Hormone-induced changes in nuclear receptor stoichiometry in HL60 cells correlate with induction of monocyte or neutrophil differentiation

P G McTernan, M C Sheppard and G R Williams¹

Department of Medicine, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK and ¹Molecular Endocrinology Group, Department of Medicine, Imperial College School of Medicine, London W12 0NN, UK

(Requests for offprints should be addressed to G R Williams, Molecular Endocrinology Group, Department of Medicine, Clinical Sciences Centre, Imperial College School of Medicine, The Hammersmith Campus, Hammersmith Hospital, Du Cane Road, London W12 0NN, UK)

Abstract

HL60 cells differentiate to monocytes or neutrophils in response to $1\alpha,25(\text{OH})_2$ -vitamin D_3 (D_3) and retinoids respectively. D_3 and retinoid actions converge since their receptors (VDR, RAR) heterodimerise with a common partner, RXR, which also interacts with thyroid hormone (T_3) receptors (T_3R). HL60 cells were treated with combinations of D_3 and retinoids to induce differentiation and to investigate whether increased VDR or RAR expression correlated with monocyte or neutrophil differentiation and whether altered receptor concentrations affected DNA-binding specificity. As assessed by Western blotting, VDR and RXR expression was unchanged in monocytes relative to controls but levels of RAR and T_3R were reduced. In contrast, only VDR expression was

reduced in neutrophils. T_3 did not promote differentiation or influence its induction by D_3 or retinoids and did not affect expression of any receptor. Gel mobility-shift analysis revealed that nuclear extracts from undifferentiated cells, monocytes and neutrophils interacted differently with VDRE-, RARE- and RXRE-binding sites. Monocyte nuclear protein/DNA complexes contain readily detectable VDR and RXR whereas neutrophil complexes contain RAR and RXR. Thus hormone-induced changes in receptor stoichiometry favour either VDR/RXR or RAR/RXR heterodimerisation and correlate with hormone-induced differentiation of HL60 cells to monocytes or neutrophils respectively.

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Introduction

Human promyelocytic leukaemia cells (HL60) differentiate to monocytes or granulocytes (neutrophils) in response to specific stimuli. In particular, treatment with $1\alpha,25$ -dihydroxyvitamin D_3 (D_3) promotes monocyte differentiation whilst retinoids induce neutrophil differentiation (Collins *et al.* 1977, Breitman *et al.* 1980). We have also shown that retinoids synergise with D_3 to promote monocyte differentiation (Brown *et al.* 1994). Furthermore, variation of D_3 and retinoid concentration ratios over an extensive range promotes differentiation along either the monocyte or neutrophil pathway (not both) or induces cellular apoptosis (Bunce *et al.* 1995). The synthetic D_3 analogues, EB1089 and KH1060, also effectively induce monocyte differentiation of HL60 cells but their relative potencies are inversely correlated with their ability to synergise with retinoids (Brown *et al.* 1994).

D_3 and retinoid actions are mediated by related nuclear receptors that are members of the steroid/thyroid hormone receptor superfamily (Evans 1988). The receptors act as ligand-inducible transcription factors which bind to

hormone response element DNA sequences in target gene promoters and regulate their transcriptional activation or repression (Katzenellenbogen *et al.* 1996). There is a single receptor for D_3 (VDR) (Ozono *et al.* 1991), three separate genes encoding receptors for all-*trans*-retinoic acid (RAR α , β , γ) (Mattei *et al.* 1991) and three encoding receptors for 9-*cis*-retinoic acid (RXR α , β , γ) (Mangelsdorf *et al.* 1992, Mangelsdorf 1994). VDR and RAR proteins preferentially bind to related response elements (VDRE and RARE) as heterodimers with their common partner, RXR, which can also form functional heterodimers with tri-iodo-thyronine (T_3) receptor (T_3R) (Kliwer *et al.* 1992, Leid *et al.* 1992, Glass 1994). In addition, VDR, RAR and T_3R proteins can heterodimerise with each other (Rosen *et al.* 1993, Schrader *et al.* 1994) or bind to DNA as homodimers (Yen *et al.* 1992, Zhang *et al.* 1992, Cheskis & Freedman 1994). Precise roles for each possible receptor dimer combination have yet to be determined and it is not clear which receptor complexes occur *in vivo*, although the high-affinity RXR-containing heterodimers are considered to be predominant and the most physiologically important. A large number of

in vitro biochemical and transfection studies have shown that the control of specific hormone responses by VDR, RAR, RXR and T_3 R is complex and hierarchical (Glass 1994, Williams 1994, Katzenellenbogen *et al.* 1996). The many receptor variants and their heterodimerisation potential leads to an array of possible receptor dimers, and the likelihood of complex interactions between their signalling pathways, in cells that co-express multiple receptors. Such interactions vary according to the target gene and cell type studied and may be modified by changes in receptor stoichiometry and the relative concentrations of activating hormones as well as by other recently characterised adaptor proteins (Williams 1994, Katzenellenbogen *et al.* 1996). It is not yet known which, if any, of these mechanisms are associated with the induction of monocyte versus neutrophil differentiation in HL60 cells by D_3 and retinoids.

Using previously defined hormone treatment combinations to induce monocyte or neutrophil differentiation of HL60 cells (Brown *et al.* 1994, Bunce *et al.* 1995), we have tested the possibility that preferred activation of a particular signalling pathway may be associated with increased expression of its cognate nuclear receptor by addressing the following hypotheses. First, that VDR-mediated stimulation of HL60 monocyte differentiation correlates with induction of VDR expression and; secondly, that retinoid-induced HL60 neutrophil differentiation correlates with increased RAR or RXR expression. We further investigated whether changes in relative concentrations of expressed nuclear receptors in hormone-induced HL60 cells were associated with altered DNA-binding specificity of nuclear extracts to hormone response elements.

Materials and Methods

HL60 cell culture and reagents

Cultures of HL60 cells were maintained in serum-free RPMI 1640 medium (Gibco, Paisley, UK) containing penicillin (100 units/ml) and streptomycin (100 µg/ml), supplemented with insulin, transferrin, Se_2O_3 , linoleic acid and BSA (ITS⁺; Advanced Protein Products, Smethwick, UK) as described (Brown *et al.* 1994). HL60 cells were maintained in exponential growth from a seeding density of 2.5×10^5 cells/ml to a maximum density of 1.25×10^6 cells/ml. Stock solutions of 3,5,3'-L-triiodothyronine (T_3) (Sigma Chemical Co., Poole, Dorset, UK) (10^{-4} M in 0.02 M NaOH), D_3 , EB1089 ((1S,3R)-dihydroxy-(20R)-(5'-ethyl-5'-hydroxyhepta-(1'E,3'E)-diene-1'-yl)-9,10-secopregna-(5Z,7E),10(19)-triene) and KH1060 ((1S,3R)-dihydroxy-(20R)-(4'-hydroxy-4'ethyl-1'-hexyloxy)-9,10-secopregna-(5Z,7E),10(19)-triene) (Leo Pharmaceutical Products Ltd A/S, Ballerup, Denmark) (4×10^{-3} M in isopropanol), all-*trans*-retinoic acid (RA) (Sigma) (10^{-2} M in methanol) and 9-*cis*-retinoic acid (9*cis*RA) (Hoffman-La Roche, Nutley, NJ, USA) (10^{-4} M in ethanol) were diluted in medium.

Induction of monocyte differentiation

The synthetic analogues EB1089 and KH1060 are more potent than the parent compound, D_3 , in suppressing proliferation of the promonocyte cell line U937 (Binderup *et al.* 1991) and in promoting monocyte differentiation of HL60 cells (Brown *et al.* 1994). Monocyte differentiation of HL60 cells was induced by addition of either D_3 or analogue alone, or in combination with either RA or 9*cis*RA, to growing cells initially seeded at 2.5×10^5 cells/ml and maintained in exponential growth to a maximum density of 1.25×10^6 cells/ml over 5 days. In time course experiments, identically seeded and maintained cells were harvested at 0, 1, 2, 3, 4 and 5 days after induction of differentiation (D_3 , 50 nM) and compared with matched untreated control cells at each time point. The concentrations of RA, D_3 and synthetic analogues used to induce monocyte differentiation were derived in previous studies (Brown *et al.* 1994, Bunce *et al.* 1995) and are indicated in the legends to Figs 2, 3 and 4.

Induction of neutrophil differentiation

Neutrophil differentiation of HL60 cells was induced by addition of RA alone, or in combination with a low concentration of D_3 which does not itself promote HL60 differentiation (Bunce *et al.* 1995), to growing cells initially seeded at 2.5×10^5 cells/ml and maintained in exponential growth to a maximum density of 1.2×10^6 cells/ml over 5 days. The concentrations of agents used to induce neutrophil differentiation were derived in previous studies (Bunce *et al.* 1995) and are indicated in the legend to Fig. 4.

Effect of T_3 on induction of monocyte and neutrophil differentiation

The effect of T_3 on induction of HL60 cell differentiation was examined by inducing monocyte or neutrophil differentiation over 5 days as above, in the absence or presence of T_3 (1 nM or 100 nM). The concentrations of agents used in individual experiments are indicated in the legend to Fig. 5. The maximum final concentrations of methanol, ethanol, isopropanol and NaOH vehicles used in hormone treatments were 1/100 000, 1/1000, 1/40 000 and 1/1000 (v/v) dilutions respectively. Control cultures treated with any vehicle at the upper concentrations showed no evidence of cell differentiation in any of the inductions described above.

Assessment of cell differentiation

Non-specific differentiation of HL60 cells to either monocytes or granulocytes (neutrophils) was assessed by measurement of the proportion of cells that could phagocytose complement-coated yeast (Toksoz *et al.* 1982). To determine whether mature HL60 cells had differentiated

to either neutrophils or monocytes, cytocentrifuged preparations of cells were stained for monocyte-specific esterase using α -naphthylacetate ester as substrate (Yam *et al.* 1971) and with May–Grunwald–Giemsa for assessment of cell morphology as previously described (Brown *et al.* 1994, Bunce *et al.* 1995).

Nuclear protein preparation

Nuclear protein preparations were obtained as previously described for osteosarcoma cells (Williams *et al.* 1994, 1995). Briefly, HL60 cells were cultured in 175 cm² flasks and maintained in RPMI 1640 medium for 5-day treatment periods. Cells were washed with PBS, pelleted and resuspended in 4 ml STM (sucrose 0.25 M, Tris–HCl 20 mM, MgCl₂ 1.1 mM, pH 7.85) + 0.5 mM phenylmethylsulphonyl fluoride (PMSF; Sigma). Cells were pelleted at 800 g, 4 °C, 10 min and resuspended in 3.5 ml STM+PMSF containing 0.5% Triton X-100 (Sigma) and incubated on ice for 10 min. This step was repeated and the final nuclear pellet resuspended in 300 µl STM+PMSF containing dithiothreitol 5 mM, KCl 0.4 M and glycerol 20% (v/v). The nuclear suspension was agitated on ice for 15 min to extract nuclear proteins and centrifuged (2900 g, 4 °C, 15 min). Supernatants containing nuclear extracts were aliquoted, protein concentrations determined, and stored at –80 °C.

Protein electrophoresis and Western blotting

Nuclear proteins (1–20 µg per lane) were separated by SDS/PAGE and electroblotted on to Immobilon P membrane (Millipore Ltd, Watford, Herts, UK) as described (Williams *et al.* 1994, 1995). Membranes were blocked (1 h, 25 °C) in PBS-T (PBS + 0.1% Tween 20; Sigma) containing 20% (w/v) non-fat milk powder (Marvel; Premier Brands (UK) Ltd, Stafford, Staffs, UK). Filters were incubated overnight at 4 °C with receptor-specific primary antibodies. T₃R antibodies were diluted 1:500 in PBS-T (0.05%). VDR, RXR and RAR α antibodies were diluted 1:1000 in PBS-T (0.05%) containing 0.25% BSA. For detection of T₃R isoforms, VDR and RXRs, the secondary antibody (horseradish peroxidase-conjugated; Amersham) was diluted 1:75 000 in PBS-T (0.05%), and for detection of RAR α the secondary antibody was diluted 1:60 000 in PBS-T (0.05%). Specific receptor proteins were detected by enhanced chemiluminescent assay (ECL; Amersham) after exposure of filters to X-ray film for 1–20 min. Filters were probed with the following primary antibodies: polyclonal against human T₃R α 1, c-erb A α 2 and T₃R β 1 proteins (Macchia *et al.* 1990, Falcone *et al.* 1992), polyclonal against rat RAR α (Ali *et al.* 1992), and monoclonal against human VDR (Pike *et al.* 1982) (purchased from Cambridge Research Biochemicals, Zeneca, Northwich, UK). A monoclonal antibody against mouse RXR, which recognises the α , β and γ

RXR isoforms (4RX 1D12, (Rochette Egly *et al.* 1994)), was used to detect RXR protein. Control experiments were included in which primary antibody was omitted and filters were exposed to secondary antibody and ECL detection only. No protein bands were detected in these control experiments using samples derived from cultures containing control or differentiated HL60 cells (data not shown).

Gel mobility-shift assays

Gel mobility-shift experiments were performed as described (Williams *et al.* 1991, Brent *et al.* 1992). Oligonucleotide probes were radiolabelled with [³²P]dTTP by Klenow fill-in reactions and column purified. Fresh nuclear protein preparations were prepared from undifferentiated HL60 cells or cells induced to differentiate to monocytes (D₃ 5 nM + 9 α sRA 2.5 nM) or neutrophils (D₃ 10^{–13} M + RA 500 nM) for analysis in gel shifts. The absence or presence of cell differentiation was confirmed in each culture before nuclear protein preparation. Nuclear extracts were not supplemented with additional hormone during gel shift analysis. Labelled probe (375 c.p.m.) was incubated at room temperature for 30 min with 5 µg nuclear protein from undifferentiated or hormone-treated cells in 30 µl reaction mixtures in the presence of 200 µg/ml poly(dI–dC) (Pharmacia), 20 mM HEPES, pH 7.8, 50 mM KCl, 1 mM dithiothreitol, 5 µg BSA (Fraction V; Sigma) and 20% glycerol. Competition incubations contained 25–1000-fold molar excess of unlabelled competitor oligonucleotide. Antibody studies involved addition of 2.5 µl of appropriate antibody to binding reaction mixtures 15 min before co-incubation with oligonucleotide probe for a further 30 min. Reaction products were resolved by non-denaturing PAGE (200 V at 4 °C). The 5% Tris/borate/EDTA (TBE) gels were prerun for 90 min at 4 °C in 1 × TBE buffer before loading. Gels were dried under vacuum and autoradiographed at –70 °C overnight.

Oligonucleotide sequences

Double-stranded oligonucleotides were synthesised with BamHI restriction site overhangs at each end and contained two perfect AGGTCA motifs arranged in a direct repeat configuration (DR) and separated by 1, 3, 4 or 5 spacer nucleotides to give the following sequences: DR+1: 5' TCAGGTCAGAGGTCAGAG 3'; DR+3: 5' TCAGGTCAGAGGTCAGAG 3'; DR+4: 5' TCAGGTCACAGGAGGTCAGAG 3'; and DR+5: 5' TCAGGTCAGAGCAGGTCAGAG 3'. The DR+1, DR+3, DR+4 and DR+5 oligonucleotides contain consensus idealised RXRE, VDRE, T₃RE and RARE sequences respectively (Umesono *et al.* 1991, Zechel *et al.* 1994, Katz *et al.* 1995). An oligonucleotide derived from the proximal promoter region of the human γ globin gene was used as a non-specific competitor and contained the

following sequence: 5' TCCAGTGAGGCCAGGGGCC GCGGCTGGCTAGGGATGA 3' (Cunningham *et al.* 1994).

Statistical analysis

Analyses of the linear quantifications of receptor expression in Western blots were performed using Cricket Graph III computer software (Computer Associates, Slough, Berks, UK). Changes in expression of receptor proteins are presented graphically as mean $\pm$ s.e.m. receptor level in differentiated cells relative to standardised controls derived from untreated undifferentiated HL60 cells. All experiments were performed on at least three separate occasions. All values were corrected relative to the total nuclear protein concentration in each individual extract. Statistical analysis was performed using an Instat v2.04a computer program (GraphPad Software, San Diego, CA, USA). Group and multiple comparisons were analysed initially by ANOVA. When statistical differences were obtained they were analysed further by Tukey-Kramer Multiple Comparison post tests.

Results

Hormone induction of HL60 cell differentiation

Treatment of HL60 cells with the following hormone combinations (D_3 (5 nM) + 9*cis*RA (2.5 nM); D_3 (5 nM) + RA (10 nM); D_3 (50 nM); KH1060 (0.3 nM); EB1089 (1.25 nM); KH1060 (0.3 nM) + RA (10 nM); EB1089 (1.25 nM) + RA (10 nM)) resulted in differentiation of 80–90% of HL60 cells to mature monocytes. Treatment with RA (500 nM) or D_3 (10^{-13} M) + RA (500 nM) resulted in differentiation of 20–30% or 58–72% of HL60 cells respectively to mature neutrophils (data not shown). These data, derived from three separate experiments, are consistent with previous studies which defined optimal conditions for monocyte (Brown *et al.* 1994) and neutrophil (Bunce *et al.* 1995) differentiation of HL60 cells.

Treatment of HL60 cells with T_3 alone (1 nM or 100 nM) did not induce differentiation along either lineage. Treatment of cells with D_3 (5 nM) + 9*cis*RA (2.5 nM) or D_3 (5 nM) + RA (10 nM) in the presence of T_3 (1 or 100 nM) resulted in differentiation of 78–94% of cells to mature monocytes; and treatment with RA (500 nM) or D_3 (10^{-13} M) + RA (500 nM) in the presence of T_3 (1 or 100 nM) resulted in differentiation of 24–29% or 62–76% of cells respectively to mature neutrophils (data not shown). The data, derived from three separate experiments, clearly indicate that T_3 did not influence D_3 - or retinoid-induced HL60 cell differentiation.

Receptor protein expression and quantitative detection in HL60 cells

Undifferentiated and hormone-induced HL60 cells expressed the following receptor proteins: VDR (57 kDa),

RXR (67 kDa), RAR α (49 kDa), T_3 R α 1 (48 kDa), c-erbA α 2 (58 kDa) and T_3 R β 1 (62 kDa). There was no change in receptor protein size after induction of cell differentiation by hormones (data not shown). Estimation of the approximate molecular masses of each protein using standard markers indicated that the expressed receptors were of appropriate size (Pike *et al.* 1982, Macchia *et al.* 1990, Ali *et al.* 1992, Falcone *et al.* 1992, Rochette Egly *et al.* 1994). The 4RX 1D12 monoclonal antibody used to detect RXR expression recognises the three RXR isoforms α , β and γ (Rochette Egly *et al.* 1994). Previous studies have shown that HL60 cells express a full-length mRNA encoding RXR α , a truncated mRNA that is RXR β -specific but no RXR γ transcript (Brown *et al.* 1994), whilst other workers have shown that RXR α is the only detectable RXR protein in HL60 nuclear extracts using isoform-specific antibodies in Western blots (Titcomb *et al.* 1994). The 67 kDa RXR protein detected in these studies is therefore likely to represent RXR α and is appropriately sized (Titcomb *et al.* 1994). In order to determine the relative levels of expression of individual receptors, the linear range for the detection of receptors using each antibody was determined (Fig. 1) as described previously for T_3 R in osteosarcoma cells (Williams *et al.* 1994). Thus the relative levels of expression of each receptor could be compared quantitatively between control and hormone-treated cells.

Hormone receptor expression in monocyte-differentiated HL60 cells

We have previously shown that synergy between D_3 or synthetic analogues of D_3 (KH1060, EB1089) and retinoids (RA or 9*cis*RA) results in an increased sensitivity of proliferating HL60 cells to the induction of monocyte differentiation (Brown *et al.* 1994). A variety of hormone combinations were used to induce monocyte differentiation, and relative levels of expression of VDR, RXR, RAR and T_3 R proteins were compared between each treatment group and undifferentiated control cells (Fig. 2). The changes in receptor expression seen in monocyte-differentiated cells compared with controls were consistent irrespective of hormone treatment. Thus in monocyte-differentiated cells, expression of VDR and RXR proteins was unchanged whilst expression of RAR and T_3 R was markedly reduced (Fig. 2). Levels of expression of RAR α and VDR were also determined during the induction of terminal monocyte differentiation at 0–5-day time points after the initiation of treatment with D_3 50 nM. In these studies, RAR α expression gradually decreased with time (Fig. 3A and D), whilst the percentage of monocyte-differentiated cells present in the cultures increased (Fig. 3B and D) and correlated negatively with RAR α expression (Fig. 3C). Expression of VDR was unchanged throughout the period of induction of differentiation (Fig. 3D).

Figure 1 Quantitative detection of receptor protein expression. Western blots showing VDR (57 kDa), RXR (67 kDa), RAR α (49 kDa), T₃R α 1 (48 kDa), *c-erb A* α 2 (58 kDa) and T₃R β 1 (62 kDa) protein expression in nuclear extracts prepared from HL60 cells. Increasing concentrations (1–20 μ g) of nuclear protein were examined for expression of the various receptors, and resulting Western blots were quantified by laser densitometry. Data from three identical experiments were plotted in optical density units versus nuclear protein concentration (μ g) to determine whether detection of receptor proteins was linear. An example is shown for quantification of T₃R β 1 in (B). Lines of best fit were calculated using Cricket Graph III computer software, and correlation coefficients (r^2) are given for quantification of each receptor. A concentration of nuclear protein (10 μ g) in the middle of the linear range was selected for subsequent experiments.

Hormone receptor expression in neutrophil-differentiated HL60 cells

Recently, we showed that induction of HL60 cell differentiation to neutrophils or monocytes can be specified by subtle variations in the ratio of retinoids to D₃. High concentrations of D₃ relative to retinoids resulted in monocyte differentiation whilst a reciprocal ratio of D₃ to retinoid resulted in the promotion of neutrophil differentiation (Bunce *et al.* 1995). Combinations of hormones were used to induce neutrophil differentiation of HL60 cells in parallel with an undifferentiated control group and a group of cells induced to differentiate to monocytes for comparison. In monocyte-differentiated cells (Fig. 4, lane 4), as before, expression of VDR and RXR proteins was unchanged relative to control undifferentiated cells (lane 1) whilst expression of RAR and T₃R proteins was markedly reduced. Changes in receptor expression shown in neutrophil-differentiated cells (lanes 2 and 3) were consistent irrespective of hormone treatment. Thus, in neutrophil-differentiated cells, expression of VDR was markedly reduced relative to controls whilst expression of RAR, T₃R and RXR proteins was unchanged (Fig. 4).

Effect of T₃ on hormone receptor expression in differentiated HL60 cells

T₃ did not induce differentiation of HL60 cells or modify induction of differentiation in response to any combinations of D₃ and retinoids. Furthermore, T₃ (100 nM) did not alter the changes in expression of any receptors seen in response to D₃ or retinoids. Thus, in monocyte-differentiated cells (Fig. 5, lanes A, D and E), expression of VDR and RXR was unchanged but expression of RAR and T₃R was reduced relative to undifferentiated control (lane B) or T₃-treated (lane C) cells; in neutrophil-differentiated cells (lanes F and G) expression of VDR was reduced but expression of RAR, T₃R and RXR was unchanged relative to undifferentiated control or T₃-treated cells.

Interaction of nuclear proteins from control and differentiated HL60 cells with hormone response elements

We next investigated whether the changes in receptor stoichiometry correlated with differences in how nuclear proteins interact with DNA. Nuclear extracts from control, monocyte- and neutrophil-differentiated HL60 cells

Figure 2 Expression of nuclear receptor proteins in monocyte-differentiated HL60 cells. Expression of VDR, RXR, RAR α , T $_3$ R α 1, c-erb A α 2 and T $_3$ R β 1 proteins in nuclear proteins (10 μ g/lane) from monocyte-differentiated HL60 cells was determined in Western blots and quantified by laser densitometry of autoradiographs. The data from three or four experiments are shown relative to standardised control undifferentiated cells in graphs plotted below a representative Western blot from a single experiment. Treatments resulting in monocyte differentiation (lanes 2–8) are shown on the x-axis (lane 1, control; 2, D $_3$ 5 nM + 9cisRA 2.5 nM; 3 D $_3$ 5 nM + RA 10 nM; 4 D $_3$ 50 nM; 5 KH1060 0.3 nM; 6 EB1089 1.25 nM; 7 KH1060 0.3 nM + RA 10 nM; 8 EB1089 1.25 nM + RA 10 nM), and mean $\pm$ S.E.M. receptor expression relative to control undifferentiated cells is shown on the y-axis. Statistical analysis was by ANOVA and Tukey-Kramer post tests; * P <0.05, ** P <0.01, *** P <0.001 compared with control.

were incubated with consensus DNA-binding sites for RXR (DR+1), VDR (DR+3) and RAR (DR+5) (Umesono *et al.* 1991, Zechel *et al.* 1994, Katz *et al.* 1995) in gel mobility-shift assays.

Control nuclear extracts formed two major specific complexes with VDRE, RARE and RXRE sequences that were not competed for by addition of 100-fold molar excess of unlabelled non-specific oligonucleotide (Fig. 6A, lanes 1 and 2, 6 and 7, 11 and 12) but were efficiently competed for by a 100-fold excess of the unlabelled response element (Fig. 6A, lanes 3, 8 and 13). Cross-competition experiments revealed that complexes bound to the VDRE were not competed for by either the RXRE or RARE, those bound to the RARE were incompletely competed for by RXRE but were competed for by the VDRE, and those bound to the RXRE were incompletely competed for by the VDRE but were competed for by the RARE (Fig. 6A, lanes 4 and 5, 9 and 10, 14 and 15).

Monocyte nuclear extracts also formed two major specific complexes. These were not competed for by

addition of 100-fold molar excess of unlabelled non-specific oligonucleotide (Fig. 6B, lanes 1 and 2, 6 and 7, 11 and 12) but were competed for by a 100-fold excess of the unlabelled response element (Fig. 6B, lanes 3, 8 and 13). Complete competition of binding of complexes to the RXRE by unlabelled RXRE competitor required a 250-fold molar excess (not shown). In cross-competition experiments, complexes bound to the VDRE were not competed for by the RXRE but were competed for by the RARE, those bound to the RARE were incompletely competed for by the RXRE but efficiently competed for by the VDRE, and those bound to the RXRE were competed for by both the VDRE and RARE (Fig. 6B, lanes 4 and 5, 9 and 10, 14 and 15), although complete competition by the RARE required a 250-fold molar excess (not shown).

Neutrophil nuclear extracts formed three specific complexes. These were not competed for by addition of 100-fold molar excess of unlabelled non-specific oligonucleotide (Fig. 6C, lanes 1 and 2, 6 and 7, 11 and 12) but were competed for by a 100-fold excess of the unlabelled

Figure 3 Expression of RAR α and VDR proteins in HL60 cells harvested at sequential time points after the onset of induction of monocyte differentiation. Expression of RAR α and VDR proteins in nuclear proteins (10 μ g/lane) from differentiating HL60 cell cultures (harvested at 0, 1, 2, 3, 4 and 5 days after initiation of treatment with 50 nM D₃ to induce monocyte differentiation) was determined in Western blots and quantified by laser densitometry of autoradiographs. Receptor expression data from three inductions are shown relative to standardised untreated control HL60 cells for each time point and expressed as optical density units (mean $\pm$ S.E.M.). The percentage of differentiated cells in each culture was determined by measurement of the proportion of cells that could phagocytose complement-coated yeast. Expression of RAR α (A) and % differentiated cells (B) are plotted versus the number of days of HL60 cell culture after initiation of treatment with D₃ (50 nM); expression of RAR α is plotted versus % differentiated cells in (C). In each case the line of best fit was calculated; the linear regression equation and correlation coefficient values (r^2) are given for each analysis. The relative levels of RAR α and VDR expression (together with the percentage of differentiated cells) are given for each day of cell culture after initiation of D₃ treatment in (D).

Figure 4 Expression of nuclear receptor proteins in neutrophil- and monocyte-differentiated HL60 cells. Expression of VDR, RXR, RAR α , T $_3$ R α 1, c-erb A α 2 and T $_3$ R β 1 proteins in nuclear proteins (10 μ g/lane) from neutrophil- and monocyte-differentiated HL60 cells was determined in Western blots and quantified by laser densitometry of autoradiographs. The data from three or four experiments are shown relative to standardised control undifferentiated cells in graphs plotted below a representative Western blot from a single experiment. Treatments resulting in neutrophil (lanes 2 and 3) or monocyte (lane 4) differentiation are shown on the x-axis (lane 1, control; 2, D $_3$ 10 $^{-13}$ M + RA 500 nM; 3, RA 500 nM; 4, D $_3$ 5 nM + 9cisRA 2.5 nM), and mean $\pm$ S.E.M. receptor expression relative to control undifferentiated cells is shown on the y-axis. Statistical analysis was by ANOVA and Tukey-Kramer post tests; * P <0.05, ** P <0.01, *** P <0.001 compared with control.

response element (Fig. 6C, lanes 3, 8 and 13). In cross-competition experiments, complexes bound to the VDRE were not competed for by the RXRE but the lower of the three complexes was competed for by the RARE, those bound to the RARE were incompletely competed for by RXRE but were efficiently competed for by the VDRE, and those bound to the RXRE were competed for by both the VDRE and RARE (Fig. 6C, lanes 4 and 5, 9 and 10, 14 and 15).

Effect of receptor antibodies on nuclear protein/DNA complexes

Gel mobility-shift experiments were performed in the presence of receptor antibodies to characterise nuclear protein/DNA complexes further. Since monocyte differentiation correlated with VDR signalling and neutrophil differentiation with retinoid signalling, nuclear extracts from monocytes were investigated with the use of VDRE and RXRE probes and extracts from neutrophils with RARE and RXRE probes.

As previously, monocyte extracts formed two major complexes with the VDRE and RXRE sequences. Complex formation with the VDRE was inhibited by co-incubation with VDR antibody but was unaffected by the RXR or RAR antibodies (Fig. 7A, lanes 1–4). In contrast, complex formation with the RXRE was partially inhibited (lower complex abolished, upper complex enhanced) by the RXR antibody but unaffected by the VDR or RAR antibodies (Fig. 7B, lanes 1–4).

Neutrophil extracts formed three major complexes with the RARE and RXRE sequences as before. Complex formation with the RARE was partially inhibited by co-incubation with RAR and RXR antibodies (only the upper complex remained) but was unaffected by the VDR antibody (Fig. 7A, lanes 5–12). Similarly, complex formation with the RXRE was also partially inhibited by co-incubation with RAR and RXR antibodies but was unaffected by the VDR antibody (Fig. 7B, lanes 5–8). Only the middle complex remained in the presence of RXR antibody and both lower complexes remained with RAR antibody.

Figure 5 Expression of nuclear receptor proteins in HL60 cells induced to differentiate in the presence of T_3 . Expression of VDR, RXR, RAR α , T_3 R α 1, c-erb A α 2 and T_3 R β 1 proteins in nuclear proteins (10 μ g/lane) from monocyte- and neutrophil-differentiated HL60 cells in the presence of T_3 was determined in Western blots and quantified by laser densitometry of autoradiographs. The data from three or four experiments are shown relative to standardised control undifferentiated cells in graphs plotted below a representative Western blot from a single experiment. Treatments resulting in monocyte (lanes A, D and E) or neutrophil (lanes F and G) differentiation are shown on the x-axis (lane A, D_3 5 nM + 9cisRA 2.5 nM; B, control; C, T_3 100 nM; D, D_3 5 nM + 9cisRA 2.5 nM + T_3 100 nM; E, D_3 5 nM + RA 10 nM + T_3 100 nM; F, D_3 10^{-13} M + RA 500 nM + T_3 100 nM; G, RA 500 nM + T_3 100 nM), and mean $\pm$ S.E.M. receptor expression relative to control undifferentiated cells is shown on the y-axis. Statistical analysis was by ANOVA and Tukey-Kramer post tests; * P <0.05, ** P <0.01, *** P <0.001 compared with control.

Discussion

We have characterised the nuclear receptor profiles in HL60 cells induced to terminally differentiate to monocytes or neutrophils by D_3 or retinoids (Collins *et al.* 1977, Breitman *et al.* 1980). We demonstrated that HL60 cells express VDR, RAR α , RXR, T_3 R α 1, c-erb A α 2 and T_3 R β 1 proteins and therefore possess all the necessary components required for convergent D_3 , retinoid and T_3 signalling pathways (Kliwer *et al.* 1992, Leid *et al.* 1992, Rosen *et al.* 1993, Glass 1994, Schrader *et al.* 1994, Williams 1994, Katzenellenbogen *et al.* 1996). Although our data support the concept that activation of RXR heterodimers containing either VDR or RAR is associated with hormone-induced monocyte or neutrophil differentiation respectively, the mechanisms whereby this may be achieved are unexpected.

HL60 cells were induced to differentiate to monocytes using several optimised combinations of D_3 , synthetic D_3

analogues and retinoids (Brown *et al.* 1994, Bunce *et al.* 1995). Regardless of the combination of hormones used, levels of VDR and RXR proteins were unchanged whilst expression of RAR and T_3 R proteins was markedly reduced in monocyte-differentiated cells compared with controls. This stoichiometric change results in an increased ratio of VDR and RXR relative to RAR and T_3 R. We propose that this change would reduce competition for RXR from RAR and T_3 R and favour VDR/RXR heterodimerisation in hormone-induced monocyte-differentiated cells. In contrast, in retinoid-induced neutrophil-differentiated cells, there were no changes in the levels of expression of RAR, RXR and T_3 R proteins regardless of hormone treatment whereas expression of VDR was markedly reduced relative to controls. Thus induction of neutrophil differentiation correlates with alteration of the stoichiometry between VDR, RAR, RXR and T_3 R in favour of RAR, RXR and T_3 R. This shift in equilibrium is predicted to optimise formation of

Figure 7 Effect of receptor antibodies on formation of nuclear protein/DNA complexes. Gel mobility-shift assay analysis of nuclear proteins (5 μ g/lane) from monocyte-differentiated HL60 cells incubated with a 32 P-labelled VDRE (A; lanes 1–4) or RXRE (B; lanes 1–4) oligonucleotide (375 c.p.m./lane) and from neutrophil-differentiated HL60 cells incubated with an RARE (A; lanes 5–8) or RXRE (B; lanes 5–8) oligonucleotide. Binding to the RARE in (A), lanes 5–8 and 9–12 is shown as short and longer exposures of the same gel. The effect of addition of 2.5 μ l receptor-specific antibodies (RXR, VDR, RAR) to incubation mixtures before electrophoretic separation is shown in A (lanes 2–4, 6–8 and 10–12) and B (lanes 2–4 and 6–8).

Figure 6 Interaction of nuclear proteins from control and differentiated HL60 cells with hormone response elements. Nuclear proteins (5 μ g/lane) from control undifferentiated (A), monocyte- (B) and neutrophil- (C) differentiated HL60 cells were incubated with 32 P-labelled VDRE (lanes 1–5), RARE (lanes 6–10) and RXRE (lanes 11–15) oligonucleotides (375 c.p.m./lane) and analysed in gel mobility-shift assays. The effect of addition of 100-fold molar excess of a non-specific (NSC), VDRE, RARE or RXRE competitor oligonucleotide to incubation reactions before electrophoretic separation is shown in lanes 2–5, 7–10 and 12–15 in each panel.

RAR/RXR heterodimers at the expense of VDR-containing complexes. The maintenance of T₃R concentrations during neutrophil differentiation suggests that formation of T₃R/RXR heterodimers is not compromised during this process.

Because of this, and the possibility that T₃R may heterodimerise with VDR (Schrader *et al.* 1994) and RAR (Glass *et al.* 1989) in addition to RXR (Kliwer *et al.* 1992), we investigated the effects of T₃ in this system. These experiments conclusively demonstrated that T₃ does not influence differentiation of HL60 cells towards either the monocyte or neutrophil lineage and does not modify nuclear receptor expression. In this regard it is important to note that HL60 cells were maintained serum-free in long-term cultures and all experiments were performed under serum-free conditions. In contrast, previous studies on HL60 cells (Ballerini *et al.* 1991) and peripheral blood monocytes (Mooij *et al.* 1994), in the presence of serum, have suggested that T₃ may influence haemopoietic cell differentiation. It remains to be determined whether such T₃ effects were absent from our experiments because of a lack of possible co-operating serum components such as cytokines or growth factors (Mooij *et al.* 1994). Our studies, however, establish that hormone-induced lineage-specific differentiation of HL60 cells is associated with discrete alterations in the stoichiometry of VDR, RXR, RAR and T₃R proteins.

We next tested whether these changes correlated with differences in DNA-binding activities of nuclear extracts prepared from monocyte- and neutrophil-differentiated cells. Unique and specific protein-DNA interactions between monocyte or neutrophil extracts and VDRE, RARE or RXRE sequences were demonstrated, indicating that each hormone response element could discriminate between immature, differentiated monocytic and granulocytic HL60 cells. We used anti-receptor antibodies to characterise receptors present in each protein/DNA complex and showed that complexes formed with monocyte extracts were only disrupted by VDR and RXR antibodies whilst those formed with neutrophil extracts were only inhibited by RAR or RXR antibodies. Despite this specificity, antibody disruption of protein/DNA binding was partial, probably resulting from one of two possibilities. First, those complexes that were incompletely disrupted may contain additional unidentified proteins which contribute to the interaction of VDR or retinoid receptors with DNA. Such proteins may include the recently described co-repressor or co-activator proteins that influence nuclear receptor activity (Cavaillès *et al.* 1995, Chen & Evans 1995, Horlein *et al.* 1995, Lee *et al.* 1995). Alternatively, complexes not disrupted by antibodies may contain receptors bound to DNA in configurations that prevent the binding of antibody to its receptor epitope. Although these issues cannot be tested by the methods employed in these studies, the gel shift data demonstrate that monocyte nuclear proteins contain read-

ily detectable VDR and RXR, but that RAR and RXR are present in neutrophils. These findings agree well with the Western blotting data and with previous observations on the specificity of D₃ and retinoid signalling in HL60 cells (Collins *et al.* 1977, Breitman *et al.* 1980, Brown *et al.* 1994, Bunce *et al.* 1995).

The studies show that D₃-induced monocyte differentiation of HL60 cells correlates with decreased expression of RAR whilst retinoid-induced neutrophil differentiation correlates with reduced expression of VDR. Since RXR is a common heterodimer partner, this indicates that the concentration of RXR is likely to be limiting in this system. Thus, in monocyte-differentiated cells, reduced expression of 'competing' RAR may facilitate VDR/RXR heterodimer formation whilst, in neutrophil-differentiated cells, the opposite stoichiometric changes may favour RAR/RXR heterodimerisation. This model predicts that hormone-induced monocyte differentiation may be related to decreased expression of the receptor that mediates neutrophil differentiation and *vice versa*. Indeed, time course studies of RAR α and VDR expression during induction of monocyte differentiation revealed that RAR α expression was reduced and negatively correlated with the onset of differentiation whilst VDR expression was unchanged. Although supportive of the above model, it is important to note, however, that no causal relationship has been demonstrated. Indeed, it is possible that decreased expression of RAR (monocyte differentiation) or VDR (neutrophil differentiation) merely occurs as a consequence of cell differentiation. In this case induction of differentiation would depend on the ligand and would not necessarily require down-regulation of 'competing' receptor. Thus the gradual decline in RAR α expression seen during the induction of monocyte differentiation may only represent the fewer mature monocytes present in cultures treated with D₃ for suboptimal time periods. To address these issues in future studies, it will be necessary to determine the temporal relationship between changes in receptor expression and differentiation in individual cells. It will also be important to establish whether such changes in receptor stoichiometry are sufficient to induce altered patterns of expression of the unknown target genes that promote HL60 cell differentiation.

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