. 8B), and 600 μsec for the DAX-1-Ad4BP/SF-1-FK group (Fig. 8C). The fluorescence intensities of the region of interest were obtained using LSM software (version 3.0), and the data were analyzed using Microsoft Excel. The fluorescence recovery is usually incomplete, probably because attenuation of fluorescence occurs during the serial scanning and also the total amount of fluorescence protein decreases, as around 20% of them have been bleached. In addition, the fluorescent intensity after bleaching is not always the same. Therefore we normalized the raw FRAP data (both intensity of each time point and the time) by the method described by Stenoien *et al.* (51). Briefly, intensity values were normalized using the equation: $It = (Xt - Y)/(Z - Y)$, where I is the intensity at time t, X is the intensity at time t, Y is the intensity immediately after the photobleach (where t is equal to 0), and Z is the intensity at the final time point. This sets the initial postbleach intensity (at $t = 0$ sec) to 0 and the final intensity to 1 using arbitrary units. The normalized intensity values were averaged and plotted against time to make the recovery curve. The $t_{1/2}$ value can be observed from the graph as the time at which the normalized intensity reaches 0.5 arbitrary units. A subgroup of FRAPed cells was traced by initially being seeded on grid-carved glass-bottom dishes (code no. 3920-035, IWAKI, Chiba, Japan), and was subsequently subjected to quantitative immunofluorescence staining to ensure that cells selected for FRAP study also overexpressed both YFP-Ad4BP/SF-1 and pRc/RSV-DAX-1 in a reasonable range as described above.

Statistics

One-way ANOVA followed by Scheffe's test was used for multigroup comparisons.

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Address all correspondence and requests for reprints to: Toshihiko Yanase, M.D., Ph.D., Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan. E-mail: yanase@intmed3.med.kyushu-u.ac.jp.

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SF-1/Ad4BP transforms primary long-term cultured bone marrow cells into ACTH-responsive steroidogenic cells

Shigeki Gondo, Toshihiko Yanase, Taijiro Okabe, Tomoko Tanaka, Hidetaka Morinaga, Masatoshi Nomura, Kiminobu Goto and Hajime Nawata*

Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka, 812-8582, Japan

Bone marrow stem cells develop into haematopoietic and mesenchymal lineages, but have not been known to participate in steroidogenic cell production. Steroidogenic factor 1 (SF-1), also designated adrenal 4 binding protein (Ad4BP), is an essential orphan nuclear receptor for steroidogenesis as well as for adrenal and gonadal gland development. In the present study, we revealed that the adenovirus-mediated forced expression of SF-1 can transform cultured primary long-term cultured bone marrow cells into steroidogenic cells, showing the *de novo* synthesis of multiple steroid hormones in response to adrenocorticotropic hormone (ACTH). This finding may provide an initial step in innovative autograft cell transfer therapy for steroid hormone deficiencies.

Introduction

Steroidogenic factor 1/adrenal 4 binding protein (SF-1/Ad4BP), formally designated NR5A1, was originally identified as a steroidogenic tissue-specific transcription factor for most steroidogenic genes (Omura & Morohashi 1995; Parker & Schimmer 1997), belonging structurally to the nuclear receptor superfamily. SF-1 is essential for steroidogenesis (Fig. 1) and steroidogenic tissue development, since the disruption of mouse SF-1 caused a lack of adrenal and gonadal development (Ingraham *et al.* 1994; Luo *et al.* 1994; Morohashi & Omura 1996). In embryonic stem cells (ESCs), stable SF-1 expression results in a steroidogenic capacity and the adenosine 3, 5-cyclic monophosphate (cAMP) or retinoic acid-dependent inducibility of cytochrome P450scc, leading to progesterone production (Crawford *et al.* 1997). However, this steroidogenic capacity was restricted to the stage of progesterone synthesis, and did not occur as *de novo* synthesis because the addition of an exogenous substrate, 20α-hydroxycholesterol, which bypasses the mitochondrial outer membranes, was required for progesterone production. Nevertheless, these results clearly indicate that SF-1 is a key factor in steroidogenic cell differentiation.

A number of recent studies have suggested that upon transplantation into different tissues bone marrow cells (BMCs) might contribute to the regeneration of haematopoietic or mesenchymal lineages in multiple organs (Petersen *et al.* 1999; Brazelton *et al.* 2000; Lagasse *et al.* 2000; Mezey *et al.* 2000; Orlic *et al.* 2001). Although these potentials might occur partly because of spontaneous cell fusion with recipient cells (Terada *et al.* 2002; Ying *et al.* 2002), BMCs surely contain pluripotent progenitor cells which differentiate into multiple lineages. Bone marrow stem cells have not been shown to participate in steroidogenic cell production. We therefore tested whether the introduction of SF-1 into BMCs produce steroidogenic cells.

Result

De novo synthesis of multiple steroid hormones from primary long-term cultured BMCs infected with Adx-bSF-1

We prepared an adenovirus construct containing bovine SF-1 (bSF-1), Adx-bSF-1. It became apparent from the results of this experiment that long-term cultured BMCs infected with Adx-bSF-1 could produce a significant amount of multiple steroids. Long-term (123 days) cultured BMCs from male GFP mice were infected with Adx-bSF-1 or Adx-LacZ as a control, and incubated for 7 days. The steroid contents in the medium accumulated

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**Correspondence*: E-mail: nawata@intmed3.med.kyushu-u.ac.jp

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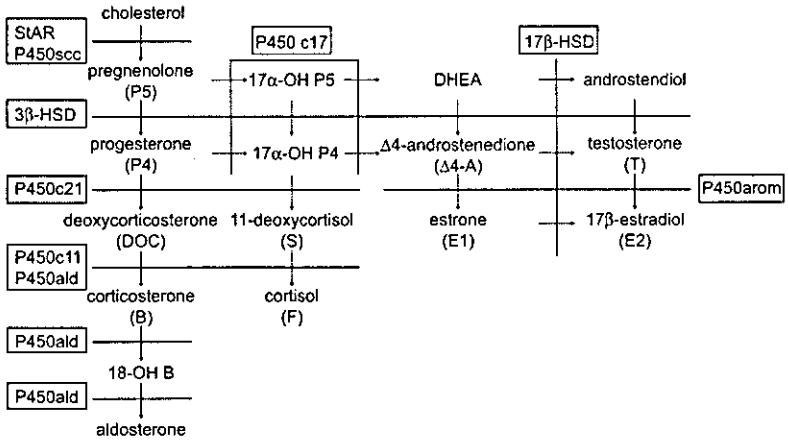

Figure 1 Steroidogenic pathways. StAR, P450scc and 3β-HSD, are expressed in both the adrenals and gonads of humans and mice. P450c21, P450c11 and P450ald are exclusively expressed in the adrenals, but not the gonads of both humans and mice. P450c17 is expressed in both the adrenals and gonads of humans, but in only the gonads of mice. 17β-HSD type 3 in mice and in humans are mostly expressed in the testis.

for the next 4 days were then measured. The BMCs infected with Adx-bSF-1 produced a significant amount of progesterone (P4), deoxycorticosterone (DOC), corticosterone (B), 17α-hydroxyprogesterone (17α-OH P4), 11-deoxycortisol (S), dehydroepiandrosterone (DHEA), Δ4-androstenedione (Δ4-A) and testosterone (T), while those infected with Adx-LacZ did not (Fig. 2A). All of the steroid contents in the medium from the control cells infected with Adx-LacZ were undetectable except S. The slight detection of S in the control medium is probably due to the cross reactivity (9.5%) of the antibodies against S with hydrocortisone in the cultured medium, considering no significant production of precursor steroids

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR) of the cells used in the above mentioned experiment revealed steroidogenic acute regulatory protein (StAR), P450scc, 3β-hydroxysteroid dehydrogenase (3β-HSD), P450c11, P450c17 and 17β-HSD type 3 mRNA expressions in the BMCs on day 11 after infection with Adx-bSF-1, but not after infection with Adx-LacZ (Fig. 2B). However, we could not prove the expression of P450ald mRNA. Adrenocorticotropic hormone receptor (ACTH-R) was expressed even in cells infected with Adx-LacZ, though the expression level was very low (2/1000 the expression in the adrenal glands), whereas it rather decreased with Adx-bSF-1 infection (Fig. 2B). Tissue-specific alternative promoter and 5′-untranslated exon usage of mouse ACTH-R gene has been reported. Namely, mouse adipose tissue exclusively contains an exon 1f, located 1.4 Kb downstream in the genome of previously reported exon 1 (exon 1a) transcribed in the mouse adrenal gland. The promoter region flanking to exon 1f does not contain SF-1 binding sites (Kubo *et al.* 2004), while adrenal-specific promoter contains multiple SF-1 sites and is surely responsive to SF-1 (Cammas *et al.* 1997). RT-PCR of BMCs revealed a predominant expression of adipose-tissue type exon 1f (data not shown), which may explain well the reason why mouse BMCs are unresponsive to SF-1.

We also observed a quite similar steroid profile of BMCs from 129SVJ mice (data not shown), suggesting little strain difference in steroidogenic capacity of BMCs.

An immunocytochemical study of cultured BMCs from 129SVJ mice confirmed the actual expression of P450scc using a specific antibody against cytochrome P450scc (Fig. 2C). The cells did not react with preimmune serum as a negative control (data not shown).

Characterization of the cell lineage of steroidogenic BMCs

The flowcytometry experiment revealed surface markers of the above mentioned steroidogenic BMCs (Fig. 3); that is, it revealed the negative expressions of CD45, which is specific to haematopoietic cells. The monocyte/macrophage marker, CD11b was also negative. Although the character of mouse mesenchymal stem cell has not been fully clarified and controversial (Jiang *et al.* 2002; Sun *et al.* 2003), one of such potential markers, CD44 was negative in our BMCs. In addition, the experiment revealed the positive expression of c-kit and Sca-1, which are haematopoietic and mesenchymal stem/progenitor markers. These results suggest that steroid-producing cells originate from multipotent and immature stem cells.

Steroidogenic responsiveness to ACTH of primary cultured BMCs infected with Adx-bSF-1

Since ACTH-R expression has been confirmed, we next tested the responsiveness of BMCs to ACTH by measuring

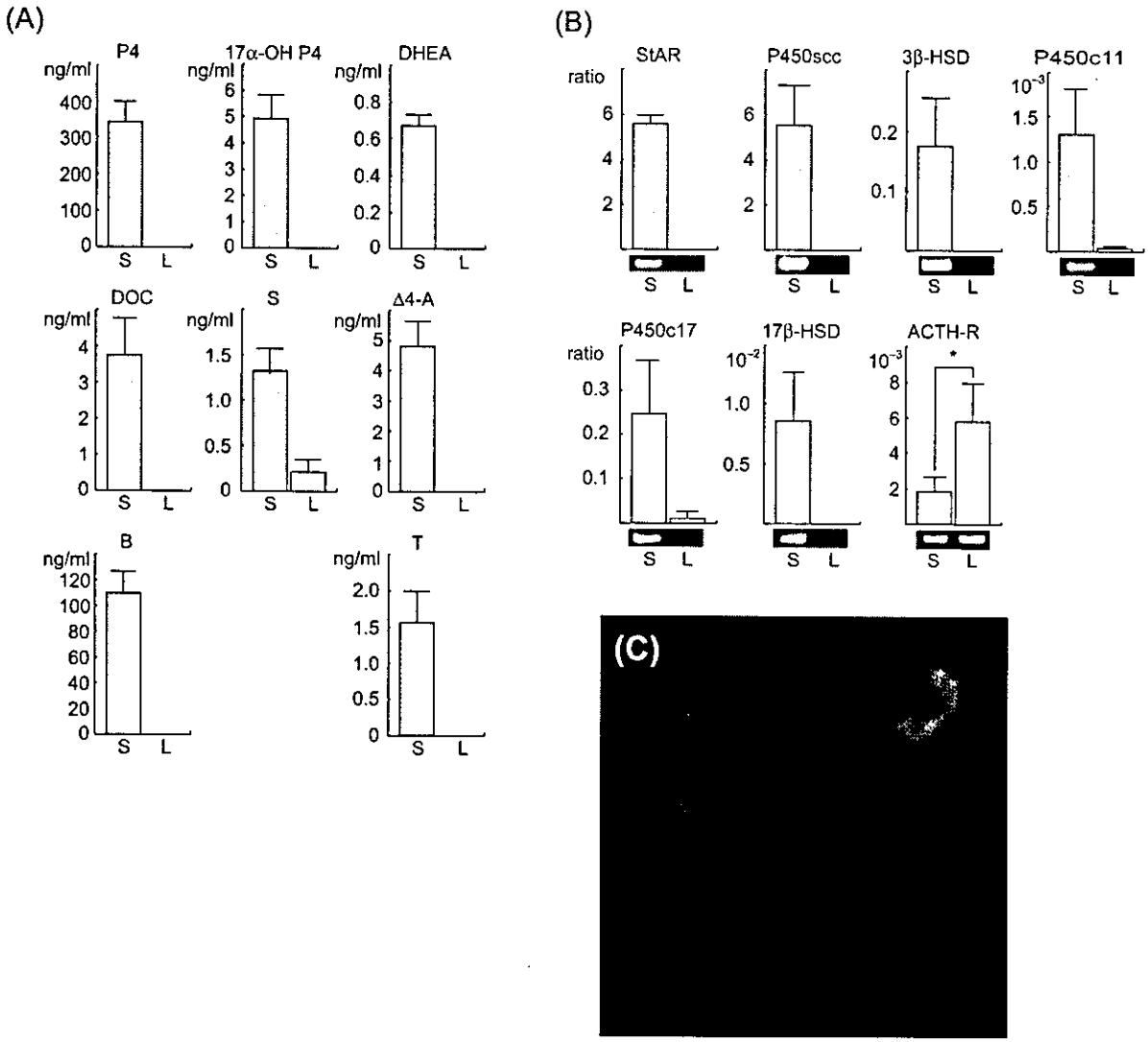

Figure 2 Character of BMCs infected with Adx-bSF-1. (A) Basal secretion of progesterone (P4), deoxycorticosterone (DOC), corticosterone (B), 17α-hydroxyprogesterone (17α-OHP4), 11-deoxycortisol (S), dehydroepiandrosterone (DHEA), Δ4-androstenedione (Δ4-A) and testosterone (T) in the medium of the long-term cultured BMCs from GFP mice. The cells were infected with Adx-bSF-1 or Adx-LacZ as a control and cultured for 7 days. The steroid contents in the medium accumulated for next 4 days were then measured. Values represent the mean ± SD ($n = 3$). The steroid contents in the medium from the control cells were undetectable except 11-deoxycortisol (S). S and L indicates BMCs transfected with Adx-bSF-1, and BMCs transfected with Adx-LacZ, respectively. (B) Real-time PCR of StAR, P450scc, 3β-HSD, P450c11, P450c17, 17β-HSD type 3 and ACTH-R. Relative mRNA expression levels were calibrated to β-actin. A relative ratio to the expression of the control Y-1 cells is expressed in the cases of StAR, P450scc and 3β-HSD; to that of the mouse adrenal is expressed in the cases of P450c11 and ACTH-R; and to that of the mouse testis is expressed in the cases of P450c17 and 17β-HSD type3. S and L indicates BMCs transfected with Adx-bSF-1, and BMCs transfected with Adx-LacZ, respectively. No significant PCR products of StAR, P450scc, 3β-HSD, P450c11, P450c17 and 17β-HSD type 3 were obtained from control cells infected with Adx-LacZ. Values represent the mean ± SD ($n = 3$). *Indicates $P < 0.05$. The actual specific PCR bands amplified by more than 40 cycles on ethidium bromide stained-agarose gel were shown as lower figures. (C) Immunocytochemical study of the BMCs from 129SVJ mice with an antibody against anti-cytochrome P450scc. Green fluorescent cells were positive for P450scc (×200).

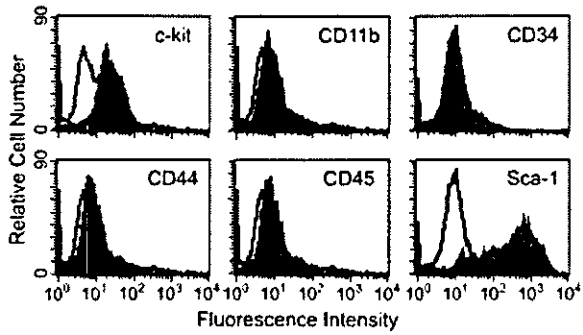

Figure 3 Flowcytometric analysis of surface marker expression in the cultured BMCs. The flowcytometry experiment was done before the infection with adenovirus using the same BMCs used in the experiment in Fig. 2A.

P4 and DOC secretion. We infected long-term (100 days) cultured BMCs from GFP mice with Adx-bSF-1 or Adx-LacZ, and then at 3–4 day intervals after infection they were stimulated with 2.4 nM–2.4 μM ACTH. After each stimulation the medium was collected for measurement of steroid content. ACTH stimulated the production of these steroids in a dose-dependent manner from the BMCs infected with Adx-bSF-1 (Fig. 4A), but not in the cells infected with Adx-LacZ (data not shown). These findings indicate that the introduction of bSF-1 into long-term cultured BMCs leads to transformation into steroidogenic cells, which in a basal state as well as in response to ACTH are capable of producing multiple steroid hormones. The induction of the mRNAs of steroidogenic enzymes, namely, P450scc, 3β-HSD, P450c21, P450c11 and 17β-HSD, by treatment with 2.4 μM

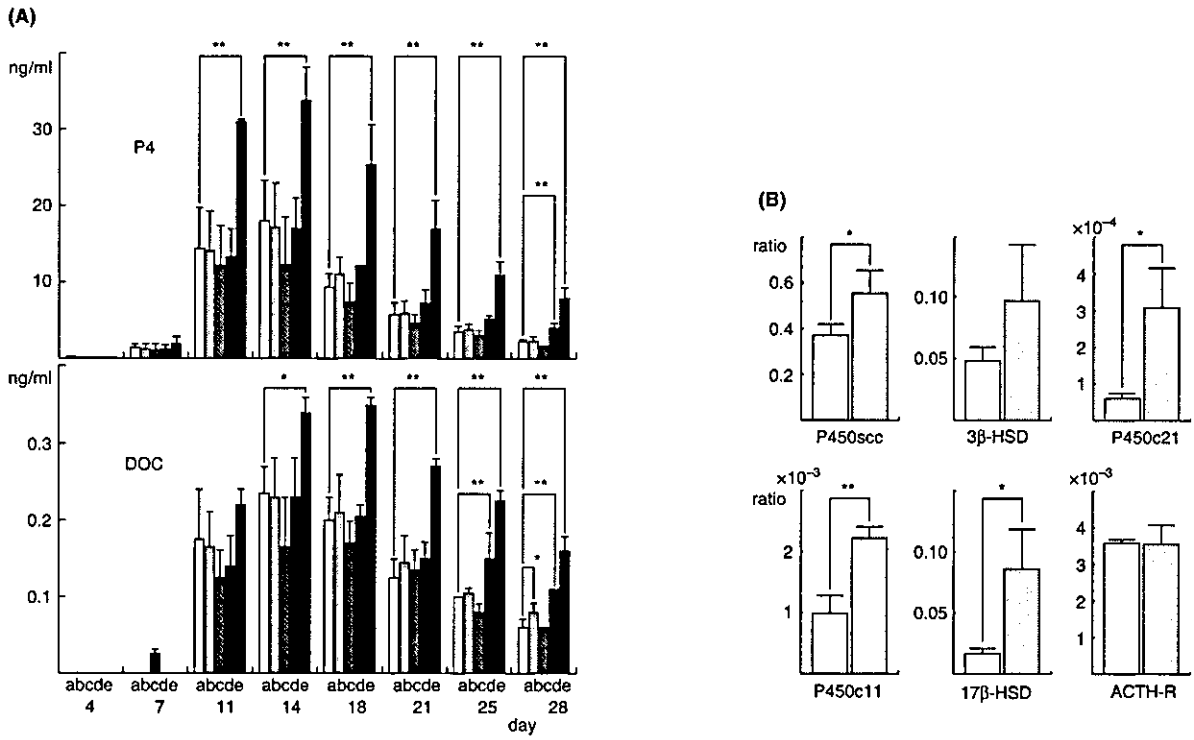

Figure 4 ACTH responsibility. (A) Effect of ACTH on the secretion of progesterone (P4) and DOC from cultured BMCs prepared from GFP mice. After infection with Adx-bSF-1 or Adx-LacZ (day 0), cells were treated with 2.4 nM to 2.4 μM ACTH on days 0, 4, 7, 11, 14, 18, 21, 25 and 28. Before the addition of ACTH, the medium was collected and the steroid concentration was measured. Values represent the mean ± SD (n = 3); a, b, c, d and e indicate 0, 2.4, 24, 240 nM and 2.4 μM ACTH, respectively. $*P < 0.05$, $**P < 0.01$ vs. control (absence of ACTH). (B) Real-time PCR of P450scc, 3β-HSD, P450c21, P450c11, 17β-HSDtype 3 and ACTH-R in the presence (▨) or absence (□) of 2.4 μM ACTH. After infection with Adx-bSF-1 (day 0), BMCs were treated with 2.4 μM ACTH on day 0, 4 and 7 and cultured for 4 days. On day 11, total RNAs of the cells were extracted and real-time PCRs were performed. Values represent the mean ± SD (n = 3). Relative mRNA expression levels were calibrated to β-actin. A relative ratio to the expression of the control Y-1 cells is expressed in the cases of P450scc and 3β-HSD; to that of the mouse adrenal is expressed in the cases of P450c21, P450c11 and ACTH-R; and to that of the mouse testis is expressed in the cases of 17β-HSD type3. $*P < 0.05$, $**P < 0.01$ vs. control (absence of ACTH).

ACTH for 4 days was also confirmed by real-time PCR (Fig. 4B). The unique induction of 17β-HSD type 3 mRNA by ACTH might support the direct stimulation of testosterone production by ACTH in foetal and neonatal mouse testis (O'shaughnessy *et al.* 2003).

Long-term steroidogenesis of primary cultured BMCs infected with Adx-bSF-1

Finally, we tested how long steroid production lasts in transformed BMCs after Adx-bSF-1 infection. We infected long-term (180 days) cultured BMCs with Adx-bSF-1 or Adx-LacZ, and then measured P4 and DOC levels in the cultured medium every 3–4 days. We observed a significant production of P4 and DOC until at least 112 days (Fig. 5A). Considering the relatively short half-life of adenovirus (2–3 weeks), this long-term steroid production is unexpected. It is reasonable to speculate the possible induction of endogenous SF-1 during culture, which might contribute to continuous long-term steroid production. However, this is unlikely because we could not prove the induction of endogenous mouse SF-1 expression during the experimental period (until 49 days after the infection) by RT-PCR, but we could detect adenovirus-induced bSF-1 expression (Fig. 5B).

Discussion

Steroidogenic properties of the cultured BMCs infected with Adx-bSF-1

Mouse adrenal produces mineralocorticoids like DOC and B because of the adrenal-specific expression of P450c11, but does not produce C19 steroids like DHEA and sex steroids because of the lack of P450c17 expression in this tissue. On the other hand, mouse gonad (testis or ovary) possesses the reverse expression profile of the above two enzymes, namely gonad-specific P450c17 expression and the absent expression of P450c11, resulting in the predominant production of C19 steroids without any productions of mineralocorticoids. In the present study, we demonstrated, for the first time, that BMCs could differentiate into steroidogenic cells in response to ACTH under condition of adenovirus-mediated forced expression of SF-1. BMCs originally expressed ACTH receptor which was a type transcribed in adipose tissue but not in adenal gland. The steroid profile of the cultured BMCs showed a mixed pattern of mouse adrenal and gonadal steroidogenesis, namely the simultaneous productions of DOC, B, DHEA, Δ4-A and T. Interestingly, P450c17 is expressed in human adrenal but not in mouse adrenal, thus the significant expression

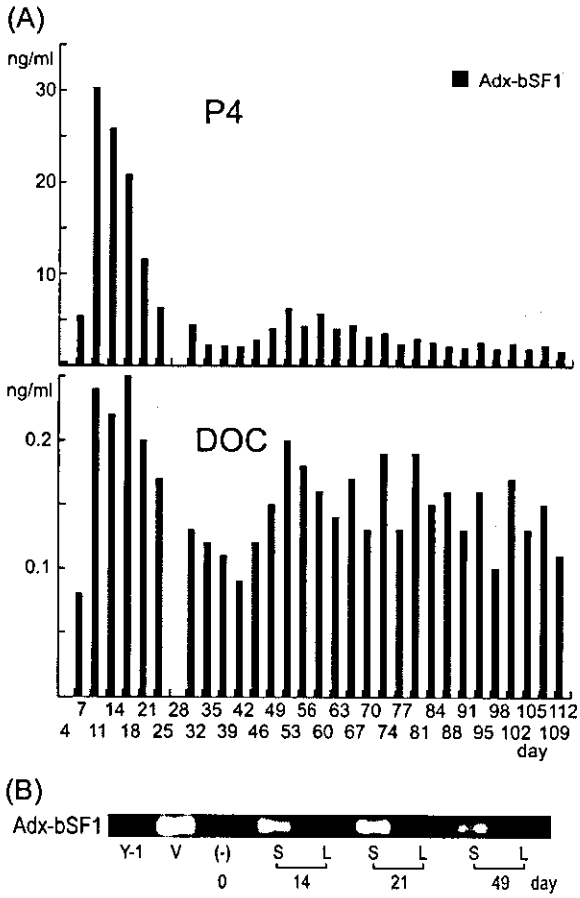

Figure 5 Long-term steroidogenesis. (A) Time course of basal progesterone (P4) and deoxycorticosterone (DOC) secretions in the medium of the long-term cultured BMCs from GFP mice. Cells were transfected with Adx-bSF-1 or Adx-LacZ and cultured as described in methods. Values represent the mean values of duplicate dishes. The black column indicates the steroids secreted from the cells infected with Adx-bSF-1. The secretions of P4 and DOC were undetectable in the medium from the BMCs infected with Adx-LacZ (data not shown). (B) Expression of Adx-bSF-1-derived bSF-1 in agarose gel. RT-PCR of bSF-1 was performed using RNA extracted from the cells obtained on days 0, 14, 21 and 49 in Fig. 4A. Electrophoresis was conducted on 1.5% agarose gel, and ethidium bromide was used for staining. Y-1, V (-), S and L indicate the Y-1 cells (negative control), Adx-bSF-1 (positive control), BMCs before infection, BMCs transfected with Adx-bSF-1, and BMCs transfected with Adx-LacZ, respectively.

of P450c17 in the BMCs and the significant production of 17α-hydroxylated steroid, S may also suggest a mixed steroid profile beyond species. These findings might suggest the multipotency of BMCs during steroidogenic cell differentiation and the possibility of a common origin for steroidogenic tissues, namely, stem cells. Although little

is known about the origin of these stem cells, a previous study on the expression profile of SF-1 indicated that the undifferentiated adrenal cortex and gonads of early stage fetuses originate from common adreno-genital primordium (Hatano *et al.* 1996).

In the long term culture, a significant production of P4 and DOC was surprisingly observed until at least 112 days. Interestingly, there was a relatively continuous production of DOC in contrast to a sharp decline in P4 production from days 18 to 25 (Fig. 5A). We need to clarify in the future whether this time-dependent change in the steroid profile might reflect a BMC differentiation process or just reflect a phenomenon due to a difference of the steroidogenic enzyme stability. Although the half-life of adenovirus is 2–3 weeks and we could not detect the induction of endogenous mouse SF-1, steroidogenesis lasted much longer than we expected. The continuous bSF-1 expression by adenovirus infection, even if at a low level, might be enough to maintain BMCs for long-term multiple steroid production. Another possibility is that SF-1 expression may be indispensable for the initiation of steroidogenesis by inducing steroidogenic enzymes, but may not be so critical for its maintenance.

The multipotency of BMCs to differentiate into either adrenal or gonadal steroidogenic cells might provide an important model for the future clarification of the mechanisms of tissue-, zone- or cell-specific adrenal and gonadal steroidogenic cell differentiation. An additional undifferentiated zone between the zona glomerulosa and zona fasciculate has been suggested as an adrenocortical stem cell zone which expresses SF-1 (Mitani *et al.* 2003). The presence of intra-adrenal stem cell has been also suggested from the success of xenotransplanted adrenocortical tissue formation from clonal or immortalized bovine adrenocortical cells in immunodeficient mice (Thomas *et al.* 1997, 2000). One plausible speculation might be that bone marrow-derived stem cells settle in the adrenocortical stem cell zone, where SF-1 expression might become possible.

Cell origin of steroidogenic BMCs

In the present study, we expanded a relatively purified BMC population by culturing the BMCs for 120–180 days (over 12–18 passages) and then the cells were subjected to the experiment to investigate steroidogenic property after infection with Adx-bSF-1. Although the BMCs in our experiment still constitute a heterogeneous population, the analysis of the cell surface markers highly suggested a possibility that the steroid-producing cells originate from multipotent and immature stem cells. Importantly, the long-term cultured BMCs differentiated into an osteoblastic phenotype by treatment with 0.05 mM ascorbic acid, 10 mM β-glycerophosphate and 0.1 μM dexamethasone (Pittenger *et al.* 1999) as shown by alkaline phosphatase staining (data not shown), suggesting that the character of the steroidogenic cells may be much closer to mesenchymal BMC lineages (Pittenger *et al.* 1999). However, the exact origin of these stroidogenic cells remains unclear and warrants further investigation.

Therapeutic potential for patients with steroid insufficiency

Steroid hormone replacement therapy has become well established and provides many benefits to patients with adrenal insufficiency or hypogonadism. However, such treatments must be continued throughout an individual's lifetime, especially in cases of adrenal insufficiency. Therefore, alternative therapies such as gene therapy or *ex vivo* steroidogenic cell transplantation might be beneficial. In addition to the above-mentioned successful reports of xenotransplantation of adrenocortical cells (Thomas *et al.* 1997, 2000), the feasibility of gene therapy for congenital adrenal hyperplasia has been demonstrated. Namely, a single intra-adrenal injection of an adenoviral vector encoding CYP21 has been shown to compensate for the biochemical and endocrine alterations in 21-hydroxylase deficient mice (Tajima *et al.* 1999). Although more extensive studies are required, autograft cell transplantation of the SF-1-induced steroidogenic BMCs presented in this study may provide another therapeutic possibility for adrenal insufficiency. To determine their applicability in therapeutic treatments, the limits and biological importance of these BM-derived steroidogenic cells need to be assessed.

Experimental procedures

Construction of the adenovirus vector

We prepared a recombinant adenovirus vector derived from the human type 5-adenovirus using a commercially available Adenovirus Expression Vector Kit (Takara, Osaka, Japan). Bovine SF-1/Ad4BP cDNA (Honda *et al.* 1993) was provided by Prof K Morohashi (National Institute for Basic Biology, Okazaki, Japan), restricted by *Bam*HI and *Eco*RI, blunt-ended, and inserted into the *Sma*I site of the recombinant cosmid vector, pAxCAwt (Takara), which contains a CAG promoter. The recombinant SF-1 adenovirus (Adx-bSF-1) was obtained according to the manufacturer's protocol based on an original report (Miyake *et al.* 1996).

Long-term bone marrow culture and adenovirus treatment

We obtained bone marrow cells (BMCs) from a 3-month old male B6-GFP (green fluorescence protein) mouse, C57BL/6Tg14

(act-EGFP) osbY01, donated by Dr Yamada (Kyoto University, Kyoto, Japan). In some experiments, we prepared BMCs from a 4 month-old male 129SVJ mouse. We cultured the BMCs according to a previous method (Dexter *et al.* 1977), with some modifications. Briefly, fresh whole bone marrow was harvested by flushing the bones from mice in medium A. Medium A includes α-MEM containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.0125 μg/mL, Amphotericin B (Sigma-aldrich, Irvine, UK), 10^{-7} M hydrocortisone (Nikkenkayaku, Japan), and 20% donor horse serum (Lot 6603F or 7307F, ICN Biochemicals, Aurora, OH, USA). We seeded the harvested cells in 75 cm^2 tissue culture flasks (Nalge Nunc, Rochester, NY, USA), incubated them at 37 °C in 5% CO_2 in air with medium A. Only the adherent cells were maintained for several weeks, trypsinized and stored in a cell banker at −80 °C until use. When needed, we cultured the stored BMCs for 120–180 days (over 12–18 passages) with medium A, aiming at the expansion of a relatively purified cell population. From this cell population, 5×10^5 BMCs were seeded again in 60 mm dish (Nunc) with medium A and when the BMCs became subconfluent, the cells were infected with adenovirus at approximately 10 plaque-forming units/cell. As a control for all experiments, we infected the BMCs with recombinant adenovirus expressing β-galactosidase (Adx-LacZ). After the infection, the cells were also maintained with medium A.

Measurements of the steroid content in the medium secreted from the BMCs

We measured the progesterone (P4), deoxycorticosterone (DOC), corticosterone (B), 17α-hydroxyprogesterone (17α-OHP4), 11-deoxycortisol (S), dehydroepiandrosterone (DHEA), Δ4-androstenedione (Δ4-A) and testosterone (T) contents secreted into the culture medium with the collaboration of SRL Co. Ltd. (Tokyo, Japan) using commercial RIA kits (Diagnostic Products Corp., LA, USA) and respective specific RIA systems developed by SRL (Den *et al.* 1978). The secretions of P4 and DOC in the medium were also confirmed in the presence or absence of synthetic 1–24 ACTH (Shionogi Co., Osaka, Japan). The detection limits of P4, DOC, B, 17α-OHP4, S, DHEA, Δ4-A and T were less than 0.1 ng/mL, 0.02 ng/mL, 20.0 ng/mL, 0.1 ng/mL, 0.04 ng/mL, 0.2 ng/mL, 0.1 ng/mL and 0.05 ng/mL, respectively.

Quantitative real-time PCR

We performed quantitative analysis of the mRNA expressions of StAR, ACTH receptor (ACTH-R) and various steroidogenic enzymes including P450scc, P450c17, P450C11, P450C21, P450ald, 3β-HSD, and 17β-HSD type 3 by real-time PCR using a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) as previously described (Mukasa *et al.* 2003). We isolated the total RNA from the cultured BMCs and Y-1 cells using a RNeasy mini kit (Qiagen), and from mouse testis and adrenals using Isogen (Wako Pure Chemical Industries, Osaka Japan). We synthesized first-strand complimentary DNA using 5 μg of total RNA as a template and carried out PCR in a LightCycler according to the manufacturer's instructions. The sense/anti-sense primers used were previously reported (Ilgar *et al.* 1997; Jennifer & Holly 2002; Mukai *et al.* 2002; Kubo *et al.* 2004): StAR 5′-TAG CTG AAG ATG GAC AGA CTT GC-3′/5′-GAC CTT GAA AGG CTC AGG AAG AAC-3′; P450scc, 5′-AAC TTG AAG GTA CAG GAG ATG CTG C-3′/5′-CAT CAG GAT GAG GCT GAA CTT GGT C; 3β-HSD, 5′-CAG ACC ATC CTA GAT GT-3′/5′-AGG AAG CTC ACA GTT TCC A-3′; P450c21, 5′-CTT CAC GAC TGT GTC CAG GAC TTG-3′/5′-CAG CAG AGT GAA GGC CTG CAG CAG-3′; P450c11, 5′-AAG AAA ACT TAG AGT CCT GGG ATT-3′/5′-GTG TCA GTG CTT CCA GCA ATG AGT-3′; P450ald, 5′-AAG AAC ATT TCG ATG CCT GGG ATG-3′/5′-GTG TCA ACG CTC CCA GCG GTG AGC-3′; 17β-HSD, 5′-CAT TTG AGT TGG CCA GAC ATG G-3′/5′-GGA GCA TTC CAA CGT TGT TGA C-3′; ACTH-receptor, 5′-CCA AGG AGA GGA GCA TTA TTG G-3′/5′-CAG GAC AAT CGG AGT TAT TTC TTG CGG-3′; ACTH-R 1a, 5′-CAG TCA TCT TGC CGA GAA AG-3′/5′-CAG ACT GCC CAA CAT GTC-3′; ACTH-R 1f, 5′-CAA GGG AGG GCA GAA ACT G-3′/5′-CAG ACT GCC CAA CAT GTC-3′; β-actin, 5′-GCA ATG CCT GGG TAC ATG GTG G-3′/5′-GTC GTA CCA CAG GCA TTG TGA TGG-3′. PCR conditions are available on request. Threshold values were obtained where fluorescent intensity was in the geometric phase of amplification, as determined with LightCycler Software Ver.3.5. Products were verified on a 2% agarose gel. We verified the nucleotide sequences of each PCR product by direct sequencing using the appropriate primers. Relative expression levels of the mRNA were calibrated to those of β-actin and its ratio to the control mouse adrenocortical Y-1 cells or mouse adrenal or testis.

Flowcytometry

The protocol essentially followed a previously described method (Hirase *et al.* 2000). Briefly, 3×10^5 BMCs were incubated with either PE (phycoerythin)-conjugated anti-mouse c-kit, CD11b, CD34, CD44, CD45, and Sca-1 monoclonal antibodies (BD Biosciences, Japan) or an isotype-matched PE-conjugated rat IgG (BD Biosciences) for 30 min at 4 °C. The cells were finally analysed on a FACScan flow cytometer (Becton Dickinson).

Immunocytochemistry

We conducted an immunocytochemical study of the BMCs from 129SVJ mice with an antibody against rabbit anti-cytochrome P450scc (RDI, NJ, USA) using Zenon Rabbit IgG labelling kits (Molecular Probes, Inc., OR, USA) or preimmune serum. The cells were plated on to collagen I-type membranes (Asahi technoglass, Tokyo, Japan) in 35 mm dishes and fixed with 4% paraformaldehyde at 4 °C for 1 h. After this, we completely followed the manufacturer's protocol. The fluorescence was observed using fluorescence microscopy (BX-51; Olympus, Tokyo, Japan).

Statistics

One-factor ANOVA was used for statistical evaluation. $P < 0.05$ was considered statistically significant.

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A Novel Nonradioactive Method for Measuring Aromatase Activity Using a Human Ovarian Granulosa-Like Tumor Cell Line and an Estrone ELISA

Ken Ohno,* Naohiro Araki,* Toshihiko Yanase, Hajime Nawata† and Mitsuru Iida*,[1]

*EcoScreen R & D Section, Endocrine Disrupting Chemical Analysis Center, Otsuka Life Science Initiative, Otsuka Pharmaceutical Co., Ltd. 224-18 Ebisuno Hiraishi, Kawauchi-cho, Tokushima, 771-0195, Japan; and †Department of Medicine and Bioregulatory Science (Third Department of Internal Medicine), Graduate School of Medicine Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan

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Aromatase is a key enzyme in steroidogenesis and plays an important role in sexual differentiation, fertility, and carcinogenesis. Importantly, a variety of chemicals in the environment may influence its activity and thereby disrupt endocrine function. In the current studies, we developed a novel nonradioactive method for measuring aromatase activity that uses a specific ELISA for estrone along with KGN human ovary granulosa-like carcinoma cells. This cell line has relatively high aromatase activity, and because it lacks 17α-hydroxylase, it secretes little or no androstenedione, 17β-estradiol, or estrone. Therefore, aromatase activity can be assayed simply by measuring the production of estrone in the culture medium after addition of the substrate, androstenedione. Furthermore, by making a slight change in the commercial ELISA kit and optimizing the experimental conditions, we developed a sensitive aromatase assay that could measure a wide range of estrone concentrations with very low interference by androgens. We used this assay to investigate the effects of 23 chemicals that have been previously reported to affect aromatase activity *in vitro*. We confirmed that 17 of 23 test chemicals had inhibitory or inducible effects, although the specific effects of some were different than previously reported. In conclusion, we have developed a simple, sensitive, and nonradioactive assay that can be used for large-scale screening of compounds that can disrupt endocrine function by influencing aromatase activity.

Key Words: aromatase; endocrine disrupter; screening assay; KGN cell line; benomyl.

A variety of environmental contaminants and chemicals used in commercial products are suspected endocrine disrupters that may lead to abnormalities in sexual differentiation, reproductive capacity, growth, and development (McLachlan, 2001). Current research has mainly focused on the interactions of these chemicals with sex hormone receptors, such as the estrogen and androgen receptors (Kelce *et al.*, 1997; Lambright *et al.*, 2000). However, these chemicals may also disrupt biological function by other mechanisms, such as altering hormone biosynthetic pathways.

Aromatase is a key enzyme in the conversion of androgens to estrogens and has an important role in maintaining a homeostatic balance between them. Some flavonoid chemicals, for example, α-naphthoflavone, apigenin, and chrysin, are known to inhibit aromatase activity *in vitro* (Campbell and Kurzer, 1993; Jeong *et al.*, 1999; Kellis and Vickery, 1984; Le Bail *et al.*, 1998; Pelissero *et al.*, 1996). In addition, the herbicide atrazine has recently been shown to induce aromatase activity, and various imidazole-like fungicides have been shown to be aromatase inhibitors (Sanderson *et al.*, 2002). Also, the biocides triphenyltin (TPT) and tributyltin (TBT), which are used in antifouling paints and wood preservatives, are suspected to inhibit aromatase activity and cause imposex in gastropods (Heidrich *et al.*, 2001; Saitoh *et al.*, 2001).

Two kinds of *in vitro* assay have been developed to measure aromatase activity: a cell-free assay using human placental microsomes (Njar *et al.*, 1995; Vinggaard *et al.*, 2000) or human recombinant aromatase protein; and a cell-based assay using mammalian cell lines, such as the human JEG-3 (Drenth *et al.*, 1998; Yue and Brodie, 1997) and JAr (Brueggemeier *et al.*, 1997) cell lines. In either case, aromatase activity is determined by measuring the amount of ^{3}H-water released upon enzymatic conversion of radiolabeled androstenedione. However, these assays require the use of radioactive materials and specialized equipment for radiometric measurement. An alternative fluorescent cell-free method has recently been developed using human recombinant aromatase protein (Stresser *et al.*, 2000), but this method cannot detect aromatase induction because it utilizes a cell-free system.

In the current study, we developed a novel nonradioactive cell-based assay that can detect both inhibition and induction of aromatase. This assay was performed using KGN cells, which are a steroidogenic human ovarian granulosa-like tumor cell line that was established from a patient with invasive granulosa cell carcinoma (Nishi *et al.*, 2001). This cell line possesses normal properties of granulosa-like cells, including relatively

[1] To whom correspondence should be addressed. Fax: 81-88-665-3613. E-mail: iidam@hq.otsuka.co.jp.

high aromatase activity that is stimulated by follicle stimulating hormone or cAMP. In addition, the KGN cells cannot synthesize androgen or estrogen by themselves due to the absence or low level of 17α-hydroxylase. Therefore, the aromatase activity can be evaluated simply by culturing the cells with androstenedione and measuring the estrone level in the culture medium with a specific enzyme-linked immunosorbent assay (ELISA). This novel assay should be useful for the high-throughput screening of chemicals for aromatase inhibition or induction.

MATERIALS AND METHODS

Cell culture. KGN cells were grown in Dulbecco's modified Eagle medium/Ham's F-12 nutrient mix (DMEM/F-12) medium (Invitrogen, Carlsbad, CA) supplemented with 5% (v/v) fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS), 50 U/ml penicillin, and 50 μg/ml streptomycin in 175-cm^2 cell culture flasks (Nalge Nunc International, Rochestar, NY) at 37°C in a humidified atmosphere containing 5% CO_2. The cells were passaged every 2 to 3 days when they reached confluence by treating them for 4 min with 0.05% trypsin and then replating them at (1.5 to 2.5) × 10^6 cells/flask.

Chemical preparations. Test chemicals (Table 1) and androstenedione (Sigma, St. Louis, MO) were dissolved in DMSO at a concentration of 10 mM and stored at −20°C. The maximum concentration of each chemical used in the experiments was 10 μM, 1 μM, or 100 nM, depending on its solubility in the cell culture medium. Then the test chemicals and the substrate were diluted with serum-free DMEM/F-12 medium by 50 and 100 folds, respectively. And there were six dose points (by 10 folds diluted from maximum concentrations) tested in this study. The final concentration of DMSO in the cell growth medium was 0.15% (v/v), and we confirmed that 0.15% DMSO had no statistically significant effect on the estrone production in KGN cells by Student's *t*-test ($p = 0.65, n = 8$). All treatments were tested in triplicate, except for the controls, which were performed in sextuplicate.

Aromatase assay. A 180-μl volume of 5 × 10^4 KGN cells/ml was added to each well of a 96-well tissue culture plate. Cells were grown at 37°C in DMEM/F-12 medium containing 5% (v/v) charcoal/dextran-treated FBS (Hyclone, Logan, UT). After 2 days, 10 μl of test chemicals were added, and cells were incubated for 24 h. Solvent control wells were dosed with media plus 0.15% DMSO. Next, 10 μl of 0.1 μM androstenedione was added to each well, and cells were incubated for another 24 h. A 120-μl sample of the culture medium was removed from each well and transferred to a second 96-well tissue culture plate. The estrone concentration in each of these wells was measured using an estrone ELISA kit (Otsuka Pharmaceuticals Co., Ltd., Tokushima, Japan), and cell

TABLE 1
Summary of Tested Chemicals and Effects on Aromatase Activity

Chemical name	Chemical structure	CAS No.	Supplier	Chemical type	Reported IC_{50} values by microsome assay	Reported IC_{50} values by cell based assay	Aromatase inhibition in this study (IC_{50} value)	Aromatase induction in our study	Maximum concentration tested in this study
α-Naphthoflavone	$C_{19}H_{12}O_2$	604-59-1	ICN	flavonoid	0.07 μM (a)		0.412 μM		10 μM
Flavone	$C_{15}H_{10}O_2$	525-82-6	Wako	flavonoid	10 μM (b)	>100 μM (c)	#		10 μM
7-Hydroxyflavone	$C_{15}H_{10}O_3$	6665-86-7	Wako	flavonoid	0.5 μM (b)	0.35 μM (c)	5.31 μM		10 μM
Chrysin	$C_{15}H_{10}O_4$	480-40-0	TCI	flavonoid	0.7 μM (d)	0.5 μM (c)	1.89 μM		50 μM
Flavanone	$C_{15}H_{12}O_2$	487-26-3	Wako	flavonoid	8 μM (b)		#		10 μM
Apigenin	$C_{15}H_{10}O_5$	520-36-5	Sigma	flavonoid	2.9 μM (e)	0.18 μM (c)	2.58 μM		50 μM
Naringenin	$C_{15}H_{12}O_5$	480-41-1	TCI	flavonoid	9.2 μM (e)	1.4 μM (c)	2.42 μM		100 μM
7-Methoxyflavanone	$C_{16}H_{14}O_3$	21785-09-1	Avocado	flavonoid	3.2 μM (d)		1.18 μM		10 μM
Fenarimol	$C_{17}H_{12}Cl_2N_2O$	60168-88-9	Wako	pesticide	10 μM (f)	2 μM (f)	2.00 μM		10 μM
Triadimefon	$C_{14}H_{16}ClN_3O_2$	43121-43-3	Wako	pesticide	32 μM (f)		3.59 μM		50 μM
Imazalil	$C_{14}H_{14}Cl_2N_2O$	35554-44-0	Wako	pesticide	0.04 μM (f)	0.1 μM (g)	0.00444 μM		50 μM
Propiconazole	$C_{15}H_{17}Cl_2N_3O_2$	60207-90-1	Wako	pesticide	6.5 μM (f)	5 μM (g)	0.968 μM		10 μM
o,p'-DDT	$C_{14}H_9Cl_5$	789-02-6	AccuStandard	agricultural chemical		no value (g)	+/- *		10 μM
p,p'-DDD	$C_{14}H_{10}Cl_4$	72-54-8	Wako	agricultural chemical		no value (h)	7.87 μM		10 μM
4-Hydroxy androstenedione (4-OHA)	$C_{19}H_{26}O_3$	566-48-3	Sigma	pharmaceutical compound	0.6 μM (i)	0.08 μM (j)	0.00115 μM		10 μM
Aminoglutethimide	$C_{13}H_{16}N_2O_2$	125-84-8	Sigma	pharmaceutical compound	1.2 μM (e)	15.8 μM (j)	2.25 μM		100 μM
Tributyltin cloride (TBT)	$C_{12}H_{27}ClSn$	1461-22-9	Wako	organotin	6.2 μM (k)	no value (l)	+/- *		(1000 ng/ml)
Triphenyltin cloride (TPT)	$C_{18}H_{15}ClSn$	639-58-7	Strem Chemicals	organotin		no value (l)	+/- *		(1000 ng/ml)
Atrazine	$C_8H_{14}ClN_5$	1912-24-9	Wako	agricultural chemical		++ (g,m)		+/- *	50 μM
Diclobutrazol	$C_{15}H_{19}Cl_2N_3O$	75736-33-3	Dr. Ehrenstorfer	agricultural chemical		++ (g)		+/- *	10 μM
Vinclozolin	$C_{12}H_9Cl_2NO_3$	50471-44-8	Wako	agricultural chemical		++ (g)		+/- *	10 μM
Benomyl	$C_{14}H_{18}N_4O_3$	17804-35-2	Dr. Ehrenstorfer	agricultural chemical		++ (h)		++	10 μM
Forskolin	$C_{22}H_{34}O_7$	66575-29-9	Wako	natural product		+++ (g,h)		++++	100 μM

Note. +, inducible; +/−, no effect; #, weak inhibitor; *, discrepancy with previous reports.

a, Kellis *et al.*, 1998; b, Ibrahim and Abul-Haji, 1990; c, Saarinen *et al.*, 2001; d, Le Bail *et al.*, 1998; e, Le Bail *et al.*, 2001; f, Vinggaard *et al.*, 2000; g, Sanderson *et al.*, 2002; h, Morinaga *et al.*, 2003; i, Njar *et al.*, 1995; j, Yue and Brodie, 1997; k, Heidrich *et al.*, 2001; l, Saitoh *et al.*, 2001; m, Heneweer *et al.*, 2004.

viability was determined using the cells remaining in the original 96-well plate. In some cases, plates were stored at −20°C prior to measurement of estrone levels.

Estrone ELISA. The estrone ELISA was carried out as described in the manufacturer's instructions, except that 3,3′,5,5′-tetramethylbenzidine solution and Stop Buffer (Scytech, Logan, UT) was used instead of the o-phenylenediamine and the stop buffer provided in the kit. The absorbance in each well was measured at 450 nm using an ARVOsx 1420 multilabel counter (Wallac, Turku, Finland), and the estrone concentration in each well was calculated based on a standard curve and using SOFTmax Pro 4.0 (Molecular Devices, Sunnyvale, CA).

Measurement of cell viability. The cytotoxicity of the various test chemicals was assessed using AlamarBlue assay (Ahmed *et al.*, 1994). An 8 µl volume of the AlamarBlue reagent (Serotec Ltd., Oxford, UK) was added to the wells, and cells were incubated for 3 to 4 h at 37°C according to the manufacture's protocol. The fluorescence was measured at 590 nm with excitation at 544 nm using the ARVOsx 1420 microplate reader. The cytotoxicity was determined by comparing the fluorescence in each well with the fluorescence of solvent control wells (0.15% DMSO).

Data and statistical analysis. Effects on aromatase activity and cell toxicity were expressed as a relative ratio of estrone concentrations at each dosing point divided by the estrone concentration in solvent controls. If there was more than 50% inhibition of aromatase activity, IC_{50} values were calculated by GraphPad PRISM ver 4.0 (GraphPad, San Diego, CA) using sigmoidal dose-response curve fitting (variable slope). Data were analyzed by one-way factorial ANOVA using SatView for Windows (SAS Institute Inc., Cary, NC).

RESULTS

Cross-Reactivity of the Estrone ELISA Kit

We first investigated the specificity of the commercially available estrone ELISA kit (Otsuka Pharmaceuticals) by examining its cross-reactivity with 17β-estradiol, estriol, testosterone, progesterone, and androstenedione (Table 2). We found less than 0.005% cross-reactivity with the aromatase substrates androstenedione and testosterone. In contrast, there was 30% cross-reactivity with estradiol and 0.4% cross-reactivity with estriol. Finally, a representative estrone standard curve using the ELISA kit is shown in Figure 1. Collectively, these results confirmed that the ELISA kit can be used to determine the estrone level in the culture medium without interference by aromatase substrates.

Optimization of the Assay Conditions

To optimize the sensitivity of the test system, we examined the relationship between the substrate concentration and estrone production. Two days after plating the cells, 10 µl of the serum-free medium was added in cell culture medium instead of samples. After 24 h, various concentrations of androstenedione were then added to the wells, and the estrone concentration in the culture medium was measured after 1, 2, 3, 6, 12, and 24 h using the estrone ELISA (Fig. 2). With the exception of 100 pM, there was a linear increase in estrone concentration with time at all androstenedione concentrations. In addition, the estrone level reached a 24-h maximum between 100 and 120 pg/ml at androstenedione concentrations above 10 nM. To be able to detect the effects of a competitive inhibitor, the concentration of substrate should be near the K_m. Therefore, we chose 5 nM androstenedione as the substrate concentration for the analysis of test chemicals because it gave a reaction rate approximately half of the maximal velocity.

Other test conditions were optimized, such as cell density, preincubation time, and incubation time with substrate, and

TABLE 2
Cross Activity of Estrone ELISA (Otsuka)

Compounds	cross activity(%)
Estrone	100.0
17β-Estradiol	30.6
Estriol	0.4
Testosterone	<0.005
Progesterone	<0.005
4-Androstene-3,17-Dione	<0.005

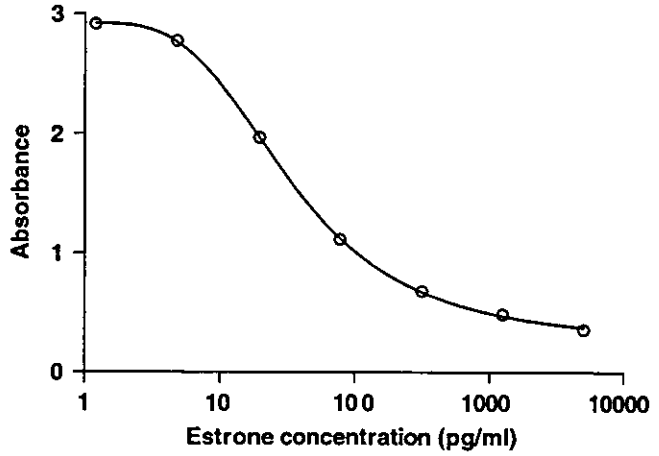

FIG. 1. Example of a standard curve for the estrone ELISA. Data from eight estrone concentrations (0, 1.2, 4.8, 12.5, 78.1, 312.5, 1250, and 5000 pg/ml) are shown.

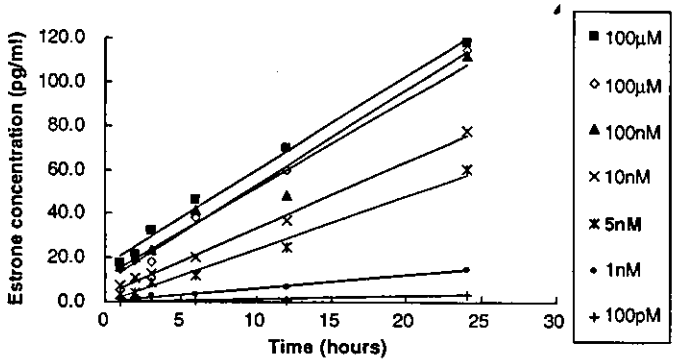

FIG. 2. Effect of substrate concentration on estrone production. KGN cells were grown in 96-well plates for 48 h. Next, 10 µl of serum-free medium was added in cell culture medium instead of samples. After 24 h, various concentrations (10 µM, 1 µM, 100 nM, 10 nM, 5 nM, 1 nM, or 100 pM) of androstenedione were added in triplicate. After an additional 24 h, the estrone concentration in the medium of each well was assayed by estrone ELISA, and the average values were plotted.

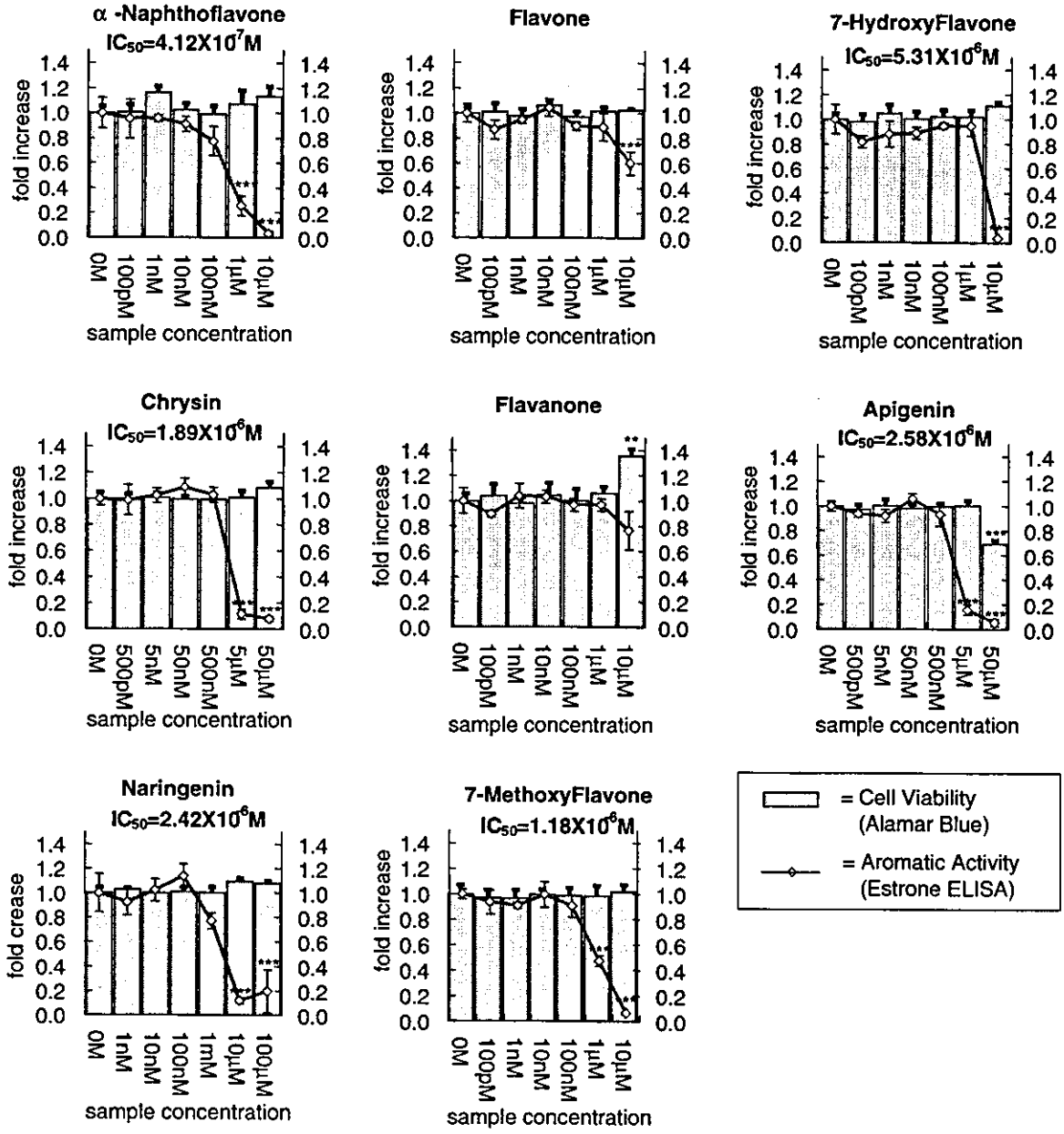

FIG. 3. Effects of flavonoid compounds on aromatase activity and cell viability. The bar graph represents the cell viability as measured by AlamarBlue reagent. The line graph represents the aromatase activity as evaluated by measurement of estrone concentration with ELISA. Values were calculated as the ratio of the measured activity versus control, and the plots show one set of triplicate measurements. The error bars show the SD from the triplicate determinations. IC_{50}s shown are the means of three investigations ± SD. Asterisks denote statistical significance compared to respective control: $^{**}p < 0.001$, $^{***}p < 0.0001$.

effects of plate position were minimized (data not shown). The final conditions included seeding the KGN cells at 9×10^3 cells per well, 2 days growth following plating, 24-h incubation with substrate, and exclusion of wells on the edge of the plate.

Effects of Test Chemicals on Aromatase and Cell Viability

Using the optimized assay, we tested the effect of 23 chemicals that had previously been reported to affect aromatase activity. Most of these were selected from a draft of a detailed review paper on aromatase by the U. S. Environmental Protection Agency (2002). Included in the 23 chemicals were flavonoids, a pesticide, pharmaceutical compounds, and organotin compounds. We first tested the eight flavonoid chemicals for effects on aromatase activity and cell viability (Fig. 3). All flavonoids were found to inhibit aromatase activity at relatively high concentrations, and flavone and flavanone were very weak inhibitors. These chemicals did not have cell toxicity on KGN

cells, excepting for apigenin, which had some cell toxicity at maximum concentration. Next, we investigated the effects of 10 pesticides, pharmaceutical compounds, and organotins (Fig. 4). With the exception of *o,p*-DDT (*o,p*-dichloro-1,1-diphenyl-2,2,2-trichloroethane), all of the chemicals inhibited the aromatase activity. The organotins also decreased aromatase activities, but they were very cytotoxic. These inhibitory effects on aromatase activity by organotins seemed to be affected by

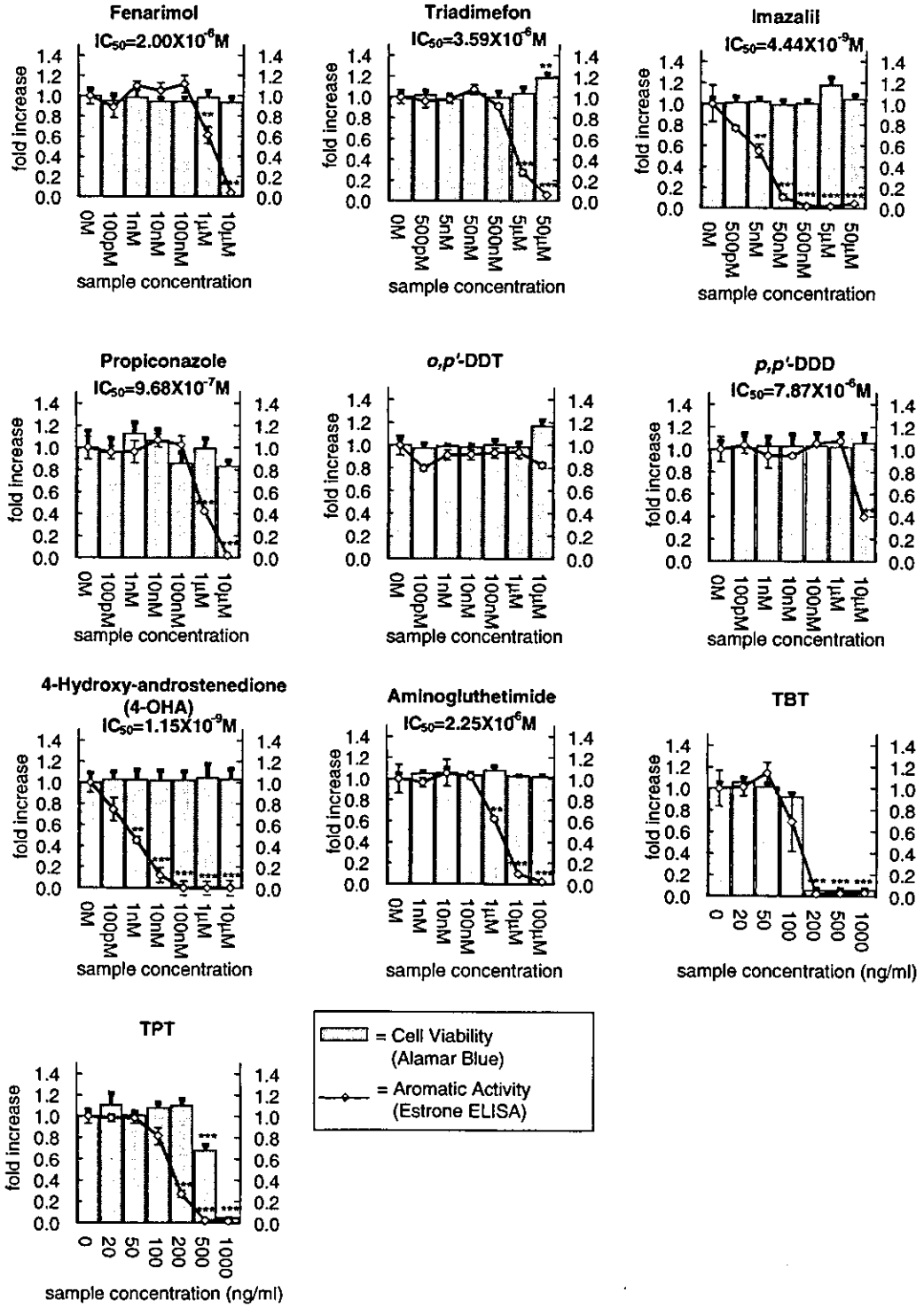

FIG. 4. Effect of various classes of suspected aromatase inhibitors on aromatase activity and cell viability. Experiments were carried out and data are presented as in Fig. 3.

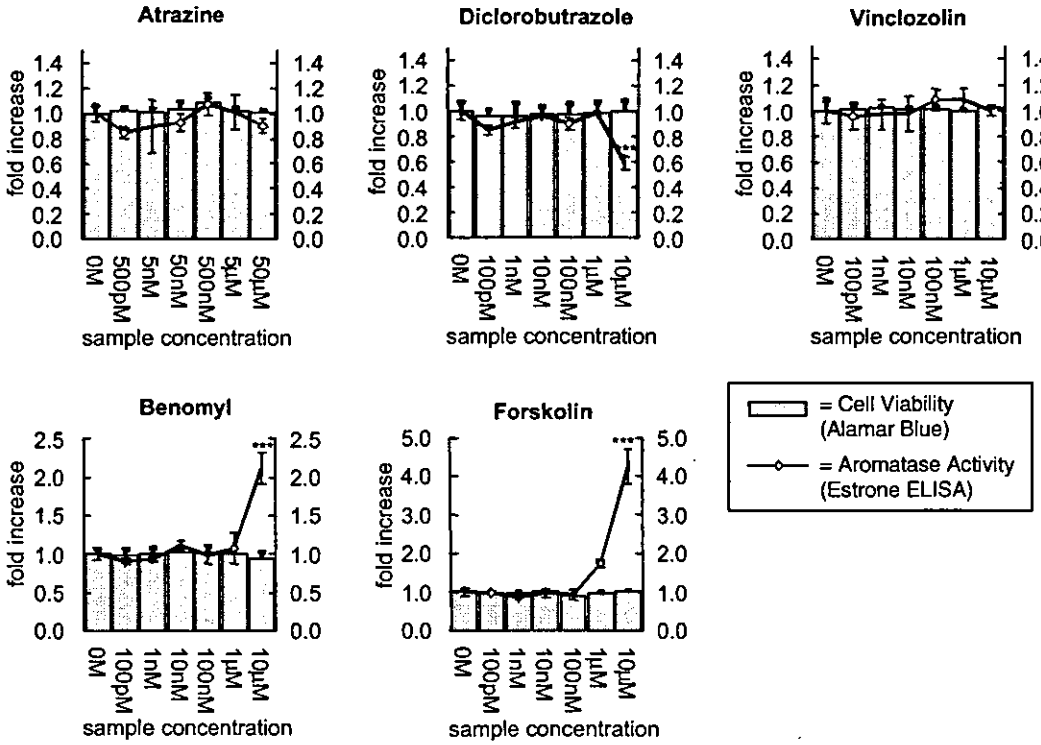

FIG. 5. Effect of suspected aromatase inducers on aromatase activity and cell viability. Experiments were carried out and data are presented as in Fig. 3.

these cell toxicities. Finally, we tested five chemicals that are suspected to induce aromatase activity (Fig. 5). We found that benomyl and forskolin induced aromatase activity, but the other three, atrazine, diclobutrazole, and vinclozolin, had no inducible effects. A summary of these results and the related chemical information is shown in Table 1. The table also shows the mean IC_{50} values (n = 3) calculated for compounds that inhibited aromatase activity as well as any previously reported IC_{50} values. Overall, the IC_{50} values increased the following order: 4-hydroxyandrostenedione (4-OHA) < imazalil ≪ α-naphthoflavone < propiconazole < 7-methoxyflavone < chrysin < fenarimol < aminoglutethimide < naringenin < apigenin < triadimefon ≪ 7-hydroxyflavone < p,p'-DDD (p,p'-dichlorodiphenyldichloroethane).

Reproducibility of this Aromatase Assay

The interassay reproducibility of the system was evaluated by calculation of coefficients of variation (CV), where CV = 100% × SD/mean. The overall average CV for aromatase activity in the chemical tests was 10.94% (n = 161), and for cell viability, the overall average CV was 6.01% (n = 161). Intra-assay reproducibility in the aromatase assay was determined by comparing the IC_{50}s calculated for each of the triplicate dose-response curves for 4-OHA and imazalil, which were chosen because their inhibitory effects covered a wide concentration range. The average calculated IC_{50} value for 4-OHA was $1.15 \pm 0.28 \times 10^{-9}$ M (CV = 24.7%), and for imazalil, $4.44 \pm 1.28 \times 10^{-9}$ M (CV = 28.9%).

DISCUSSION

In this study, we developed a novel nonradioactive cell-based assay that can measure both inhibition and induction of aromatase by chemical compounds. This was made possible by the use of KGN cells in combination with an estrone ELISA. The ELISA kit used in this study had a low cross-reactivity toward substrate compounds, and only 17β-estradiol, which is not an aromatase substrate, showed significant (30%) cross-reactivity. If there is a relatively high 17β-hydroxysteroid dehydrogenase (17β-HSD) activity in KGN cells, most of the estrone would be converted to 17β-estradiol, which could interfere with quantitative measurement of aromatase activity. Nishi *et al.* (2001) reported that estrone (E_1) and 17β-estradiol (E_2) were produced in a ratio of approximately 1:1.7 when KGN cells were incubated with 10 μM androstenedione, a concentration 2000-fold higher than we used in this study. However, using an ELISA for 17β-estradiol, we previously found that incubation of KGN cells with E_1 for 24 h resulted in only 10% or less conversion to E_2 (data not shown). Therefore, the activity of 17β-HSD in KGN cells may not be enough to interfere with in the measurement of estrone. However, both of these experiments were used the immunoassay technique; a more careful consideration was

required for cross-reactivity to various steroids. Further studies using HPLC or LC/MS/MS may be necessary to confirm this problem, but in general, the assay appears to reflect actual aromatase activity.

Our testing of potential endocrine disrupters showed that the eight flavonoids, which are weak inhibitors of aromatase *in vitro* (Ibrahim and Abul-Hajj, 1990; Kao *et al.*, 1998; Kellis *et al.*, 1984; Le Bail *et al.*, 2001; Saarinen *et al.*, 2001), also inhibited aromatase activity in our cell-based assay. In addition, flavone and flavanone, which are weaker aromatase inhibitors than other flavonoids, could be detected using our assay, indicating that the method has high sensitivity. However, IC_{50} values were not calculated for these two compounds because the highest concentrations tested produced less than 50% inhibition.

Among agricultural chemicals, pesticides, and fungicides suspected to be inhibitors of aromatase, fenarimol, triadimefon, imazalil, propiconazole, and *p,p'*-DDD had inhibitory effects in our assay, whereas *o,p'*-DDT had little or no effect at the concentration range we tested (100 pM–10 μM). On the contrary, *o,p'*-DDT was reported to inhibit aromatase activity at 10 μM in H295R cells (Sanderson *et al.*, 2002). Although the reason for this discrepancy is unclear, it may be due to the difference in cell type or origin. We also found that the pharmaceutical compounds 4-OHA (also called formestane) and imazalil strongly inhibited the aromatase activity. In fact, the IC_{50} of imazalil was lower in our assay than in previous reports (Sanderson *et al.*, 2002). Sanderson *et al.* (2002) investigated imazalil at concentration from 0.1 μM to 1 mM, and their IC_{50} value of imazalil was 0.1 μM in H295R cell. Our estimated IC_{50} value of imazalil was about 20-folds lower than that of H295R results (Table 1).

Organotin compounds were very toxic to the KGN cells. In fact, both TBT (tributyltin chloride) and TPT (triphenyltin chloride) had previously been reported to have strong cell toxicity (Saitoh *et al.*, 2001). They showed TBT and TPT had inhibitory effect on aromatase activity in KGN cells with no cell toxicity at concentration lower than 20 ng/ml. In this study, decrease of the cell viability was also observed in TBT and TPT (Fig. 4). In TBT, the reduction of aromatase activity was also observed, however the cell viability was described in parallel. Therefore we considered reduction of aromatase activity in TBT mostly depended on its cell toxicity. TPT also had cell toxicity at concentration of 1000 ng/ml; however aromatase activity at 200 ng/ml was clearly decreased without cytotoxic effect. TPT could inhibit aromatase activity, but that range was very limited. We have used 24 h for preincubation of chemicals with KGN cells and an additional 24-h incubation for aromatase reaction with 96-well plate; meanwhile Saitoh's have 48 h and 6 h incubation with petri dish, respectively. Cell culture methods and exposure time with KGN cells and/or enzyme reaction time may have contributed to the difference about organotin compound between two studies. Although Bettin *et al.* (1996) concluded that the induction of imposex in gastropods by organotin compounds may be due to inhibition of aromatase (Bettin *et al.*, 1996), our results as well as those of Sanderson *et al.* (2000) and Morcillo and Porte (1999) suggest that the induction of imposex in gastropods by TBT or TPT occurs via mechanisms other than inhibition of aromatase activity.

In our investigation of aromatase inducers, we found that forskolin, a known inducer of cAMP, strongly enhanced aromatase activity. This is not surprising, because cAMP promotes aromatase gene expression by binding to a cAMP response element upstream of the aromatase gene. At 10 mM, benomyl, one of fungicides, induces aromatase activity twofold, which is consistent with previously reported results in KGN cells (Morinaga *et al.*, 2004). They further reported that benomyl and its metabolite carbendazim induce aromatase activity through stimulation of *CYP19* (aromatase) expression. The mechanism was unclear, although it was confirmed to not be through elevation of intracellular cAMP. This suggests that long-term exposure of wildlife and humans to chemicals like benomyl might lead to estrogen-mediated pathologies, such as tumor promotion, inappropriate sexual differentiation, and inappropriate feminization. Therefore, it is urgent to further investigate the physiological effects of this and similar compounds.

In contrast to forskolin and benomyl, there was no enhancement of aromatase by atrazine, *o,p'*-DDT, diclobutrazole, or vinclozolin, even though they have been reported to increase aromatase activity in H295R cells (Sanderson *et al.*, 2002). Particularly, atrazine has been reported to cause over twofold induction on aromatase activity at 30 μM in H295R cells (Heneweer *et al.*, 2004; Sanderson *et al.*, 2002). However we could not observe such inducible effects at 5 μM or 50 μM in KGN cells. Because the H295R cell line is derived from a human adrenocortical carcinoma cell, the observed discrepancy may be due to the different origins of the cells. This could also be due to alternative transcriptional mechanisms; for example, the promoter sites in the aromatase (CYP19) gene are different in various tissues and are regulated by cellular conditions or signaling pathways (Bulun *et al.*, 2004; Simpson, 2004). In fact, one study reported that atrazine induces aromatase activity in H295R but not rat R2C (Heneweer *et al.*, 2004). Furthermore, another report using KGN cells and a radioisotope-based aromatase assay showed no enhancement of activity by 10 μM of atrazine (Morinaga *et al.*, 2004). Also, although atrazine has been reported to enhance cAMP levels in H295R cells (Sanderson *et al.*, 2002), it had no effect on aromatase in KGN cells despite the fact that forskolin promoted aromatase in our assay. Although the reason for this is unclear, again, it could be due to alternative promoter site usage. Considering these results, if the objective is to screen endocrine disruptors, it would be better to use cells from the reproductive tissue origin.

In summary, in the current studies we introduced a novel method for measuring aromatase activity. Whether KGN cells are the most suitable for aromatase screening remains to be determined, but we expect that this assay will be very useful

for the screening of large numbers of samples because the assay is simple, nonradioactive, and adaptable to high-throughput screening. Therefore, it should be possible to perform large-scale screening for identification of chemicals that can affect aromatase activity and thereby lead to abnormal steroidogenesis.

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The KGN cells are available from the Riken Cell Bank as the stock number RCB No.1154 (http://www.brc.riken.go.jp/).

SUPPLEMENTARY DATA

Supplementary Data is available online.

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□ REVIEW ARTICLE □

Coregulator-Related Diseases

Toshihiko YANASE, Masahiro ADACHI, Kiminobu GOTO, Ryoichi TAKAYANAGI* and Hajime NAWATA

Abstract

Coregulators are a group of proteins, which modulate the nuclear receptor transactivation function. In this study, a new "coregulator disease" concept was proposed from observations of a case of androgen insensitivity syndrome (AIS) and cases involving Rubinstein-Taybi syndrome and X-linked dementia and hypothyroidism syndrome. In addition, coregulators are thought to be closely associated with the pathogenesis of several diseases such as hormone-dependent cancers and leukemia. Based on these observations, the clinical disorders associated with some coregulator abnormalities were reviewed. (Internal Medicine 43: 368–373, 2004)

Key words: coregulator, coactivator, corepressor, androgen insensitivity syndrome (AIS), Rubinstein-Tabi sundrome, Refetoff syndrome, hormone-dependent cancer, leukemia

Introduction

Steroid hormone receptors such as the androgen receptor (AR), estrogen receptor (ER) and glucocorticoid receptor (GR) are ligand-dependent transcription factors that belong to the nuclear receptor superfamily. Nuclear receptors bind to their cognate response elements in the promoter region of target genes, and regulate their expressions (1, 2). Some abnormalities in the structures of transcription factor cause the disturbed transactivation of target genes, leading to various physiological abnormalities. Such a disease state is well established as a "transcription factor disease." However, there are several cases that show no mutations in the corresponding nuclear receptors, although clinical and biochemical profiles completely match the disease concept. Coregulators are most likely associated with such a mechanism since coregulators interact with various nuclear receptor proteins and modulate the transcriptional activity (2–4). We have proposed a new disease concept of coregulator disease from a patient with androgen insensitivity syndrome (AIS) who showed no *AR* gene mutation (5). Rubinstein-Taybi syndrome and X-linked dementia and hypothyroidism syndrome, which are known to be caused by abnormalities of CBP and TR associated protein (TRAP) 230, respectively, have already been established as a coregulator disease (6, 7). This review focuses on several clinical disorders, which have been proven or are thought to be related to coregulator abnormalities.

Mechanism of Steroid Receptors

Nuclear receptors share a common structure, which consists of a transcription active domain, a DNA-binding domain and a ligand-binding domain. There are two transcription activation domains; the activation function-1 (AF-1) domain in the N-terminal region and the activation function-2 (AF-2) domain in the C-terminal region. While the AF-2 domain is relatively conserved among nuclear receptors, the AF-1 domain differs widely (Fig. 1) (1, 2). When a ligand is bound to a receptor, the receptor changes in structure, translocates from the cytoplasm to the nucleus and then binds to the promoter region of the target gene. Coregulator proteins bind to the nuclear receptors and modulate the transcriptional activity of the nuclear receptors in a promoter- and cell-specific manner (Fig. 2). There are two types of coregulators, coactivator proteins, which activate transcription, and corepressor proteins, which repress transcription. CBP/p300, p160 family (steroid receptor coativator (SRC) -1/NcoA1, transcriptional intermediary factor (TIF) 2/GRIP etc), VDR interacting protein (DRIP)/TRAP and others have been reported as typical coactivators, and nuclear receptor corepressor (NcoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT), as typical corepressors (2, 3). Although most coregulators are AF-2 binding proteins, p300/CBP and SRC-1 interact with both AF-1 and AF-2. In addition, several AF-1 binding coregulators such as breast cancer susceptibility gene 1 (BRCA1), SRA cyclin E and AR N-terminal domain transactivating protein-1 (ANT-1) (8) have also been identified. Interaction of the AF-1 and AF-2 domains is important

From the Department of Medicine and Bioregulatory Science and *Department of Geriatric Medicine, Graduate School of Medical Science, Kyushu University, Fukuoka

Reprint requests should be addressed to Dr. Toshihiko Yanase, the Department of Medicine and Bioregulatory Science, Graduate School of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582