RAR-independent RXR signaling induces t(15;17) leukemia cell maturation

Gérard Benoit¹, Lucia Altucci²,
Maria Flexor¹, Sandrine Ruchaud¹,
Johan Lillehaug^{1,3}, Wolfgang Rappsberger²,
Hinrich Gronemeyer^{2,4} and Michel Lanotte^{1,4}

¹INSERM U496, Centre G.Hayem, Hôpital Saint-Louis, 1, Avenue Claude Vellefaux, 75010 Paris and ²Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC)/CNRS/INSERM/ULP, BP 163, 67404 Illkirch Cedex, C.U. de Strasbourg, France

³Present address: Department of Molecular Biology, Bergen High Technology Centre (BHTC), University of Bergen, N-5020, Norway

⁴Corresponding authors
e-mail: mlanotte@jupiter.chu-stlouis.fr or hg@igbmc.u-strasbg.fr

Although retinoic acid receptor alpha (RAR α) agonists induce the maturation of t(15;17) acute promyelocytic leukemia (APL) cells, drug treatment also selects leukemic blasts expressing PML–RAR α fusion proteins with mutated ligand-binding domains that no longer respond to all-*trans* retinoic acid (ATRA). Here we report a novel RAR α -independent signaling pathway that induces maturation of both ATRA-sensitive and ATRA-resistant APL NB4 cells, and does not invoke the ligand-induced alteration of PML–RAR α signaling, stability or compartmentalization. This response involves a cross-talk between RXR agonists and protein kinase A signaling. Our results indicate the existence of a separate RXR-dependent maturation pathway that can be activated in the absence of known ligands for RXR heterodimerization partners.

Keywords: differentiation/leukemia/nuclear receptors/protein kinase A/retinoids/signal transduction

Introduction

Acute promyelocytic leukemia (APL), originating from the generation of a retinoic acid receptor fusion protein (PML–RAR α) due to a t(15;17) chromosomal translocation (Rowley *et al.*, 1977; Borrow *et al.*, 1990; de Thé *et al.*, 1990, 1991; Goddard *et al.*, 1991; Kakizuka *et al.*, 1991; Kastner *et al.*, 1992), is the prototype of a cancer treated by differentiation therapy using all-*trans* retinoic acid (ATRA; Huang *et al.*, 1988; Castaigne *et al.*, 1990; Fenaux *et al.*, 1997). Although the mechanism triggering APL cell maturation remains unclear, it is generally admitted that the formation of PML–RAR α causes a differentiation block that can be relieved by RAR α agonists (reviewed by Slack and Gallagher, 1999). PML–RAR α acts as a ligand-responsive transcription regulator, which forms heterodimers with RXRs (Perez *et al.*, 1993) and interacts with nuclear receptor coregulators. It has been proposed that increased corepressor binding efficiency to PML–RAR α causally relates to the differentiation block of APL blasts [Hong *et al.*, 1997; Grignani *et al.*, 1998;

Guidez *et al.*, 1998; He *et al.*, 1998; Lin *et al.*, 1998; the structural and functional implications of nuclear receptor/coregulator interactions have been reviewed by Moras and Gronemeyer (1998) and Xu *et al.* (1999)]. *In vitro* studies (Kizaki *et al.*, 1996; Kitamura *et al.*, 1997; Shao *et al.*, 1997) and clinical observations (Imaizumi *et al.*, 1998) have shown that mutations in PML–RAR α at the ligand-binding domain abrogate ATRA-induced APL cell maturation. At present it is unclear whether ATRA simply de-represses a PML-dependent or activates an RAR α -dependent signaling pathway, and whether the ATRA-induced association of PML–RAR α with nuclear bodies correlates with, or is required for, blast cell maturation. In fact, *in vivo* RAR α is apparently not required for granulocyte differentiation, as RAR α ^{−/−} mice have a normal granulocyte compartment (Labrecque *et al.*, 1998). This suggests that *in vivo* RAR α is either not required for normal granulopoiesis or that alternative signaling pathways exist. To investigate the possibility of inducing APL cell maturation by an RAR α -independent signaling pathway, we analyzed comparatively the cellular consequences of RAR (retinoid) and RXR ('rexinoid'; Mukherjee *et al.*, 1997) ligands on triggering maturation of the prototypic t(15;17) APL NB4 cell line (Lanotte *et al.*, 1991) and ATRA-resistant NB4 subclones (Duprez *et al.*, 1992; Ruchaud *et al.*, 1994).

Results

Differential actions of RAR- and RXR-specific agonists on NB4 cells: cross-talk between rexinoid and protein kinase A signaling induces cell maturation

As reported previously (Chen *et al.*, 1996), all RAR α agonists (ATRA, 9-*cis* retinoic acid, BMS753; the activities of all the ligands discussed are summarized in Table I) induce maturation of NB4 cells (Figure 1A, top; Figure 1B, lanes 7 and 12; Figure 1C, lanes 5 and 9). In keeping with the critical role of PML–RAR α and/or the resident non-affected RAR α allele, neither RAR β -(BMS641) nor RAR $\beta\gamma$ -(BMS961) selective retinoids induced differentiation as assessed by morphological criteria (Figure 1A, top), NBT reduction (Figure 1B, lanes 18 and 20) and CD11c integrin expression (Figure 1C, lanes 13 and 15). That RAR pan-agonists also induce NB4 maturation through RAR α activation is demonstrated by the observations that the RAR α -selective antagonist BMS614 dramatically decreased the maturation induced by 9-*cis* retinoic acid (9-*cis* RA; Figure 1B, lane 8; Figure 1C, lane 6; similar data were obtained for ATRA, not shown). As expected, the RAR α agonist BMS753-induced differentiation was abrogated by BMS614 or the RAR pan-antagonist BMS009 (Figure 1B, lanes 13 and 14; Figure 1C, lane 10). In contrast to RAR agonists, RXR

Table I. Functional features of synthetic retinoids

Ligand	RAR AF-2			RXRAF-2	(RXR) ₂ DR1	RAR α -RXR DR5
	α	β	γ			
ATRA	+	+	+	0	0	+
9- <i>cis</i> RA	+	+	+	+	+	+
BMS 753	+	0	0	0	0	+
BMS 614	–	0	0	0	0	–
BMS 641	0	+	0	0	nd	nd
BMS 961	0	(+)	+	0	0	0
BMS 749	–	–	–	+	+	0
BMS 009	–	–	–	0	0	0
SR 11237	0	0	0	+	+	0
HX 531	0	0	0	–	–	0

The transcriptional activities of synthetic retinoids were determined for the AF-2 functions of RAR α , β , γ and RXR α using reporter cells (Figure 3A; Chen *et al.*, 1995). RXR α homodimer activity on a DR1-tk-CAT or RAR α -RXR α heterodimer activity on a DR5-tk-CAT reporter were assessed by transient RXR transfections. '+', agonist; '(+)', weak agonist; '–', antagonist; '0', no activity; 'nd', not determined.

agonists (SR11237; Lehmann *et al.*, 1992) alone were completely inactive (Figure 1A, top; Figure 1B, lane 23; Figure 1C, lane 17; Kitamura *et al.*, 1997).

Surprisingly, however, the SR11237 rexinoid agonist, which is devoid of any RAR activity (Lehmann *et al.*, 1992; Chen *et al.*, 1996), induced full maturation of NB4 cells in the presence of the protein kinase A (PKA) agonist 8CPT-cAMP, which on its own did not exhibit any differentiating activity. Morphological changes, CD11c integrin expression and NBT reduction assays all demonstrated that the RXR-PKA cross-talk induced bona fide granulocytic maturation of NB4 cells as does ATRA (Figure 1A, lower; Figure 1B, lane 24; Figure 1C, lane 18). Similar results were obtained with other rexinoid agonists (BMS749; see below). Importantly, neither RAR α -selective (BMS614) nor RAR pan-antagonists (BMS009; identical results were obtained with other RAR pan-antagonists, such as BMS493, data not shown) affected significantly the RXR-PKA synergism for NB4 maturation, irrespective of whether 9-*cis* RA or SR11237 was used as RXR agonist (Figure 1B and C). This excludes any contribution of serum-borne traces of retinoic acid, as well as unrecognized weak RAR agonist cross-reactivity of SR11237, since RAR-agonist-induced differentiation is inhibited efficiently by these antagonists (see above). Excess of the RXR antagonist HX531 (Vivat *et al.*, 1997) largely decreased the rexinoid-PKA agonist-induced differentiation of NB4 cells, as shown by NBT assays (Figure 1B), as well as morphological criteria and CD11c expression (data not shown). Note that the incomplete inhibition of differentiation by HX531 is most likely due to its weak binding affinity (compare also Vivat *et al.*, 1997). As expected, no synergy was observed between the RXR antagonist and 8CPT-cAMP (Figure 1B).

These data indicate the existence of a novel NB4 cell-differentiation pathway that requires agonist-bound, transcriptionally competent RXR and, in contrast to the classical ATRA-induced pathway, is operative in the presence of RAR antagonists.

Nuclear body disintegration and PML-RAR α stability are not affected during rexinoid-PKA agonist-induced differentiation

Although RXR-PKA synergy induces NB4 cell maturation, several cellular and biochemical events, previously considered to be key features of APL cell differentiation, are not observed. In particular, this concerns the reorganization of nuclear structures and the stability of the PML-RAR α protein.

It is well documented that ATRA exposure of APL blasts or NB4 cells reverses the destabilization of specific nuclear structures, referred to as nuclear bodies or PODs (Hodges *et al.*, 1998), caused by the PML-dependent targeting of PML-RAR α to these structures (Dyck *et al.*, 1994; Koken *et al.*, 1994, 1997; Weis *et al.*, 1994; Duprez *et al.*, 1996a). Notably, no reorganization of nuclear bodies was seen when NB4 cell maturation was induced by rexinoid (SR11237)-PKA cross-talk (Figure 2A). The absence of nuclear body reorganization was confirmed using antibodies directed against PML (Figure 2A) or SP100 (data not shown), thus excluding the possibility that a subset of nuclear bodies harboring PML-RAR α could exist that would not reorganize upon maturation. Concomitant with the reformation of nuclear bodies, ATRA induces PML-RAR α degradation (Duprez *et al.*, 1996b; Raelson *et al.*, 1996; Yoshida *et al.*, 1996). In keeping with the persistence of destabilized nuclear bodies, exposure of NB4 cells to SR11237 plus 8CPT-cAMP did not affect PML-RAR α stability under conditions in which efficient maturation was observed (Figure 2B). Taken together, the above results indicate that the PML-RAR α fusion-induced differentiation block in t(15;17) APL cells can be relieved by rexinoid-PKA cross-talk without affecting PML-RAR α stability or reversing its effects on nuclear body integrity.

Evidence for (PML-)RAR-RXR heterodimer-independent rexinoid signaling

To provide additional evidence that it is not the (PML-)RAR-RXR heterodimer whose PKA cross-talk induces differentiation, we took advantage of the recently discovered retinoid, BMS749. This retinoid has the rather exceptional characteristics that it acts as a strong RXR AF-2 (GAL-RXR α ; Figure 3A) and RXR homodimer (Figure 3B, lane 3) agonist on 17mer- (Chen *et al.*, 1995) and DR1-based reporters, respectively, whereas it is a pan-antagonist for RAR AF-2s (Figure 3A), and almost inactive with RAR-RXR heterodimers on a DR5-based reporter (Figure 3B, lane 7). Despite being virtually inactive on its own, BMS749 did not inhibit, but rather synergized with, ATRA-induced transactivation of DR5 reporters (Figure 3B, lane 8). In keeping with its inefficiency in activating the RAR-RXR heterodimer, BMS749 alone did not exert any differential effect on NB4 cells (Figure 3C). However, in the presence of 8CPT-cAMP this retinoid induced maturation with a potency similar to 9-*cis* RA (Figure 3C). Importantly, this activity was also seen in the presence of a transactivation-incompetent RAR α , as it could not be inhibited by RAR α -selective (BMS614) or pan-antagonists (Figure 3C; data not shown).

Fig. 1. Differential effects on NB4 cells of RAR- and RXR-specific agonists. Evidence for positive cooperation between the retinoid and PKA signaling pathways. The compounds used were as follows: ATRA, 1 μ M; other retinoid receptor agonists, 0.2 μ M; RAR antagonists, 2 μ M; RXR antagonist HX531, 8 μ M; cell permeable cAMP analog (8CPT-cAMP), 100 μ M. (A) Morphological features of NB4 cells in response to treatments (after 72 h). (B) Nitroblue tetrazolium enzymatic reduction in retinoid-treated NB4 cells (after 72 h; percentage of positive cells). (C) Flow-cytometry analysis of CD11c integrin expression following treatment of NB4 cells by retinoids and cAMP (after 48 h). Pan-agonists (ATRA, 9-*cis* RA) and RAR α agonists (BMS753), but not RAR β (BMS641) and RAR $\beta\gamma$ (BMS961) agonists, induce NB4 cell maturation. On its own the RXR pan-agonist SR11237 failed to trigger NB4 cell maturation. However, consistent with an RXR-ligand-dependent signaling, co-treatment with SR11237 and cAMP induces maturation of NB4 cells through a pathway that is not inhibited by the RAR antagonists BMS614 or BMS009.

Fig. 2. Distinct actions of retinoid (ATRA) and rexinoid (SR11237)-PKA signaling on nuclear body reorganization and PML-RAR α stability. (A) Confocal microscopy analysis of PML nuclear bodies in NB4 cells after 72 h exposure to the compounds indicated; at this time ATRA and SR11237 plus 8CPT-cAMP induced a fully mature phenotype. Note that ATRA, but not SR11237 plus 8CPT-cAMP, treatment induces nuclear body reorganization. (B) Western blot analysis of PML-RAR α during NB4 cell treatment with ATRA and SR11237 in the presence and absence of 8CPT-cAMP as indicated. Cell maturation was evaluated by morphological criteria, NBT staining and CD11c expression are classified as ‘-’, negative; ‘+/-’, weak; ‘+’, positive; ‘++’, very positive.

No evidence for the implication of RXR heterodimers with TR, VDR and PPAR in RXR-PKA cross-talk

RXR is a promiscuous heterodimerization partner for a great number of nuclear receptors (Gronemeyer and Laudet, 1995; Mangelsdorf *et al.*, 1995; Chambon, 1996) and NB4 differentiation could potentially be mediated by heterodimers other than RAR-RXR. To this end we tested whether thyroid hormone, vitamin D3 or PPAR agonists in the presence of either the RXR agonist SR11237 or 8CPT-cAMP would induce the differentiation of NB4 cells. However, combinations of TR or PPAR pan- or isotype-selective (provided by Bristol-Myers Squibb) agonists with SR11237 or 8CPT-cAMP did not induce any maturation (data not shown). Only vitamin D3, which was inactive on its own or in the presence of SR11237, cross-talked at a μ M concentration with 8CPT-cAMP to produce a moderate granulocytic maturation (according to NBT staining), which was $\sim$ 20% of the effect exerted by SR11237 in the presence of the PKA agonist. The vitamin D3-PKA effect was additive to that exerted by rexinoid-PKA synergism, suggesting that the two cross-talks operate independently of each other. As none of the above heterodimers efficiently induced NB4 cell differentiation in response to the cognate ligand of the RXR partner when

co-exposed to 8CPT-cAMP, it is unlikely that the rexinoid acted through one of the above heterodimers. This is further supported by the apparently distinct gene programs initiated by vitamin D3 and the rexinoid-PKA agonist synergy (see below and Figure 4). No cross-talk between the PKA pathway was seen with rexinoid antagonists (Figure 1B), such as the RXR-selective pan-antagonist HX531 (Vivat *et al.*, 1997), indicating that synergism requires RXRs to be in a transcription-activating conformation.

ATRA and rexinoid-PKA agonist-induced signaling pathways trigger distinct gene programs

The above data suggest that two distinct pathways exist for NB4 cells to undergo maturation. One involves (PML-)RAR α -RXR heterodimers and can be activated by RAR α agonists, whereas the second does not involve such heterodimers and depends on the cross-talk between RXR and PKA agonists. Monitoring cytokine signaling of NB4 cells by multiplex RNase protection assays demonstrated that the two pathways do indeed regulate different, albeit overlapping, gene programs. Most remarkably, the expression of granulocyte colony stimulating factor (G-CSF), which is weakly induced by the PKA agonist 8CPT-cAMP (Figure 4A, lane 9), is boosted dramatically by SR11237 (lane 15), even though SR11237 on its own does not affect G-CSF expression (lanes 10–12). The synergy factor was >50 -fold, leading to a huge stimulation of G-CSF gene expression, which was undetectable in the non-induced state. Notably, ATRA was incapable of stimulating G-CSF expression (Figure 4A, lanes 1–3), but induced the expression of macrophage colony stimulating factor (M-CSF; lane 3) and the C-X-C chemokine interferon-inducible protein 10 (IP10; Figure 4B, lane 3), which were not seen with SR11237 and/or 8CPT-cAMP. GM-CSF expression was triggered selectively by the RXR-PKA cross-talk (Figure 4A, lane 15) and SR11237 further stimulated 8CPT-cAMP induction of the expression of interleukin 8 (IL-8), monocyte chemoattractant protein 1 (MCP1) and the C-C chemokines macrophage inflammatory proteins MIP1 α and MIP1 β (Figure 4B, lanes 9 and 15). IL-8 and MCP1 expression was also induced by ATRA (Figure 4B, lanes 2 and 3). Notably, some cytokines were induced by SR11237 on its own, as the *c-kit* ligand stem cell factor (SCF), IL-8 and MCP1 (Figure 4A and B, lanes 11 and 12). The only other tested condition leading to SCF induction was exposure to vitamin D3 (Figure 4A, lanes 5 and 6), which also induced IL-8 expression (Figure 4B, lanes 5 and 6). The C-C cytokine RANTES, which is constitutively expressed in NB4 cells, is inhibited by the PKA agonist. This inhibition was compensated for by co-exposure to SR11237, leading only to a transient decrease at 24 h (Figure 4B, compare lanes 7–9 with lanes 13–15).

RXR-PKA cross-talk induces maturation of retinoid-resistant cells

That two distinct pathways can induce NB4 cell differentiation prompted us to test whether ATRA-resistant cells would still respond to the RXR-PKA cross-talk. Indeed, NB4-R2 cells (Duprez *et al.*, 1992, 2000; Ruchaud *et al.*, 1994), which are resistant to ATRA due to the mutation of Gln₄₁₁ to an in-phase stop codon in the ligand-

Fig. 3. Features of BMS749 as a bifunctional rexinoid. (A) Reporter cell assays; the RAR and RXR AF-2 agonistic and antagonistic activities of BMS749 are displayed as a false color illustration of the luciferase activity emanating after retinoid induction (from 1 nM, '9' to 1 μ M, '6') from 96-well plates containing equal numbers of cells (Chen *et al.*, 1995). Monitoring and quantitation was carried out using a photon-counting camera. (Δ), Signals in the presence of the retinoid (agonist activity; '-' indicates the signal for the vehicle alone); ($\blacktriangle$), signals in the presence of both 10 nM ATRA ('-', only ATRA) and increasing amounts of the retinoid (antagonistic activity, if the ATRA-induced signal decreases). Note that the RXR α reporter cell line also contains the inactive dnRXR α Δ AB, which lacks both AF-1 and AF-2, to sequester endogenous RARs that silence RXR in the absence of RAR agonists (Vivat *et al.*, 1997). (B) Transient transactivation of RXR α homodimers on a DR1 reporter in the presence of BMS749 (relative to 0.1 μ M 9-*cis* RA, standardized to 100) and of RAR α -RXR α heterodimers (relative to 10 nM ATRA, standardized to 100). The small DR5 activation in lane 7 may originate from the weak interaction of RXR homodimers with DR5 elements (Mader *et al.*, 1993). (C) BMS749 is a highly potent partner of PKA signaling to trigger NB4 cell maturation. Morphological and histochemical features of NB4 cells treated with BMS749, with or without 8-CPT-cAMP as indicated. Comparative analysis of the synergistic action of cAMP and 9-*cis* RA or BMS749 to elicit NBT response and CD11c expression in NB4 cells is shown on the right.

binding domain of PML-RAR α (Figure 5A), ceased growth in the presence of SR11237 or BMS749 plus 8CPT-cAMP (note that NB4, but not NB4-R2, cells are growth inhibited by BMS753; data not shown) and underwent granulocytic maturation, as confirmed by morphological criteria, NBT reduction (Figure 5B) and CD11c integrin expression (data not shown). Note that nuclear bodies, which are disrupted in NB4-R2 cells (Duprez *et al.*, 1996b), did not reform under these conditions (data not shown). Moreover, the rexinoid-PKA cross-talk induced the same pattern of cytokine expression in NB4 and NB4-R2 cells, whereas ATRA was unable to induce IP10, MCP1 or M-CSF expression in the resistant cells, as it did in the parental NB4 cells (Figure 4 and data not shown). No differentiation of NB4-R2 was seen in the presence of the rexinoid or PKA agonists alone, or in the presence of ATRA or 9-*cis* RA (Figure 5B). It is worth noting that, in the presence of 8CPT-cAMP, BMS753 induced some differentiation of NB4-R2 cells (lane 15), suggesting that the non-modified RAR α allele can synergize with PKA agonists to induce some markers of differen-

tiation even in the absence of morphological cell alterations (Figure 5B, top).

Discussion

RXR-PKA cross-talk constitutes a novel signaling pathway

Several arguments support our conclusion that NB4 cell differentiation can be achieved through two different signaling paradigms, i.e. either RAR α agonists through (PML)-RAR α -RXR heterodimers or through rexinoid-PKA agonist synergism. Owing to 'RXR subordination' the RAR-RXR heterodimer cannot respond to pure RXR agonists, such as SR11237, unless RAR is liganded (Lehmann *et al.*, 1992; Kurokawa *et al.*, 1994; Forman *et al.*, 1995; Vivat *et al.*, 1997). In addition, BMS749, which is a RXR homodimer agonist and a RAR antagonist, was unable to activate RAR-RXR heterodimers efficiently on its own. However, together with the PKA agonist, but not on their own, both SR11237 and BMS749 efficiently induced NB4 cell differentiation. Note that we have

Fig. 4. Retinoid-PKA cross-talk induces a cytokine expression program that differs from that induced by ATRA (A and B). Modulation of cytokine expression in NB4 cells was assessed by multiplex RNase protection assays. Cells were exposed to the agents displayed at the top for 0, 24 and 48 h (subsequent lanes for each treatment). For comparison the effect of vitamin D3 (D3) was also studied. In (A) the intact probes run on the same gel are shown on the left (L32 and GAPDH are the invariant internal controls used for calibration) and the positions of the protected fragments are indicated. In (B) only the positions of the protected fragments are shown.

excluded the possibility that 8CPT-cAMP simply relieves 'RXR subordination' in RAR-RXR heterodimers, since we did not observe any SR11237-induced transactivation of a DR5-based reporter by co-expressed RAR and RXR in the presence of the PKA agonist (data not shown). The degradation of PML-RAR α and the reorganization of PML nuclear bodies during ATRA treatment are regarded as key events during APL cell maturation. This work shows that reversion of the known PML-RAR α -induced effects is not obligatory for APL cell maturation, thus further supporting the idea of a distinct pathway. Cytokine/chemokine expression analysis confirms that SR11237 plus 8CPT-cAMP is not simply an alternative to induce the same gene program as RAR α agonists. Transgenic mice expressing a dominant-negative RXR in myeloid cells exhibited maturation defects at the promyelocytic stage (Sunaga *et al.*, 1997) indicating that RXR is not just a silent heterodimerization partner of RAR and plays an active transcriptional role either in hetero- or homodimers that is crucial for myelopoiesis. NB4 mutant cells, that do not respond to ATRA, do differentiate efficiently via the retinoid-PKA pathway. Whether this PML-RAR-RXR heterodimer-independent pathway operates through synergy between the PKA pathway and RXR homodimers or via an as yet unidentified 'RXR permissive' heterodimer remains to be established. Also note that we use the terms 'retinoid-PKA cross-talk' phenomenologically and do not distinguish between: (i) a direct action of one pathway on components of the other; and (ii) two separate signaling pathways whose actions complement each other with respect to cell differentiation.

Implications for APL therapy

Our data pave the way for alternative therapies for APL patients to inhibit the outgrowth of ATRA-resistant pools of leukemic blasts and eliminate such cells from relapsed

Benoit *et al.* Figure 5

Fig. 5. A distinct RAR-ligand-independent signaling pathway triggers maturation of NB4-R2 cells resistant to RAR agonists. (A) Molecular definition of the mutated PML-RAR α in NB4-R2 cells. NB4-R2 is an ATRA-resistant subclone isolated from bone marrow leukemic cells of a ATRA-resistant patient by a selection with both cAMP and ATRA (Duprez *et al.*, 1992; Ruchaud *et al.*, 1994). Sequencing of the PML-RAR α cDNA reveals a point mutation generating a stop codon at position 411 in the retinoid-binding domain of PML-RAR α (L) of NB4 cells, thus generating a truncated chimeric protein. (B) Morphological and functional maturation of NB4-R2 cells in response to synergistic treatment by retinoids and cAMP. NB4-R2 cells were treated with protocols similar to those used for NB4 cells (Figure 1 legend). Note that cooperation between cAMP and retinoid signaling allows maturation of ATRA-resistant cells.

patients. The observations that: (i) ATRA augments the expression levels of the receptors for G-CSF (Tkatch, 1995) and GM-CSF (de Gentile *et al.*, 1994); (ii) G-CSF and GM-CSF are superinduced by the PKA and retinoid agonists (Figure 4); and (iii) G-CSF combined with ATRA can increase granulocyte counts without leukemia regrowth in patients who developed neutropenia (Usuki *et al.*, 1996), suggest that a combination therapy using retinoid, retinoid, which in human clinical trials did not manifest side-effects that are normally associated with retinoid therapy (Miller *et al.*, 1997), and PKA agonists (or drugs elevating cAMP levels) may provide a third therapeutic tool, in addition to combination therapies with ATRA plus chemotherapy and ATRA plus arsenic trioxide (Soignet *et al.*, 1998; Lallemand-Breitenbach *et al.*, 1999).

Materials and methods

Ligands, cell lines, cell cultures and granulocytic maturation assays

Retinoids and PPAR α and γ ligands were generously provided by Bristol-Myers Squibb. NB4 and NB4-R2 cells were cultured as described previously (Lanotte *et al.*, 1991; Duprez *et al.*, 1992; Ruchaud *et al.*, 1994). Essentially, cultures were established with log phase growing cells at a concentration (10^5 cells per ml) that allowed exponential growth of untreated control cultures over 3 days without medium replenishment. Retinoids (provided by Bristol-Myers Squibb; dissolved in ethanol as stock solution at 10^{-3} M) and 8-CPT-cAMP (Sigma; dissolved in saline at 10^{-2} M) were added at the indicated concentrations. Cultures were light-protected. Cell morphology was analyzed after May-Grünwald staining. A histological NBT reaction was carried out as described previously (Lanotte *et al.*, 1991; Duprez *et al.*, 1992). Morphology analysis and the NBT reaction were performed 72 h after treatment. At least 300 cells were analyzed for each treated culture. The data given are typical of one experiment, each treatment was repeated at least three times. The expression of CD11c integrin was analyzed by direct immunofluorescence, essentially as described by Lanotte *et al.* (1991). Briefly, after incubation (48 h), cells were washed in phosphate-buffered saline (PBS) and labeled with an anti-human CD11c fluorescein isothiocyanate (FITC) mouse monoclonal antibody (mAb; Becton-Dickinson). Cells were then washed twice in PBS and fixed in 1% paraformaldehyde/PBS solution. Cells were analyzed using a Coulter flow cytometer.

Cellular extracts and SDS-PAGE analysis

Cytoplasmic and nuclear protein extracts were as reported previously (Duprez *et al.*, 1996a). Protein extracts (5 μ g) were loaded onto SDS-polyacrylamide gels, electrophoresed and blotted onto poly(vinylidene difluoride) (PVDF) membranes (Immobilon P; Millipore). Membranes were blocked with 5% non-fat milk in PBS pH 7.6, 0.1% Tween 20 (PBS-T), then incubated with a specific antiserum raised against human RAR α [R α (F)] in PBS-T/0.5% milk for 18 h at 4°C. Membranes were incubated with horseradish peroxidase (HRP)-coupled anti-rabbit antibody (Jackson Laboratories) for 30 min at 25°C. Each step was followed by three 10 min washes in PBS-T. Detection was performed as described in the ECL Kit (Amersham).

Confocal immunofluorescence analyses

For immunofluorescence studies, the cells were smeared onto histological glass slides using a cytocentrifuge (Cytospin, Shandon). After drying overnight, they were fixed in acetone at 4°C for 10 min and allowed to air dry for 20 min. The slides were incubated for 15 min with PBS and for 1 h with polyclonal rabbit antiserum raised against PML (Duprez *et al.*, 1996a) or SP100 (generously provided by Drs M.Koken and H.de Thé) at a dilution of 1:500 in PBS. After three washes in PBS, the slides were incubated for 30 min with fluorescein-coupled anti-rabbit antibody (Bioss) at a dilution of 1:200 in PBS. After three washes in PBS, slides were mounted with 5 μ l of fluorescent mounting medium (Dako). All incubations were carried out at room temperature. Preparations were examined by confocal laser scanning microscopy (MRC-600 Confocal Imaging System; Bio-Rad Microscience Ltd, Hertfordshire, UK), mounted on an optiphot II Nikon microscope. Images were collected using an oil immersion lens (60 $\times$, NA 1.4 plan apochromat). For fluorescein excitation an argon ion laser (Ion Laser Technology Inc., Salt Lake City, UT, USA) operating with the 488 nm line was used.

RNase protection assay

Total RNA was extracted with Trizol (Gibco-BRL). The ribonuclease protection assay was performed according to the supplier's instructions (Pharmingen, San Diego, CA, USA). Briefly, human cytokine 4 and 5 template sets (45034P; 45035P) were labeled with α - 32 P uridine triphosphate. RNA (4 μ g) and 6×10^5 c.p.m. of labeled probes were used for hybridization. After RNase treatments, the protected probes were resolved on a 5% urea-polyacrylamide-bis-acrylamide gel.

Sequencing PML-RAR α cDNA of NB4 and NB4-R2 cells

Total RNA from NB4 and NB4-R2 cells was purified and PML-RAR α -specific mRNA was isolated using Dynal magnetic beads (M-280 streptavidin) coupled to a biotinylated PML-RAR α oligonucleotide (specific for the sequence at the recombination site). This mRNA preparation was used for RT-PCR with oligod(N)6 as the primer and AMV reverse transcriptase at 42°C for 60 min. One-twentieth of the RT

reaction was used for PCR amplification with the primers R2L (CTG CCC CTG GAG ATG GAT GAT) and R2H (GCG GAG GGC GAG GGC TGT GTC) and the following conditions: 5 min at 95°C, cycle 1 min at 95°C, 2 min at 65°C, 1 min 30 s at 72°C and then 10 min at 72°C. The PCR product was gel isolated and cloned into the pCRTM2.1 AT sequencing vector. Clones were selected and PCR-tested for the presence of the insert. Positive clones were selected, plasmids were purified from these clones and DNA was sequenced with SP6 and T7 primers using the Perkin-Elmer Big Dye kit and ABI 377 automatic sequencer.

Acknowledgements

The authors would like to thank Drs Koken and de Thé for anti-SP100 antibodies, the Bristol-Myers Squibb chemists for providing the synthetic retinoids and PPAR agonists, and the photography and artwork services for their help. L.A. and H.G. greatly acknowledge the excellent technical help of Michèle Lieb. L.A. was supported by a fellowship from the Rotary foundation, W.R. was supported by a Marie Curie Research Training grant. G.B. was the recipient of a fellowship from Association pour la Recherche contre le Cancer (ARC). M.F. was supported by Association Claude Bernard. J.L. was supported for a sabbatical year by the University of Bergen (Norway). This work was supported by funds from the Institut National de la Santé et de la Recherche Médicale, the Centre National de la Recherche Scientifique, the Ligue Nationale contre le Cancer, the Hôpital Universitaire de Strasbourg, the Norwegian Research Council (grant to J.L.), the EC BIOMED programme (grant BMH4-96-0181 to H.G.), and Bristol-Myers Squibb.

References

- Borrow,J., Goddard,A.D., Sheer,D. and Solomon,E. (1990) Molecular analysis of acute promyelocytic leukemia breakpoint cluster region on chromosome 17. *Science*, **249**, 1577–1580.
- Castaigne,S., Chomienne,C., Daniel,M.T., Ballerini,P., Berger,R., Fenaux,P. and Degos,L. (1990) All *trans*-retinoic acid as a differentiation therapy for acute promyelocytic leukemia: I. Clinical results. *Blood*, **76**, 1704–1709.
- Chambon,P. (1996) A decade of molecular biology of retinoic acid receptors. *FASEB J.*, **10**, 940–954.
- Chen,J.Y., Penco,S., Ostrowski,J., Balaguer,P., Pons,M., Starrett,J.E., Reczek,P., Chambon,P. and Gronemeyer,H. (1995) RAR-specific agonist/antagonists which dissociate transactivation and AP1 transrepression inhibit anchorage-independent cell proliferation. *EMBO J.*, **14**, 1187–1197.
- Chen,J.Y., Clifford,J., Zusi,C., Starrett,J., Tortolani,D., Ostrowski,J., Reczek,P.R., Chambon,P. and Gronemeyer,H. (1996) Two distinct actions of retinoid-receptor ligands. *Nature*, **382**, 819–822.
- de Gentile,A. *et al.* (1994) Induction of high-affinity GM-CSF receptors during all-*trans* retinoic acid treatment of acute promyelocytic leukemia. *Leukemia*, **8**, 1758–1762.
- de Thé,H., Chomienne,C., Lanotte,M., Degos,L. and Dejean,A. (1990) The t(15;17) translocation of acute promyelocytic leukemia fuses the retinoic acid receptor α gene to a novel transcribed locus. *Nature*, **347**, 558–561.
- de Thé,H., Lavau,C., Marchio,A., Chomienne,C., Degos,L. and Dejean,A. (1991) The PML-RAR- α fusion mRNA generated by t(15;17) translocation in acute promyelocytic leukemia encodes a functionally altered RAR. *Cell*, **66**, 675–684.
- Duprez,E., Ruchaud,S., Houge,G., Martin-Thouvenin,V., Valensi,F., Kastner,P., Berger,R. and Lanotte,M. (1992) A retinoic acid 'resistant' t(15;17) acute promyelocytic leukemia cell line: isolation, morphological, immunological and molecular features. *Leukemia*, **6**, 1281–1287.
- Duprez,E., Lillehaug,J.R., Naoe,W. and Lanotte,M. (1996a) cAMP signal is decisive for recovery of nuclear bodies (PODs) during maturation of RA-resistant t(15;17) promyelocytic leukemia NB4 cells expressing PML-RAR α . *Oncogene*, **12**, 2451–2459.
- Duprez,E., Lillehaug,J.R., Gaub,M.P. and Lanotte,M. (1996b) Differential changes of receptor (RXR α) and its RAR α and PML-RAR α partners induced by cAMP and retinoic acid distinguish maturation sensitive and resistant t(15;17) promyelocytic leukemia NB4 cells. *Oncogene*, **12**, 2443–2450.
- Duprez,E., Benoit,G., Flexor,M., Lillehaug,J. and Lanotte,M. (2000) A mutated PML/RAR α found in the retinoid maturation resistant NB4

- subclone, NB4-R2, blocks RARA and wild-type PML/RARA transcriptional activities. *Leukemia*, **14**, in press.
- Dyck, J.A., Maul, G.G., Miller, W., Jr, Chen, J.D., Kakizuka, A. and Evans, R.M. (1994) A novel macromolecular structure is a target of the promyelocyte-retinoic acid receptor oncoprotein. *Cell*, **76**, 333–343.
- Fenaux, P., Chomienne, C. and Degos, L. (1997) Acute promyelocytic leukemia: biology and treatment. *Semin. Oncol.*, **24**, 92–102.
- Forman, B.M., Umesono, K., Chen, J. and Evans, R.M. (1995) Unique response pathways are established by allosteric interactions among nuclear hormone receptors. *Cell*, **81**, 541–550.
- Goddard, A.D., Borrow, J., Freemont, P. and Solomon, E. (1991) Characterization of a zinc finger gene disrupted by the t(15;17) in acute promyelocytic leukemia. *Science*, **254**, 1371–1374.
- Grignani, F. et al. (1998) Fusion proteins of the retinoic acid receptor- α recruit histone deacetylase in promyelocytic leukaemia. *Nature*, **391**, 815–818.
- Gronemeyer, H. and Laudet, V. (1995) Transcription factors 3: nuclear receptors. *Protein Profile*, **2**, 1173–1308.
- Guidez, F., Ivins, S., Zhu, J., Soderstrom, M., Waxman, S. and Zelent, A. (1998) Reduced retinoic acid-sensitivities of nuclear receptor corepressor binding to PML- and PLZF-RAR α underlie molecular pathogenesis and treatment of acute promyelocytic leukemia. *Blood*, **91**, 2634–2642.
- He, L.Z., Guidez, F., Tribioli, C., Peruzzi, D., Ruthardt, M., Zelent, A. and Pandolfi, P.P. (1998) Distinct interactions of PML-RAR α and PLZF-RAR α with co-repressors determine differential responses to RA in APL. *Nature Genet.*, **18**, 126–135.
- Hodges, M., Tissot, C., Howe, K., Grimwade, D. and Freemont, P.S. (1998) Structure, organization, and dynamics of promyelocytic leukemia protein nuclear bodies. *Am. J. Hum. Genet.*, **63**, 297–304.
- Hong, S.H., David, G., Wong, C.W., Dejean, A. and Privalsky, M.L. (1997) SMRT corepressor interacts with PLZF and with the PML-retinoic acid receptor α (RAR α) and PLZF-RAR α oncoproteins associated with acute promyelocytic leukemia. *Proc. Natl Acad. Sci. USA*, **94**, 9028–9033.
- Huang, M.E., Ye, C.Y., Chen, S.R., Chai, J.C., Lu, J.X., Zhou, L., Gu, L.G. and Wang, Z. (1988) Use of all-trans retinoic acid in the treatment of acute promyelocytic leukemia. *Blood*, **72**, 567–572.
- Imaizumi, M. et al. (1998) Mutations in the E-domain of RAR portion of the PML/RAR chimeric gene may confer clinical resistance to all-trans retinoic acid in acute promyelocytic leukemia. *Blood*, **92**, 374–382.
- Kakizuka, A., Miller, W.H., Umesono, K., Jr, Warrell, R.P., Franckel, S.R., Murty, V.S., Dmitrovsky, E. and Evans, R.M. (1991) Chromosomal translocation t(15;17) in human acute promyelocytic leukemia fuses RAR α with a novel putative transcription factor, PML. *Cell*, **66**, 653–674.
- Kastner, P., Perez, A., Lutz, Y., Rochette-Egly, C., Gaub, M.P., Durand, B., Lanotte, M., Berger, R. and Chambon, P. (1992) Structure, localization and transcriptional properties of two classes of retinoic acid receptor α fusion proteins in acute promyelocytic leukemia (APL): structural similarities with a new family of oncoproteins. *EMBO J.*, **11**, 629–642.
- Kitamura, K., Kiyoi, H., Yoshida, H., Saito, H., Ohno, R. and Naoe, T. (1997) Mutant AF-2 domain of PML-RAR α in retinoic acid-resistant NB4 cells: differentiation induced by RA is triggered directly through PML-RAR α and its down-regulation in acute promyelocytic leukemia. *Leukemia*, **11**, 1950–1956.
- Kizaki, M. et al. (1996) Establishment and characterization of a novel acute promyelocytic leukemia cell line (UF-1) with retinoic acid-resistant features. *Blood*, **88**, 1824–1833.
- Koken, M.H. et al. (1994) The t(15;17) translocation alters a nuclear body in a retinoic acid-reversible fashion. *EMBO J.*, **13**, 1073–1083.
- Koken, M.H. et al. (1997) Leukemia-associated retinoic acid receptor α fusion partners, PML and PLZF, heterodimerize and colocalize to nuclear bodies. *Proc. Natl Acad. Sci. USA*, **94**, 10255–10260.
- Kurokawa, R., DiRenzo, J., Bohem, M., Sugarman, B., Gloss, M.G., Rosenfeld, M.G., Heyman, R.A. and Glass, C.K. (1994) Regulation of retinoid signalling by receptor polarity and allosteric control of ligand binding. *Nature*, **371**, 528–531.
- Labrecque, J., Allan, D., Chambon, P., Iscove, N.N., Lohnes, D. and Hoang, T. (1998) Impaired granulocytic differentiation *in vitro* in hemopoietic cells lacking retinoic acid receptors α 1 and γ . *Blood*, **92**, 607–615.
- Lallemand-Breitenbach, V., Guillemin, M.C., Janin, A., Daniel, M.T., Degos, L., Kogan, S.C., Bishop, J.M. and de Thé, H. (1999) Retinoic acid and arsenic synergize to eradicate leukemic cells in a mouse model of acute promyelocytic leukemia. *J. Exp. Med.*, **189**, 1043–1052.
- Lanotte, M., Martin-Thouvenin, V., Najman, S., Ballerini, P., Valensi, F. and Berger, R. (1991) NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). *Blood*, **77**, 1080–1086.
- Lehmann, J.M., Jong, L., Fanjul, A., Cameron, J.F., Lu, X.P., Haefner, P., Dawson, M.I. and Pfahl, M. (1992) Retinoids selective for retinoid X receptor response pathways. *Science*, **258**, 1944–1946.
- Lin, R.J., Nagy, L., Inoue, S., Shao, W., Miller, W., Jr and Evans, R.M. (1998) Role of the histone deacetylase complex in acute promyelocytic leukaemia. *Nature*, **391**, 811–814.
- Mader, S., Leroy, P., Chen, J.Y. and Chambon, P. (1993) Multiple parameters control the selectivity of nuclear receptors for their response elements. Selectivity and promiscuity in response element recognition by retinoic acid receptors and retinoid X receptors. *J. Biol. Chem.*, **268**, 591–600.
- Mangelsdorf, D.J. et al. (1995) The nuclear receptor superfamily: the second decade. *Cell*, **83**, 835–839.
- Miller, V.A. et al. (1997) Initial clinical trial of a selective retinoid X receptor ligand, LGD1069. *J. Clin. Oncol.*, **15**, 790–795.
- Moras, D. and Gronemeyer, H. (1998) The nuclear receptor ligand-binding domain: structure and function. *Curr. Opin. Cell Biol.*, **10**, 384–391.
- Mukherjee, R. et al. (1997) Sensitization of diabetic and obese mice to insulin by retinoid X receptor agonists. *Nature*, **386**, 407–410.
- Perez, A., Kastner, P., Sethi, S., Lutz, Y., Reibel, C. and Chambon, P. (1993) PML-RAR α homodimers: distinct DNA binding properties and heterodimeric interactions with RXR. *EMBO J.*, **12**, 3171–3182.
- Raelson, J.V., Nervi, C., Rosenauer, A., Benedetti, L., Monczak, Y., Pearson, M., Pelicci, P.G. and Miller, W., Jr (1996) The PML/RAR α oncoprotein is a direct molecular target of retinoic acid in acute promyelocytic leukemia cells. *Blood*, **88**, 2826–2832.
- Rowley, J.D., Golomb, H.M. and Dougherty, C. (1977) 15/17 translocation: a consistent chromosomal change in acute promyelocytic leukaemia. *Lancet*, **1**, 549–550.
- Ruchaud, S., Duprez, E., Gendron, M.C., Houge, G., Genieser, H.G., Jastorff, B., Doskeland, S.O. and Lanotte, M. (1994) Two distinctly regulated events, priming and triggering, during retinoid-induced maturation and resistance of NB4 promyelocytic leukemia cell line. *Proc. Natl Acad. Sci. USA*, **91**, 8428–8432.
- Shao, W., Benedetti, L., Lamph, W.W., Nervi, C. and Miller, W., Jr (1997) A retinoid-resistant acute promyelocytic leukemia subclone expresses a dominant negative PML-RAR α mutation. *Blood*, **89**, 4282–4289.
- Slack, J.L. and Gallagher, R.E. (1999) The molecular biology of acute promyelocytic leukemia. *Cancer Treat. Res.*, **99**, 75–124.
- Soignet, S.L. et al. (1998) Complete remission after treatment of acute promyelocytic with arsenic trioxide. *N. Engl. J. Med.*, **339**, 1341–1348.
- Sunaga, S., Maki, K., Lagasse, E., Blanco, J.C., Ozato, K., Miyazaki, J. and Ikuta, K. (1997) Myeloid differentiation is impaired in transgenic mice with targeted expression of a dominant negative form of retinoid X receptor β . *Br. J. Haematol.*, **96**, 19–30.
- Tkatch, L.S., Rubin, K.A., Ziegler, S.F. and Tweardy, D.J. (1995) Modulation of human G-CSF receptor mRNA and protein in normal and leukemic myeloid cells by G-CSF and retinoic acid. *J. Leuk. Biol.*, **57**, 964–971.
- Usuki, K., Nishizawa, Y., Endo, M., Osawa, M., Kitazume, K., Iki, S., Watanabe, M. and Urabe, A. (1996) Administration of granulocyte colony-stimulating factor during remission induction therapy with all-trans retinoic acid for acute promyelocytic leukemia. *Int. J. Hematol.*, **64**, 213–219.
- Vivat, V. et al. (1997) A mutation mimicking ligand-induced conformational change yields a constitutive RXR that senses allosteric effects in heterodimers. *EMBO J.*, **16**, 5697–5709.
- Weis, K., Rambaud, S., Lavau, C., Jansen, J., Carvalho, T., Carmo-Fonseca, M., Lamond, A. and Dejean, A. (1994) Retinoic acid regulates aberrant nuclear localization of PML-RAR α in acute promyelocytic leukemia cells. *Cell*, **76**, 345–356.
- Xu, L., Glass, C.K. and Rosenfeld, M.G. (1999) Coactivator and corepressor complexes in nuclear receptor function. *Curr. Opin. Genet. Dev.*, **9**, 140–147.
- Yoshida, H., Kitamura, K., Tanaka, K., Omura, S., Miyazaki, T., Hachiya, T., Ohno, R. and Naoe, T. (1996) Accelerated degradation of PML-retinoic acid receptor α (PML-RARA) oncoprotein by all-trans-retinoic acid in acute promyelocytic leukemia: possible role of the proteasome pathway. *Cancer Res.*, **56**, 2945–2948.

Received August 12, 1999; revised and accepted October 22, 1999