An RPE cell line as a useful in vitro model for studying retinoic acid receptor β: expression and affinity

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Synopsis
Retinoids mediate their biological effect by interacting with specific nuclear receptors. Of the several known RAR (retinoic acid receptor) subtypes, RAR-β is of particular interest, since its expression is silenced in many cancers and it is believed to be a tumour suppressor. Specific ligands of RAR-β can potentially be used in anti-cancer therapy. In the present study, we have investigated the feasibility of using HRPE cells (human retinal pigment epithelial cells) as an experimental model for characterizing RAR-β–ligand interaction. RT–PCR (reverse transcription–PCR) and Western blot analyses show that HRPE cells specifically express only RAR-β and none of the other receptor subtypes. In addition, we show that the expression of RAR-β increases with increasing passage number of the cells. Interestingly, the increase in RAR-β expression is not associated with telomere shortening, a typical biomarker of cellular senescence. In the present study, we also describe a protocol for characterizing RAR-β–ligand interactions using nuclear extract from late passage HRPE cells as a source of endogenous RAR-β. Using [3H]CD367 as the ligand, RAR-β in HRPE cells showed an affinity of 9.6 ± 0.6 nM and a Bmax of 780 ± 14 fmol/mg of protein. We have confirmed the feasibility of using this assay to detect the interaction of ligands with RAR-β by investigating the ability of certain flavonoids to inhibit the binding of [3H]CD367 to nuclear extracts from HRPE cells. The inhibition constant of the flavonoids for RAR-β was between approx. 1–30 μM, showing that the flavonoids interact with RAR-β with low affinity.

Key words: culture passage, human retinal pigment epithelial cell (HRPE cell), retinoic acid receptor β (RAR-β), senescence, telomere

INTRODUCTION

Retinoic acid and its natural derivatives (retinoids) are important vitamin A metabolites that regulate cellular growth and differentiation [1]. Intracellularly, retinoic acid specifically interacts with two distinct types of protein. In the cytoplasm, retinoic acid interacts with CRABPs (cellular retinoic-acid-binding proteins), which are primarily involved in the storage, intracellular transport and metabolism of retinoic acid [2]. In the nucleus, retinoic acid interacts with nuclear RARs (retinoic acid receptors), which are proteins that function as ligand-dependent transcription factors that are related to the steroid and thyroid hormone receptor family [3]. Upon ligand binding, the receptors are activated and induce the transcription of target genes by binding to RAREs (retinoic acid responsive elements) in the promoter regions. There are two distinct classes of nuclear RARs: RAR [or NR (nuclear receptor) 1A] and the RXR (retinoid X receptor) (or NR2B) [2,4]. Both RAR and RXR have three subtypes (α, β and γ) that are encoded by separate genes located at distinct chromosomal loci [5]. The nuclear RAR subtypes are expressed differentially both spatially as well as temporally [5–7]. In humans, RAR-α is expressed in most tissues; RAR-β is expressed mainly in neural tissues with limited expression in skin; RAR-γ is expressed predominantly in...
skin; RXR-α is expressed in the liver, kidney, spleen and skin; RXR-β is expressed in almost all tissues; and RXR-γ is expressed mostly in muscle and brain.

Among the nuclear RARs, the RAR-β gene is of particular interest to cancer biologists because it regulates the metabolic pathways associated with tumour-suppressive effects mediated by retinoids [8,9]. The RAR-β gene contains a RARE and thus the expression of RAR-β is regulated by retinoic acid. Though loss of expression of several of the nuclear retinoid receptors has been detected in various cancer cell lines, and loss of RAR-β is most common in a variety of cancers, including cancers of the head and neck, breast, lung, oesophagus, pancreas, cervix and prostate, and its expression correlates inversely with cancer progression and tumour grade [10]. Epigenetic modifications of the RAR-β promoter have been linked to the loss of RAR-β expression in cancer cells [9]. Previous studies by Geisen et al. [8,11] have shown that RAR-β may also have a role to play in cellular senescence. They observed that both basal and retinoic acid-induced RAR-β mRNA levels in normal cells have a tendency to increase with senescence. Similar observations were made by Lee et al. [12], in aging fibroblasts with decreased proliferative capacity, who observed a selective up-regulation of RAR-β expression in response to retinoic acid and its derivatives in these cells. This suggests that genetic events important in regulating cellular senescence may also play a significant role in tumour suppression in humans. Indeed, RAR-β expression is up-regulated with serial passage in senescent normal mammary epithelial cells, whereas it is down-regulated in mammary cancer cell lines [1]. RAR-β, therefore, is of great interest as a potential target in the design of anti-tumoral drugs and as a tool to study the mechanisms of their effects [9]. Availability of a standardized and sensitive method to characterize RAR-β-specific ligand binding would therefore be of great use. However, the accuracy and reproducibility of different methods for studying the binding affinities of specific compounds to RARs can be compromised by several factors, which can be summarized as: (i) a low abundance of RARs in natural cells and tissues, resulting in a low ratio of specific compared with non-specific binding sites; (ii) the chemical instability of the natural ligand, retinoic acid; and (iii) the difficulty of designing a perfect method to separate free from bound ligand [13].

In the present study, we report an effective method to perform RAR-β-specific ligand-binding studies using HRPE cells (human retinal pigment epithelial cells), which overcomes most of the drawbacks listed above. Since RPE cells (retinal pigment epithelial cells) perform a number of physiological functions essential for vision, namely the uptake, processing, transport and release of vitamin A (retinol) and its visual cycle intermediates, retinoids [14], we first investigated the expression of retinoid receptors in HRPE cells. The results presented here show, for the first time, that HRPE cells natively and selectively express appreciable levels of only RAR-β. We also provide evidence to show that RAR-β expression in these cells increases with increasing passage of the cells. We then standardized an RAR-β receptor assay using nuclear extracts from late passage HRPE cells and [3H]CD367, a stable synthetic ligand for the RAR subtypes [15]. The efficacy of our method has allowed us to distinguish between different levels of native RAR-β expression in serially passaged HRPE cells and to characterize the affinity of several flavonoids to RAR-β.

**EXPERIMENTAL**

**Materials**

[3H]CD367 (specific activity 42 Ci/mmol) and CD367 were provided by Galderma. [3H]9-cis-retinoic acid (specific activity 43 Ci/mmol) was obtained from Amersham Biosciences. Genistein, quercetin, daidzein, equol, 9-cis-retinoic acid, DTT (dithiothreitol), dextran, BSA, DEPC (diethyl pyrocarbonate), and the protease inhibitors PMSF, aprotinin and leupeptin were purchased from Sigma. Glycerol, thioglycerol, isopropyl alcohol and activated charcoal were obtained from Merck. All tissue-culture media, FBS (fetal bovine serum) and RT–PCR (reverse transcription–PCR) reagents were purchased from Invitrogen.

**HRPE cell culture**

The HRPE cell line was routinely grown at 37 °C in an atmosphere of 5% CO₂/95% air, in a 1:1 (v/v) mixture of Ham’s F12 medium and DMEM (Dulbecco’s modified Eagle’s medium) supplemented with 10% (v/v) FBS, 100 μg/ml streptomycin and 10 i.u./ml penicillin. The population doubling time was 24 h. Cells were seeded every 4 to 5 days by trypsin/EDTA dispersion and grown to confluence. Early and late passage HRPE cells were defined here as those cultures receiving less than 5 trypsin passages and more than 35 trypsin passages respectively.

**Semi-quantitative RT–PCR**

Total RNA was isolated from HRPE cells using TRIZol (1 ml/10⁶ cells) following the manufacturer’s instructions. Total RNA was treated with 1 unit/μl DNase I (Invitrogen) and used in oligo(dT)-primed first strand cDNA synthesis (SuperScript II reverse transcriptase; Invitrogen). Primers and PCR conditions for the different RARs used are presented in Table 1. The relative amounts of RAR mRNA were determined by semi-quantitative RT-PCR as described previously [16]. The PCR products were resolved by electrophoresis through a 1.5% agarose gel containing ethidium bromide, and the intensity of the cDNA bands relative to the background was determined using the Quantity One system (Bio-Rad). The linear range of PCR was empirically determined in initial experiments where PCR was performed with each primer set with a different number of amplification cycles (15–40 cycles). The relationship between the intensity of the signal and the PCR cycle number was analysed to determine the linear range for PCR product formation. The intensities of the signals within the linear range were used for data analysis. RAR-β amplified linearly between cycles 28–34. When quantitative RT–PCR was performed, 18S rRNA (linear amplification between 18–22 cycles) was used as a control.
was amplified simultaneously, with its specific primers being added after the first 10 cycles and used for normalization of target gene values throughout experiments because its level did not vary significantly in relation to passage number [16]. Under the PCR conditions described above, the amount of PCR product obtained showed a linear correlation with the amount of cDNA added to the PCR mixture, i.e. the ratio of RAR-β mRNA to 18S mRNA remained almost constant. In each PCR, a reaction without reverse transcriptase (to rule out amplification of genomic DNA) and DEPC-treated water instead of cDNA were used as negative controls.

### Western blot analysis

Protein extracts were obtained from early and late passage HRPE cells at 80% confluency using Passive Lysis Buffer (Promega). Crude extracts (40 μg) were analysed by SDS/PAGE (12.5% gels). Proteins were transferred electrophoretically (2 h at 0.3 A) to nitrocellulose filters using an immunoblot transfer apparatus (Bio-Rad). After transfer, the filter was incubated for 3 h at room temperature (25°C) in 10% (w/v) non-fat dried skimmed milk powder to block non-specific binding. The blot was incubated overnight at 4°C with 10% (w/v) non-fat dried skimmed milk powder in TBS supplemented with 0.05% Tween 20 to block non-specific binding. The blot was incubated overnight at 4°C with 10% (w/v) non-fat dried skimmed milk powder in TBS supplemented with 0.05% Tween 20 containing rabbit polyclonal anti-(RAR-β) antibody (Santa Cruz Biotechnology) at a dilution of 1:1000. After three washes with TBS containing 0.05% Tween 20, the blot was incubated for 60 min at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Calbiochem) diluted at 1:2000 in 10% (w/v) non-fat dried skimmed milk powder in TBS supplemented with 0.05% Tween 20. Antibodies were visualized using a chemiluminescence detection system (Western blotting Luminol reagent; Santa Cruz Biotechnology).

### Telomere length

Genomic DNA from HRPE cells was isolated using the Wizard genomic DNA purification kit (Promega). Telomere length comparisons were performed using the Telo TAGGG Telomere Length Assay kit (Roche). Genomic DNA (2 μg) was digested with Rsal and HinfI restriction enzymes, size-fractionated on a 0.8% agarose gel, and transferred to nylon membranes in 20× SSC buffer (1× SSC is 0.15 M NaCl/0.015 M sodium citrate) by capillary blotting. Controls were DIG (digoxigenin)-labelled DNA ladder markers, and large and small unlabelled DNA marker. The blotted DNA fragments were hybridized on to a DIG-labelled TAGGG probe and incubated with a DIG-specific antibody covalently coupled to alkaline phosphatase. The immobilized telomere probe was visualized using CDP-Star, a highly sensitive chemiluminescence substrate of alkaline phosphatase. Blots were documented and analysed using the GelDoc imaging system.
system and Quantity One software from Bio-Rad. The mean length of the telomere restriction fragment was calculated according to the manufacturer’s instructions.

Preparation of cytosolic and nuclear extracts

The preparation of nuclear and cytosolic extracts was based on the procedure described by Nervi et al. [17], with several modifications. Briefly, HRPE cells (1 x 10^5–5 x 10^5 cells) at 35 or higher passages from 10–15 confluent 150 cm² tissue-culture dishes were trypsinized and collected in growth medium by centrifugation at 1000 g for 5 min and the cell pellet was rinsed twice with ice-cold PBS containing 2 mM EDTA. The pellet was then resuspended in 3 ml of ice-cold lysis buffer [10 mM Tris/HCl (pH 7.6), 1.5 mM EDTA, 1 mM DTT and 10% (v/v) glycerol] in the presence of protease inhibitors (1 mM PMSF, 20 μg/ml aprotinin and 20 μg/ml leupeptin) and incubated on ice for 15 min. Cells were then homogenized with a glass Dounce homogenizer (pestle B) and the homogenate was centrifuged at 30,000 rev./min for 30 min at 4 °C. The resulting supernatant was the cytosolic extract. The pellet, containing the nuclei, was resuspended in extraction buffer [10 mM Tris/HCl (pH 8.0), 1.5 mM EDTA, 1 mM DTT, 10 mM thioglycerol, 0.8 M KCl and 10% (v/v) glycerol] containing the protease inhibitors at a volume equal to 2/3 of the cell pellet volume. The suspension was incubated for 1 h on ice with repeated resuspension every 10 min, rehomogenized with the Dounce homogenizer (pestle B) and then centrifuged at 30,000 rev./min for 1 h at 4 °C. The resulting supernatant was referred to as the nuclear extract. Both the cytosolic and nuclear extracts were used either immediately or after storage at −80 °C, and aliquots of both were assayed for protein concentration following the method of Lowry et al. [18], with BSA used as a standard.

Dextran-charcoal adsorption-binding assay

Nuclear or cytosolic extracts containing 200 μg of protein were incubated in 200 μl of 20 mM Tris/HCl (pH 8.0), 150 mM NaCl and 1 mM DTT at 4 °C for 4 h. Saturation experiments were carried out using 10 different concentrations of [3H]CD367 (1–80 nM). Displacement experiments were performed using nine different concentrations of non-radioactive drug in the presence of 10 nM [3H]CD367. Binding assays with [3H]9-cis-retinoic acid were performed at a final concentration of 10 nM. Non-specific binding was measured in the presence of a 200-fold excess of unlabelled CD367 or 9-cis-retinoic acid. Separation of bound from free radioligand was performed essentially as described by Sablonnière et al. previously [13]. Briefly, 100 μl of ice-cold charcoal/dextran suspension [3% (w/v) water-washed activated charcoal/0.3% dextran in 20 mM Tris/HCl (pH 8.0), 1 mM EDTA, 1 mM DTT and 0.1% BSA] was added to the incubation mixture. After 10 min of incubation at 4 °C, the samples were centrifuged at 6000 g for 5 min and 150 μl of the supernatant was counted for radioactivity. In all assays, the total bound radioligand never exceeded 10% of the radioligand added. The percentage of specific binding was approx. 53% and 35% of the total bound radioactivity for [3H]CD367 and [3H]9-cis-retinoic acid respectively. Saturation experiment data (Kd and Bmax values) were obtained by computer analysis of saturation curves. Linear regression of the Scatchard plot was performed according to the equation [Bound]/[Free] = Bmax.Kd/[Bound], where Kd is 1/Kd and [Bound] is the specific binding values of the radiolabelled ligand obtained at the [Free] incubation concentrations.

The concentrations of the unlabelled drugs needed to displace 50% of the bound radioligand (IC50 values) were obtained by non-linear regression analysis of the displacement curves. Inhibitory binding constants (Ki values) were calculated from the IC50 values according to the Cheng and Prusoff [19] equation Kd = IC50/[(1+[C*])/Kd*], where [C*] is the concentration of the radioligand and Kd* is the dissociation constant. All binding data were analysed using the GraphPad Prism software (GraphPad).

Statistical analyses

Results are means ± S.E.M. Statistical significance was determined by Student’s t test (Prism 4.0; GraphPad). P < 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Expression of RAR-β in HRPE cells

Expression of the six nuclear retinoid receptors in HRPE cells was first investigated by RT–PCR using primers validated previously [14,20]. Only RAR-β-specific primers produced a 400 bp amplification product of the expected size (Figure 1). Primer pairs specific for RAR-α, RAR-γ, RXR-α, RXR-β and RXR-γ did not yield any amplification products (results not shown), suggesting that only RAR-β is expressed in HRPE cells. As mentioned earlier, expression of nuclear retinoid receptors is not ubiquitous. For instance, haematopoietic cells have been reported to express RAR-α exclusively, whereas RAR-β transcripts are particularly abundant in the brain and areas undergoing programmed cell death [6]. This intriguing phenomenon suggested that cell propagation conditions should be considered a significant variable in influencing the biological behaviour. The replicative senescence of established cell lines has been well characterized [21]. Proteins which show a simple increase or a simple decrease may not always provide a significant contribution to the passage number, we next evaluated the
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Effect of passage number on RAR-β expression in HRPE cells. To study this, we measured the steady-state levels of RAR-β mRNA in early and late passage HRPE cells by semi-quantitative RT-PCR. The transcription of RAR-β was significantly up-regulated in late passage HRPE cells compared with early passage HRPE cells (Figure 2). The expression of RAR-β was also confirmed by immunoblot analysis using an anti-(RAR-β) antibody as described in the Experimental section (Figure 3). Crude extracts of HRPE cells obtained from the 5th and 35th cell passages and analysed for RAR-β expression showed an increase in the expression levels of RAR-β in extracts from the 35th passage, compared with the level of RAR-β obtained from the 5th passage. Thus the increased level of RAR-β observed by Western blot analysis in late passage HRPE cells was consistent with increased levels of transcript detected by semi-quantitative RT-PCR in RNA isolated from corresponding cells.

Effect of cell passage on telomere length

The shortening of telomeres has been proposed previously to be the most probable mechanism of cellular senescence [25,26], but not all phenotypes of senescent cells can be ascribed to the shortening of telomeres [27]. We next investigated the relationship between RAR-β expression and telomere length in HRPE cells. The effect of replicative aging of HRPE cells in multiple passages was evaluated by a telomere length assay at either early or late passages to determine telomeric DNA loss because of cell divisions in multiple passages [28]. Figure 4 shows a representative autoradiograph of smears of genomic DNA isolated from early and late passage HRPE cells hybridized with a DIG-labelled Telo TAGGG probe. The HRPE cells displayed a stabilized telomere at approx. 3.1 kb in both low and high passage cells, indicating that the length of the telomere is not affected by passage number in HRPE cells. When telomeres reach a critical shortening in length, senescence is induced, resulting in permanent proliferation arrest [29]. In the present study, for the first time, a typical biomarker of cellular senescence, such as RAR-β, has been shown to be up-regulated by repeated passage in a well-established cell line. Furthermore, this up-regulation is not due to or associated with shortening of telomeres. Taken together, these results suggest that the increase in RAR-β mRNA and protein levels detected in late passage HRPE cells does not appear to be due to a senescence condition.

![Figure 1 RT-PCR of RAR-β isoform expression relative to the constitutive gene 18S](image)

Total RNA was reverse-transcribed, and the resultant cDNA underwent PCR (35 cycles) using the primers described in Table 1. The RNA samples used were obtained from HRPE cells at the 35th cell passage. M, DNA ladder marker.

![Figure 2 Semi-quantitative RT-PCR of RAR-β](image)

cDNA of RAR-β and 18S rRNA, derived from HRPE cells at the 5th and 35th passages, were co-amplified by semi-quantitative RT-PCR. (A) The intensity of the RAR-β transcript is higher in HRPE cells at the 35th passage. (B) RAR-β mRNA expression in HRPE cells at the 5th and 35th passages was normalized against 18S RNA expression in separate RT-PCR and co-amplified RT-PCR. Results are means ± S.E.M. (n = 3). *P < 0.001. M, DNA ladder marker; P, passage.
In vitro binding assay

We first measured the specific binding of retinoic acid in cytosolic and nuclear extracts isolated from HRPE cells at 35th or higher passages by performing assays with 10 nM [3H]9-cis-retinoic acid (an agonist of both RAR and RXR subtypes) and saturation experiments with various concentrations (1–80 nM) of [3H]CD367 (an agonist of RAR subtypes only). No detectable binding was observed in cytosolic extracts using both of the radioligands (results not shown). This could be explained by the assumption that HRPE cells do not contain detectable levels of CRABPs. Indeed, it was already reported that RPE cell cultures established from adult donors, maintained in DMEM with FBS on a tissue-culture substrate, lose RPE-specific properties, such as pigmentation and expression of tyrosinase, CRABP and bestrerin [30]. Moreover, it was demonstrated that CRABP is expressed in adult human RPE, but not in the derived RPE cell lines [31]. A similar loss of CRABP was also reported to occur in HL-60 cells, where total retinoic acid binding was found to be associated with nuclear extracts, which were found to contain only nuclear RAR-α and no cytosolic binding proteins [17].

On the other hand, specific binding was observed with both [3H]9-cis-retinoic acid and [3H]CD367. The specific binding observed for [3H]9-cis retinoic acid was 133 ± 9 fmol/mg of protein (results not shown). Figure 5 shows the saturable binding of [3H]CD367 to the late passage HRPE cell nuclear extract. Scatchard analysis (Figure 5, inset) of the results was linear in the concentration range investigated (r = 0.982, P < 0.0001, n = 10) and, analogously, computer analysis of the saturation experiments suggested a one-site, rather than a two-site binding model. The dissociation binding constant (Kd) and Bmax values derived from the saturation experiments of [3H]CD367 were 9.6 ± 0.6 nM and 780 ± 14 fmol/mg of protein respectively.

The binding results obtained with radiolabelled ligands corroborates the specific expression of RAR-β in HRPE cells. 9-Cis-retinoic acid is known to be a high-affinity ligand of both RAR and RXR subtypes [32,33]; however RT–PCR and Western blotting results reported above show that HRPE cells selectively express the RAR-β subtype. Taking into account that CD367 is a relatively stable ligand and more specific to the RAR subtype than [3H]9-cis-retinoic acid [13], we have therefore chosen [3H]CD367 to characterize the binding properties of nuclear RARs. The linearity of the Scatchard plot reported in Figure 5 suggests that [3H]CD367 is binding to only one RAR subtype in nuclear extracts from HRPE cells, which, based on our RT–PCR and Western blot results, is RAR-β. The CD367 affinity (Kd = 9.6 nM) found in HRPE cells appears to be in agreement with the values obtained by previous binding studies towards RARs [15]. Furthermore, the Bmax value found in HRPE cells (approx. 800 fmol/mg of protein) is of the same order of magnitude as that obtained from COS cells expressing RAR-β cDNA (900 fmol/mg of protein) [15]. We have performed the
binding studies on nuclear extracts from HRPE cells at 35th or higher cell passage, when the cells show a significant up-regulation of RAR-β, as demonstrated by RT–PCR (Figure 2) and Western blot analysis (Figure 3) respectively. Taken collectively, our results indicate that the HRPE cell is a good model of an established cell line for characterizing RAR-β ligand specificity. Since the expression of RAR-β increases with increasing passage of the cells, the problem of poorly detectable RAR-β in native cells can be overcome by using late passage HRPE cells instead of overexpressing RAR-β by recombinant techniques.

To confirm the feasibility of using HRPE cells to characterize RAR-β ligands, we have measured the affinity of several flavonoids for RAR-β. We chose these compounds because of their xenestrogenic properties and their presence in natural food sources. Retinoids are considered to be agents of chemoprevention and differentiation in several proliferative diseases, including benign and malignant breast diseases. Knowledge of how retinoids interrupt proliferative processes pharmacologically or through diet, and how they induce differentiation and programmed cell death, will probably lead to new treatment strategies, especially in the prevention of breast cancer [20]. Therefore to investigate the interaction of flavonoids with RAR-β, we studied the effect of these flavonoids on the binding of [3H]CD367 to nuclear extracts from HRPE cells (Figure 6). The results obtained suggest flavonoids do interact with RAR-β, but only weakly. The $K_i$ values obtained from the analysis of the curves are presented in Table 2. It can be observed that the affinity of retinoic acid for RAR-β was in the nanomolar range (9.1 ± 0.6 nM), whereas the binding of the flavonoids was relatively weak, with $K_i$ values ranging from 0.9 ± 0.5 μM to 30 ± 2 μM, and thus may not be able to significantly interfere with the endogenous activity of RAR-β. But what is important is that the methodology that we report in this study can be utilized to characterize even relatively weak interactions between flavonoids and RAR-β.

In conclusion, we report the specific expression of RAR-β in HRPE cells, and this expression increases with increasing passage of the cells. The increased RAR-β expression in aging cells is not associated with a decrease in telomere length. We also show that the increased expression in high passage cells can be exploited to develop a specific assay to characterize RAR-β–ligand interactions.

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