Volume 273, number 1,2, 19–22 FEBS 08983 October 1990

The biological activity of retinoids in melanoma cells

Induction of expression of retinoic acid receptor-β by retinoic acid in S91 melanoma cells

Christopher P.F. Redfern, Ann K. Daly*, Julie A.E. Latham and Carole Todd

Medical Molecular Biology Group, Department of Dermatology, Medical School, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne, NE4 2HH, UK

Received 30 July 1990

The expression of mRNA for retinoic acid receptor beta (RAR-β) was induced by *all trans*-retinoic acid in murine S91 melanoma cells. The induction of RAR-β was dose-dependent, rapid and insensitive to cycloheximide. Both 13-*cis*-retinoic acid and 3,4-didehydro-*all trans*-retinoic acid also induced expression of RAR-β but were only effective at concentrations 100-fold greater than *all trans*-retinoic acid. The expression of RAR-α and RAR-γ was unaffected by retinoic acid.

Retinoic acid; Retinoic acid receptor; Melanoma; RAR-β; 3,4-Didehydroretinoic acid

1. INTRODUCTION

The biological effects of retinoic acid and its derivatives are likely to be mediated, at least in part, by retinoic acid receptors (RAR), a class of nuclear retinoic acid binding proteins [1,2] closely related to steroid and thyroid hormone receptors [3–6]. Three types of RAR, RAR-α, RAR-β and RAR-γ, have so far been identified in rodents and humans and are highly conserved between species [7]. At least two transcripts for each type of RAR can be identified by Northern blotting [8,9] and in the case of RAR-γ the two major transcripts represent alternatively spliced products differing in sequence at the amino terminus of the predicted protein [9]. Patterns of expression of RAR transcripts differ markedly between tissues [8,10] and the diversity of biological effects of retinoic acid is thus presumably mediated by different patterns of RAR-gene expression and different splicing patterns in different tissues and cell types.

Retinoic acid inhibits the proliferation of many cell types in vitro and this may be accompanied by the expression of different cell phenotypes and differentiation markers, such as in the case of some teratocarcinoma and melanoma lines [11,12]. For example, S91 murine melanoma cells respond to retinoic acid by a reduction in proliferation and an increase in pigmentation [12]. Here, we show that retinoic acid also induces RAR expression in murine S91 melanoma cells and, using this response as a bioassay, we have compared the biological potency of retinoic acid with its 13-*cis* isomer and the 3,4-didehydro derivative.

Correspondence address: C.P.F. Redfern, Medical Molecular Biology Group, 4th Floor, Cookson Building, Medical School, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK

**Present address:* Department of Pharmacological Sciences, University of Newcastle upon Tyne, Medical School, Framlington Place, Newcastle upon Tyne, UK

2. MATERIALS AND METHODS

2.1. *Cell culture*

S91 melanoma cells (strain M3) were from Flow Laboratories, Irvine, Scotland, and were cultured in an atmosphere of 5% CO_2 in air at 37°C in Dulbecco's minimal essential medium (DMEM), containing 10% foetal calf serum (FCS). Cells were seeded at a density of 1.1 × 10^5 cells per cm^2 growth area in either 75 or 25 cm^2 tissue culture flasks; solutions of *all trans*-retinoic acid (Sigma), 13-*cis*-retinoic acid (Roche Products, Welwyn Garden City, Herts) or 3,4-didehydro-*all trans*-retinoic acid (Hoffmann-La Roche, Basel) in ethanol were added to final concentrations within the range 0.1–1000 nM after allowing the cells to attach overnight. An equal volume (< 5 μl per 20 ml medium) of ethanol was added to control cultures. Concentrations of the stock solutions for all 3 retinoids were estimated using an extinction coefficient of 36,500 at 343 nm. Cycloheximide was used at a concentration of 10 $\mu g \cdot ml^{-1}$. Actinomycin D was dissolved in methanol and added to a final concentration of 5 $\mu g \cdot ml^{-1}$.

For experiments with cAMP, dibutyryl cAMP was dissolved in DMEM and added to cells to a final concentration of 1 mM; isobutylmethylxanthine (IBMX) was dissolved in 1 M NaOH at 100 mM and added to cells to give a final concentration of 1 mM; α-melanocyte stimulating hormone (α-MSH) was dissolved in DMEM and added to cells to a final concentration of 0.1 μM.

2.2. *RNA extractions and Northern blotting*

In some initial experiments, total cellular RNA was prepared by the guanidinium isothiocyanate/caesium chloride method [13]. Other-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/90/$3.50

wise, total cytoplasmic RNA was prepared by the 'miniprep' method of Wilkinson [14]. RNA (30 μg per track for 1 cm wide slots) was size-fractionated on 1.2% agarose/formaldehyde gels and transferred by vacuum blotting with 1.8 M NaCl, 0.01 M EDTA, 0.1 M sodium phosphate, pH 7.4, (10× SSPE) to nylon membranes (Schleicher and Schuell). Membranes were hybridised [15] at 42°C with ^{32}P-labelled probe using 50% formamide, 6× SSPE, 0.2% w/v Ficoll 400, 0.2% w/v polyvinylpyrrolidone, 0.2% bovine serum albumin (fraction V), 0.5% SDS, 5% Dextran Sulphate and 100 μg/ml single-stranded carrier DNA [16] as the prehybridization and hybridization buffer. After hybridisation, membranes were washed 3–4 times in 1× SSPE, 0.1% SDS for >15 min each at 68°C and exposed to X-ray film with intensifying screens at −70°C. For quantitative autoradiography, X-ray film was preflashed [15] and the autoradiographs scanned using an LKB laser scanning densitometer.

2.3. *Probes*

The RAR-α probe was a *KpnI/SacI* fragment (503 bp) from the 5′ end of the human RAR-α cDNA [3]. The human RAR-β probe consisted of the complete cDNA insert (1400 bp) of the plasmid pCOD20 [5]. The human RAR-γ probe was the full length cDNA insert (1500 bp) [7]. To control for RNA loading, membranes were reprobed with a rat β-actin probe consisting of a 1200 bp *BglI* fragment of the pRβA-1 cDNA clone isolated by P. Gunning. Probes were labelled with [^{32}P]dCTP (Amersham International, 3000 Ci·mmol^{-1}) to a specific activity of approximately 10^9 dpm·μg^{-1} [17]. The human RAR-α and RAR-γ cDNA probes were provided by Martin Petkovich and Pierre Chambon, Strasbourg, France and the RAR-β probe by Anne Dejean, Paris, France.

3. RESULTS

3.1. *Expression of mRNA for RAR-α, RAR-β and RAR-γ in S91 melanoma cells*

We have previously reported [2] that RAR-α and RAR-β mRNA are present in poly-(A)$^+$ RNA from S91 cells, although the signal intensity for RAR-α was very low by comparison with some other cell lines. In subsequent studies on S91 cells using total cytoplasmic RNA, 2.8 and 3.6 kb RAR-α transcripts and a single 3.4 kb RAR-β transcript were clearly detectable (Fig. 1). The RAR-γ probe detects transcripts approximately 3.3 kb in size in human [7] and rat tissues (Rees and Redfern, unpublished results); in S91 melanoma cells, the RAR-γ probe detected transcripts of a similar size, but these were expressed at a low level compared to RAR-α and RAR-β. When S91 cells were treated with *all trans*-retinoic acid, there was a marked stimulation of expression of RAR-β (Fig. 1). A similar induction of RAR-β

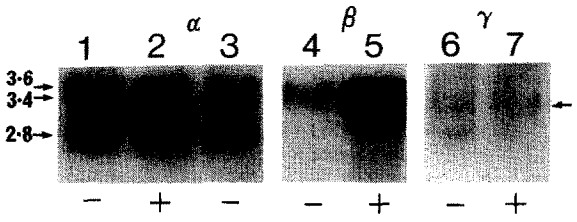

Fig. 1. Northern blot of total cytoplasmic RNA from S91 cells treated with 10^{-6} M retinoic acid (lanes 2, 5 and 7: '+') or ethanol as control (lanes 1, 3, 4 and 6: '−') and probed with RAR-α (lanes 1, 2 and 3), RAR-β (lanes 4 and 5) and RAR-γ (lanes 6 and 7).

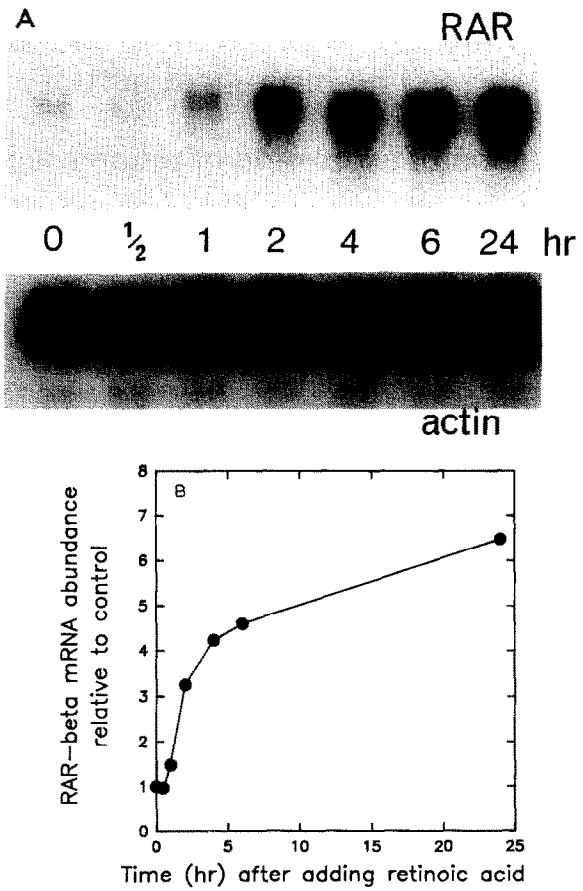

Fig. 2. Time course (hours) of induction of RAR-β in response to 10^{-7} M retinoic acid. (A) Northern blot of RNA probed for RAR-β (top panel) and β-actin (bottom panel). (B) Induction of RAR-β with time as a proportion of zero-time control, corrected for β-actin signal intensity.

in response to retinoic acid was also observed in the B16 F1 and F10 murine melanoma sublines (data not shown). Although there was also an apparent induction of a novel RAR-α transcript running just ahead of the 3.6 kb RAR-α transcript (Fig. 1), this coincides in position with the RAR-β transcript, was not detectable in blots washed at higher stringency and may therefore be due to cross hybridisation of the RAR-α probe with

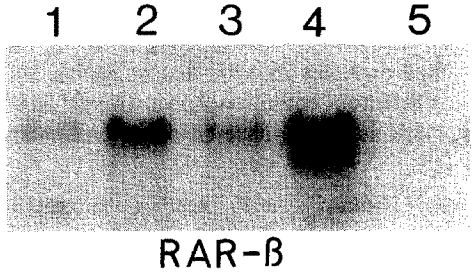

Fig. 3. Induction of RAR-β after treating S91 cells for 8 h with retinoic acid (10^{-6} M) in the presence of absence or cycloheximide (10 μg · ml^{-1}). Northern blot probed for RAR-β. Lane 1, control; lane 2, retinoic acid (10^{-6} M); lane 3, cycloheximide alone; lane 4, cycloheximide and retinoic acid; lane 5, retinoic acid and actinomycin D.

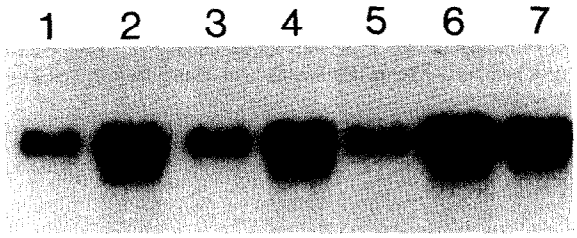

Fig. 4. Induction of RAR-β in response to 10^{-7} M retinoic acid in the presence of dibutyryl cAMP, IBMX (phosphodiesterase inhibitor) and α-MSH. Northern blot probed for RAR-β. Lane 1, control; lane 2, retinoic acid alone; lane 3, dibutyryl cAMP alone; lane 4, retinoic acid and dibutyryl cAMP; lane 5, IBMX alone; lane 6, retinoic acid and IBMX; lane 7, retinoic acid and IBMX in the presence of α-MSH.

RAR-β transcripts. There was no evidence for induction of RAR-γ in response to retinoic acid (Fig. 1).

3.2. *Induction of expression of RAR-β is rapid and independent of protein synthesis*

The induction of RAR-β in S91 cells is a rapid response to retinoic acid and was detectable within 1–2 h of addition of *all trans*-retinoic acid, producing a 6–7-fold increase in RAR-β expression by 24 h (Fig. 2). This response was not inhibited by cycloheximide (Fig. 3). However, the presence of cycloheximide did induce a low level of expression of a smaller RAR-β transcript. In the presence of actinomycin D, the induction of RAR-β in response to retinoic acid was completely abolished (Fig. 3).

RAR-β is reportedly induced by retinoic acid in F9 embryonal carcinoma cells and this effect is inhibited by culturing the cells with the cAMP analogue, dibutyryl cAMP [18]. However, in S91 cells, incubation with dibutyryl cAMP or the phosphodiesterase inhibitor IBMX in the presence and absence of αMSH, treatments known to produce a 4–8-fold increase in intracellular cAMP levels in S91 cells [19], had no detectable effect on the induction of RAR-β by retinoic acid (Fig. 4).

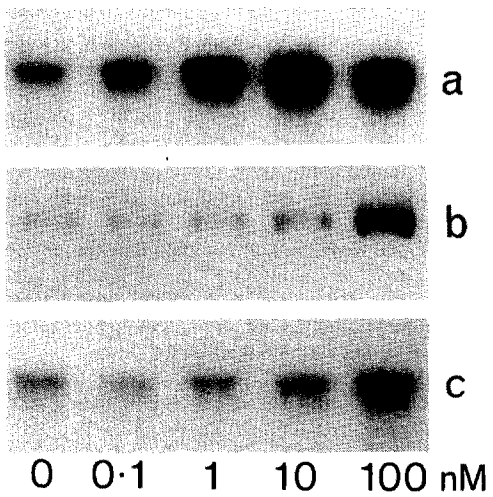

Fig. 5. Dose-dependence of the induction of RAR-β in response to incubation for 6 h with 0.1 nM, 1 nM, 10 nM, 100 nM *all trans*-retinoic acid (panel a), 13-*cis*-retinoic acid (panel b) and 3,4-didehydroretinoic acid (panel c). Control (no retinoic acid) = 0. Northern blot probed for RAR-β.

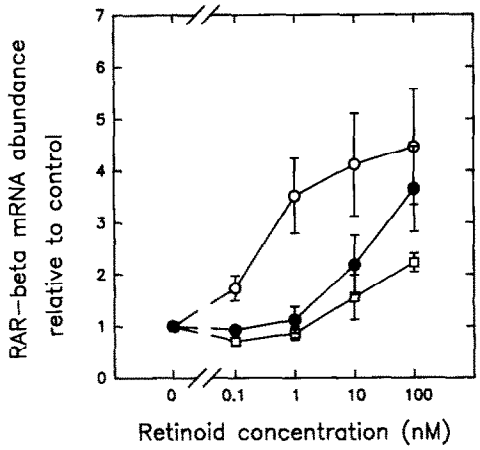

Fig. 6. Dose-dependence of RAR-β induction expressed as a proportion of the untreated control and corrected for the β-actin signal intensity. Error bars: ± SE. *all trans*-retinoic acid (o, $n=3$), 13-*cis*-retinoic acid (•, $n=4$). 3,4-didehydroretinoic acid (□, $n=2$).

3.3. *Dose-dependent induction of RAR-β by* all trans-*retinoic acid, 13-*cis-*retinoic acid and 3,4-dide-hydro-*all trans-*retinoic acid*

The induction of RAR-β was dose-dependent (Fig. 5), detectable with 0.1 nM *all trans*-retinoic acid, and gave a response 50% of maximal within the concentration range 0.1–1 nM (Fig. 6). The 13-*cis* isomer of retinoic acid was much less effective, inducing a detectable response at a concentration of 10 nM with a response 50% of maximal within the range 10–100 nM. The 3,4-didehydro derivative, recently reported to be 6-fold more abundant than *all trans*-retinoic acid in chick limb buds [20], only detectably induced RAR-β expression at concentrations above 10 nM and gave a poorer response than 13-*cis*-retinoic acid (Fig. 6).

4. DISCUSSION

S91 melanoma cells are clearly similar to human hepatocarcinoma cells with respect to the retinoic acid-mediated induction of RAR-β [21]. The rapidity of the response and insensitivity to cycloheximide suggests that, as in hepatocarcinoma cells [21], the induction of RAR-β is a direct transcriptional effect. A retinoic acid-mediated induction of RAR-β has also been observed in F9 teratocarcinoma cells but in this cell type the response may be less rapid than in either S91 or hepatocarcinoma cells and, unlike S91 cells, is inhibited by cAMP [18]. The retinoic acid-mediated induction of RAR-β shows considerable cell type specificity and also occurs in normal dermal fibroblasts but not in HL60 promyelocytic leukaemia cells or normal human keratinocytes (Daly and Redfern, unpublished data).

Although a retinoic acid response element (RARE) has been identified at the 5′ end of the RAR-β gene [22], it is clear that other factors also regulate the expression of RAR-β. Both RAR-α and RAR-β are expressed at a low level in unstimulated S91 cells and it is not yet clear whether the retinoic acid-mediated induction of RAR-β represents a positive autoregulatory loop, or is mediated by RAR-α, RAR-γ or by an as yet unknown mechanism.

The dose response studies reported here are in agreement with K_D estimates of 10^{-10} to 10^{-9} M for RAR-β [5]. The 13-*cis* isomer is much less effective than *all trans*-retinoic acid in inducing expression of RAR-β. In addition to nuclear retinoic acid receptors, a low molecular weight, cytosolic retinoic acid binding protein (CRABP) is present in many retinoic acid responsive cell lines and could act as a shuttle protein delivering retinoic acid to nuclear receptors (for review see [23]). Since 13-*cis*-retinoic acid has a 25-fold lower affinity for CRABP than does *all trans*-retinoic acid [24] the existence of such a system could result in a poor response to 13-*cis*-retinoic acid. However, the role of CRABP is not known and in S91 (clone M3) cells CRABP has been reported to be below detectable limits [25], although we have found low levels of CRABP in S91 cells (Daly and Redfern, unpublished data). In F9 cells, where CRABP is present at a level comparable to S91 cells (Daly and Redfern, unpublished data), it has been shown that there is a good correlation between the biological potency of retinoids and affinity for nuclear retinoic acid receptors [26]; it is thus likely that the differential response of S91 cells to the two retinoic acid isomers reflects a true difference in their respective affinities for RARs.

3,4-didehydroretinoic acid has recently been identified in chick limb buds where it is present at concentrations 6-fold higher than *all trans*-retinoic acid and is reportedly equipotent with *all trans*-retinoic acid in disrupting normal morphogenesis [20]. However, in S91 cells this retinoid has a much lower activity than *all trans*-retinoic acid in inducing expression of RAR-β. If this response is indeed RAR-mediated, the high degree of similarity of the ligand binding regions between the different RAR types [7] argues that 3,4-didehydroretinoic acid itself may not be an important RAR ligand in vivo. Nevertheless, a distinct class of retinoid receptor, designated RXR, has recently been described [27]. Although retinoic acid can activate RXR, the high concentrations of retinoic acid required [27] suggest that it is not the true ligand. It is thus possible that a distinct class of retinoid receptor, such as RXR, may exist for which 3,4-didehydroretinoic acid is the natural ligand.

Acknowledgements: This research was supported by a grant from the North of England Cancer Research Campaign to CPFR. We thank Pierre Chambon, Martin Petkovich and Anne Dejean for gifts of RAR probes.

REFERENCES

[1] Daly, A.K. and Redfern, C.P.F. (1987) Eur. J. Biochem. 168, 133–139.
[2] Daly, A.K., Rees, J.L. and Redfern, C.P.F. (1989) Exp. Cell Biol. 57, 281–366.
[3] Petkovich, M., Brand, N., Krust, A. and Chambon, P. (1987) Nature 330, 444–450.
[4] Giguere, V., Ong, E.S., Segui, P. and Evans, R.M. (1987) Nature 330, 624–629.
[5] Brand, M., Petkovich, M., Krust, A., Chambon, P., de The, H., Marchio, A., Tiollais, P. and Dejean, A. (1988) Nature 332, 850–853.
[6] Benbrook, D., Lernhardt, E. and Pfahl, M. (1988) Nature 333, 669–672.
[7] Krust, A., Kastner, P., Petkovich, M., Zelent, A. and Chambon, P. (1989) Proc. Natl. Acad. Sci. USA 86, 5310–5314.
[8] Rees, J.L., Daly, A.K. and Redfern, C.P.F. (1989) Biochem. J. 259, 917–919.
[9] Kastner, P., Krust, A., Mendelsohn, C., Garnier, J.M., Zelent, A., Leroy, P., Staub, A. and Chambon, P. (1990) Proc. Natl. Acad. Sci. USA 87, 2700–2704.
[10] Zelent, A., Krust, A., Petkovich, M., Kastner, P. and Chambon, P. (1989) Nature 339, 714–717.
[11] Strickland, S. and Mahdavi, M. (1978) Cell 15, 393–403.
[12] Lotan, R., Neumann, G. and Lotan, D. (1981) Ann. NY Acad. Sci. 359, 150–170.
[13] Chirgwin, J.M., Przybyla, A.E., McDonald, R. and Rutter, W.J. (1979) Biochemistry 18, 5294–5299.
[14] Wilkinson, M. (1988) Nucleic Acids Res. 16, 10933.
[15] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd edn., Cold Spring Harbour Laboratory Press, Cold Spring Harbour.
[16] Sargent, T.D. (1987) Methods Enzymol. 152, 423–432.
[17] Feinberg, A.P. and Vogelstein, B. (1983) Anal. Biochem. 132, 6–13.
[18] Hu, L. and Gudas, L. (1990) Mol. Cell. Biol. 10, 391–396.
[19] Friedmann, P.S. and Gilchrest, B.A. (1987) J. Cell. Physiol. 133, 88–94.
[20] Thaller, C. and Eichele, G. (1990) Nature 345, 815–819.
[21] de The, H., Marchio, A., Tiollais, P. and Dejean, A. (1989) EMBO J. 8, 429–433.
[22] de The, H., Vivanco-Ruiz, M., Tiollais, P., Stunnenberg, H. and Dejean, A. (1990) Nature 343, 177–180.
[23] Roberts, A.B. and Sporn, M.B. (1984) in: The Retinoids (Sporn, M.B., Roberts, A.B. and Goodman, D.S. eds) vol. 2, pp. 209–286.
[24] Daly, A.K. and Redfern, C.P.F. (1988) Biochim. Biophys. Acta 965, 118–126.
[25] Haussler, M.R., Donaldson, C.A., Kelly, M.A., Mangelsdorf, D.J., Bowden, G.T., Meinke, W.J., Meyskens, F.L. and Sidell, N. (1984) Biochim Biophys Acta 803, 54–62.
[26] Darmon, M., Rocher, M., Cavey, M.-T., Martin, B., Rabilloud, T., Delescluse, C. and Shroot, B. (1989) Skin Pharmacol. 1, 161–175.
[27] Mangelsdorf, D.J., Ong, E.S., Dyck, J.A. and Evans, R.M. (1990) Nature 345, 224–229.