and in the Russian Cancer Research Centre for Moscow. All information about the progress of the project is collected for the relevant area. The central coordinating office is the Cancer Unit at the World Heath Organization Headquarters in Geneva, assisted by the WHO Collaborating Center for Cancer Biostatistics Evaluation, Boston, U.S.A. and the WHO Collaborating Centre for Evaluation of Screening for Cancer, Toronto, Canada.

WHO SPONSORSHIP OF THE PROJECT

The study was designed and has been implemented under the sponsorship and with the direct help of the WHO. The Cancer and Palliative Care Unit at the headquarters, Geneva is responsible for the overall coordination of activities on the implementation, management and evaluation of the study. For the 15 years of study activities, collecting cases of breast cancer and their follow-up, regular meetings for evaluation of the progress of the study are organised in Geneva and Russia.

WHO, through its Collaborating Centres for Cancer Biostatistics in Boston, U.S.A. and for Evaluation of Screening for Cancer in Toronto, Canada will ensure consultations on statistical analysis of data and final presentation of the results. All paper records will be preserved in order to facilitate a review of the data when the study is completed.

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Feature Articles

Retinoid Receptors and Acute Promyelocytic Leukaemia

Ian D. Trayner and Farzin Farzaneh

INTRODUCTION

ACUTE PROMYELOCYTIC leukaemia (APL, M3 by FAB classification) represents a deregulated clonal expansion of haemopoietic progenitor cells arrested at the promyelocyte stage of differentiation. It is recognised clinically by a preponderance of granular promyelocytes in the bone marrow, and sometimes in the peripheral blood, together with a haemorrhagic syndrome.

APL cells carry a balanced t(15;17)(q22;q11.2-q12) chromosomal translocation which has become diagnostic for the disease [1]. The breakpoint on chromosome 17 lies within the retinoic acid receptor-α (RAR-α) gene locus [2–6] and results in the splicing of RAR-α to a novel gene of as yet unknown function, termed PML (promyelocytic leukaemia), on chromosome 15 [7–11]. Characteristically, promyelocytic cells with the t(15;17) translocation can be induced to differentiate into granulocytes by treatment with all-*trans* retinoic acid (RA), either *in vitro* or *in vivo*. This provides a very effective differentiation therapy for APL patients (for reviews see [12, 13]).

RA INDUCES DIFFERENTIATION OF APL CELLS

Several isomers of RA are produced from retinol (vitamin A) which occurs naturally in the diet as β-carotene and acyl retinol esters. Different retinoids display subsets of the physiological effects of vitamin A (for reviews see [14, 15]). RA is a powerful morphogen and teratogen which is thought to play a role in the development of the embryonic limb bud [16] and central nervous system [17]. These properties may be related to the ability of RA to regulate the sequential expression of homeobox genes [18]

Correspondence to F. Farzaneh.
The authors are at the Molecular Medicine Unit, The Rayne Institute, King's College School of Medicine and Dentistry, 123 Coldharbour Lane, London SE5 9NU, U.K.
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and induce the differentiation of embryonal carcinoma and germ cell lines [19–21].

The ability of RA to induce the differentiation of certain myeloid leukaemia cells in culture has been known for some time [22, 23]. More recently, all-*trans* RA was shown to induce complete remission (CR) in most APL patients [24–26]. The all-*trans* isomer is more than 10-fold more effective than 13-*cis* RA, both in inducing remission in patients and differentiation of APL cells in culture [27]. However, when all-*trans* RA is used as the sole form of therapy relapse usually occurs within a few months, and trials combining all-*trans* RA and chemotherapy are currently under way. One considerable advantage of all-*trans* RA is that it prevents the haemorrhagic syndrome which is the major cause of rapid death in APL patients receiving only chemotherapy. The most serious side-effect is the "retinoic acid syndrome" due to hyperleukocytosis. This occurs in a proportion of patients and can be managed with chemotherapy [28] or high-dose corticosteroids [29].

Although leukaemic cells carrying the t(15;17) translocation cannot be detected cytogenetically in patients during CR, residual disease can be monitored by polymerase chain reaction (PCR) using primers which specifically amplify across the PML/RAR-α junction, and the results are a useful guide to prognosis [30, 31]. Clearly, a small number of leukaemogenic cells often avoid differentiation though it is not clear whether they are promyelocytes or less mature cells (the chromosomal translocation has only been detected in promyelocytes). Plasma levels of RA fall markedly in patients during treatment, and at the time of relapse, increased dosage does not significantly increase plasma RA levels or induce remission, although the leukaemic cells are still responsive to RA *in vitro* [32]. All-*trans* RA is not useful in maintenance therapy [33].

The expression of PML/RAR-α in nearly all APL patients suggests that it plays an important role in the pathogenesis of this leukaemia. However, the precise molecular contribution of PML/RAR-α to the generation and/or progression of APL remains unclear. Models seeking to explain the molecular pathology of APL must explain the contribution made by the translocation and chimaeric protein expression, as well as the responsiveness of the patients to all-*trans* RA. This requires better understanding of the functions of both PML and RAR-α, as well as of the chimaeric PML/RAR-α.

RETINOID RECEPTORS AND THE STEROID/THYROID HORMONE RECEPTOR SUPERFAMILY

The receptors for RA, thyroid hormone (T_3) and vitamin D_3 are homologous and functionally related members of the steroid/thyroid hormone receptor superfamily. There are two families of RA receptors in mice and humans, the RARs (α, β and γ) and the retinoid X receptors (RXR-α, -β and -γ) [34–38]. The RARs have comparable affinities for all-*trans* RA and 9-*cis* RA, and are activated by both retinoids. In contrast, the RXR are only bound and activated by 9-*cis* isomers of RA. The apparent affinities of the RARs for 9-*cis* RA are approximately 20-fold higher than those of the RXRs [39]. Newts have RAR-α and -β, and a third RAR which differs sufficiently from human and mouse RAR-γ to be called RAR-δ [40]. There are two thyroid hormone receptor (TR) genes, α and β, and only one vitamin D_3 receptor (VDR) gene. Most of these genes give rise to multiple RNA transcripts due to alternative promoter usage, multiple poly-A addition sites and alternative splicing (for reviews see [35, 41]). The two human isoforms of RAR-α (RAR-α1 and RAR-α2) differ only in the A domain, which is encoded by alternative first exons. All these receptors are nuclear ligand-activated transcription regulators (for reviews see [42–44]).

All members of the steroid/thyroid hormone receptor family consist of a number of functional domains, similar to RAR-α (Fig. 1). The C domain contains a cysteine-rich DNA-binding region which contains two conserved zinc finger structures. DNA binding specificity is defined by three amino acids at the base of the first finger in a region called "the knuckle" or the P-box [45–47] and changes to these amino acids can alter the specificity of the receptor for target response elements. The E domain contains the ligand binding site and a region which is required both for dimerisation with other receptors and efficient transcriptional activation. The DNA- and ligand-binding domains are highly conserved between different receptors. The A, B, D and F domains are conserved less between receptors, but are highly conserved for the same receptor between different species, suggesting that they contain important functional information.

Steroid hormones are thought to activate transcription of the genes which they regulate by causing the dimerisation of their receptors on appropriate response elements [48]. These response elements are defined as sequences within gene promoters which

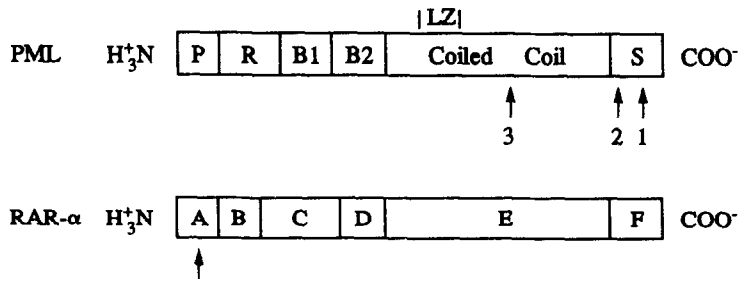

Fig. 1. Schematic diagram of the PML and RAR-α proteins. PML contains a proline-rich region (P); a cysteine-rich RING finger (R); two cysteine-rich clusters called B box 1 and 2 (B1 and B2); a coiled-coil region containing a sequence with homology to leucine zipper structures (LZ); and a serine-rich C-terminal region (S). RAR-α contains 6 domains A–F. The A and B domains contain a transcription modulating function, the C domain contains two cysteine-rich zinc finger structures required for DNA-binding, the E domain is required for ligand-binding, dimerisation and transcriptional activation, and the F domain has no identified functions. Chimaeric PML/RAR-α proteins which are expressed in the leukaemic cells of APL patients, consist of N-terminal PML sequences up to the arrows numbered 1, 2 or 3 (depending on whether the PML gene breakpoint is in *bcr*-1, 2 or 3, respectively) fused to the C-terminal B–F domains of RAR-α (from the unlabelled arrow). Reciprocal RAR-α/PML proteins are also expressed. Alternative splicing of the PML part of the primary transcript gives rise to multiple protein isoforms (see text for details).

mediate (in *cis*) transcription in response to stimulation by a specific hormone or signalling pathway. This is, of course, a functional rather than a structural definition. Response elements of steroid hormones generally consist of inverted hexamer repeats of a conserved sequence, separated from each other by a few, apparently random, nucleotides. Hormone binding causes receptor homodimerisation on appropriate response elements, with one receptor molecule binding to each hexamer motif [49].

Steroid hormone receptors contain two activation functions: one in the A/B region (AF-1) and the other in the ligand-binding domain (AF-2). The activities of these depend on cell type and target gene promoter [42]. RARs and RXRs also have a constitutive activation function in their ligand-binding (E/F) domain, which is named AF-2 by analogy, and deletion of this completely abrogates transcriptional activity [50]. Deletion of the A and B domains does not prevent transcription, but this region modulates activity in a cell type- and promoter-specific manner [50]. Response elements for RA, T_3 and vitamin D_3 also consist of conserved hexamer sequences but they are usually arranged as direct repeats separated by short sequences of variable length. By contrast to the steroid receptors, these receptors bind their target response elements in the absence of ligand [3, 51–53]. Because of these differences with classical steroid receptors, RARs, RXRs, TRs and VDR are considered to make up a subfamily within the steroid/thyroid hormone receptor superfamily.

The efficiency and specificity of response elements is a function of hexamer sequence, number, orientation and spacing [43, 54, 55] and is strongly influenced in *cis* by flanking sequences [50] and in *trans* by multiple cell type-specific nuclear factors [56, 57]. The distance between hexamer motifs does not define specificity [57], although earlier data suggest there could be a simple "3–4–5 rule" [55]. This suggestion arose from the observation that a thyroid hormone response element (TRE) containing two direct repeats of AGGTCA separated by four nucleotides was converted into a retinoic acid response element (RARE) by lengthening the spacer to five nucleotides, and into a vitamin D_3 response element (DRE) by shortening it to three nucleotides [55]. Different members of the RAR and RXR families (and even different isoforms of the same receptor) vary markedly in their transcriptional activity on different response elements and between different cell types [50]. The consensus sequences of TREs and RAREs are AGGTCA and A/GGTTCA, respectively. There are also negative TREs which inhibit transcription in response to T_3 [58] in a position- and promoter-dependent manner. Indeed, a change in position can transform a negative TRE into a positive one [59].

Members of the RAR/TR/VDR subfamily almost certainly function *in vivo* as heterodimers, either with other members of the subfamily or with other as yet uncharacterised proteins. This has been confirmed with a range of response elements for many family members using DNA binding and functional studies in a number of cell types. Receptor activation and dimerisation both require the conserved heptad repeats located in the E domain [54, 60–62]. Thus TRs and RARs cooperate in their binding to TREs but not RAREs [54, 63] although the binding of both to their respective response elements is even more strongly stimulated by a number of nuclear proteins, and it appears that heterodimerisation with these is functionally more important [54, 56]. Some of these nuclear proteins have been identified as members of the RXR family [41, 64, 65].

RXRs strongly cooperate with TRs, RARs and the VDR in binding to response elements, and RXR-α is much more efficient in cooperating with RAR-α than TR [52, 61, 62, 65–69]. There is a good correlation between synergy in DNA binding and transcriptional activation for some, but not all response elements suggesting that undefined factors are also important. Indeed, RXR-α requires a nuclear factor for efficient binding to at least one RXR response element (RXRE) [61]. The degree of transactivation observed is response element- and cell-type-specific for any combination of receptors ([70, 71, reviewed in 41]). Most of these results were obtained using all-*trans* RA, and since then it has been established that RXRs are preferentially activated by 9-*cis* RA [37, 38] which is itself produced in cells from all-*trans* RA [15]. In reticulocyte lysates, 9-*cis* RA induces homodimerisation of *in vitro*-synthesised RXR-α or -β on RAREs but not TREs [38], although whether RXR homodimerisation is physiologically relevant remains to be established.

There is evidence for at least two functionally different classes of DRE, of which only one requires an RXR for efficient transcription [72]. The expression from DREs related to the osteopontin gene promoter are activated by D_3 and 9-*cis* RA synergistically, apparently via the formation of VDR/RXR heterodimers. In contrast, DREs related to the osteocalcin promoter are only induced by D_3, and are completely independent of both RXR expression and the presence or absence of 9-*cis* RA [72].

Some receptors can inhibit the ability of other members of the family to stimulate transcription. RAR-γ1 inhibits transcription from RARE-containing promoters by RAR-β and RAR-γ2 in CV-1 cells [73] although all three receptors stimulate transcription from TREs. RAR-γ1 differs from RAR-γ2 by having a different A domain, but this region is insufficient to confer the repressive function to an RAR-γ1/RAR-β fusion protein. Nagpal *et al.* [50] also found that the modulating function in the A/B domain interacts differently with AF-2 regions from different receptors.

The dominant negative activity of mutant receptors also requires a functional dimerisation domain [48], as does inhibition between certain members of the RAR/TR/VDR subfamily. The heptad repeats of RAR-α are required for the cooperative binding of RAR-α and RXR-α to an RXRE from the cellular retinol binding protein II promoter (CRBPII-RXRE), and also for the inhibition (by RAR-α) of RXR-mediated transcription from this element in CV-1 cells [61]. In reticulocyte lysates, the cooperative binding of *in vitro*-translated RARs and RXRs to a RARE is consistently dependent on the presence of functional dimerisation domains [67]. The authors also provide evidence that efficient heterodimerisation between RARs and RXRs on RAREs often requires the DNA-binding domain of both receptors [67]. Both the dominant positive and dominant negative effects of normal and mutant chicken TR-α and -β on rat TR-mediated transcription also require the dimerisation domain [60].

The v-*erb*A gene product from the avian erythroblastosis leukaemia virus is often used as an example of an oncoprotein having a dominant negative function. This protein is a fusion product of a viral *gag* gene and a highly mutated chicken TR-α, and it can repress T_3-induced transcription when expressed in sufficient amounts. However, although v-*erb*A protein cannot bind T_3, the mechanism(s) which mediates its dominant negative effect is not firmly established, and requires both the DNA-binding and dimerisation domains (for review see [74]). A recent report demonstrates that the v-*erb*A product is a very efficient repressor of transcription from TREs (but not RAREs) when complexed with RXR-α [66].

Mutant TRs having dominant negative activity also occur in patients with generalised resistance to thyroid hormone (GRTH). This is a syndrome in which cells and tissues lack sensitivity to T_3 and it is caused by mutations in one of the TR-β alleles. These mutations usually lead to single amino acid changes in the ligand-binding domain which lessen or abrogate T_3 binding, and it has been proposed that mutated receptors form inactive complexes with normal TR-α and TR-β proteins on TREs. Recent evidence suggests that two alternative mechanisms may contribute to GRTH. Both normal and mutant TRs are able to inhibit basal transcription from TREs in the absence of T_3; and this silencing function is in the C-terminal region [75]. T_3 binding allows TR-α1 homodimers, TR-β1 homodimers and TR-α1/T-β1 heterodimers to dissociate from TREs, but dissociation of heterodimers consisting of TR (α or β) and either RXR-β or a distinct thyroid hormone receptor auxiliary protein (TRAP) does not occur. Since both of these factors greatly enhance the induction of transcription by T_3 it is likely that these (or similar) complexes mediate the induction of transcription by T_3 [51]. A mutant TR-β1 (Gly 345 to Arg) called Mf-1 (from a family with GRTH) readily forms dimers with either TR-α1 or TR-β1 on TREs, and these dissociate normally in the presence of T_3. However, Mf-1 preferentially forms homodimers on TREs and these are not removed by T_3, potentially causing a constitutive inhibition of transcription by competition for response elements. Mf-1 also heterodimerises on TREs with TRAP and RXR-β in a T_3-independent manner, and if these complexes are transcriptionally inactive (as might be expected by the low affinity of Mf-1 for T_3), this might also contribute to the dominant negative effect [51].

Mutant forms of RAR-α with dominant negative activity have also been reported. A RAR-α which is prematurely truncated at amino acid 391 and lacks the C-terminal 70 amino acids has been cloned from a correct RA-resistant P19 embryonal carcinoma cell line (RAC65). This receptor cannot mediate RA-stimulated transcription from a TRE within the context of the murine mammary tumour virus (MMTV) promoter [76] and inhibits the activity of normal RARs (α, β and γ). Interestingly, expression of the antisense transcript of the mutated receptor had the same effect, but this may be due to antisense-mediated inhibition of normal RAR expresssion. The RA-resistance of RAC65 cells can be reverted by over-expression of exogenous RARs (α, β and γ), therefore, the mutation appears to account for the resistant phenotype [20]. The truncated receptor binds to a RARE in mobility-shift assays.

Another recombinant RAR-α containing all of the A, B and C domains and part of the D domain (up to amino acid 189) also acts as a dominant negative RAR and inhibits the differentiation of F9 EC cells [19]. Interestingly, this receptor entirely lacks the ligand-binding and heterodimerisation domains yet is able to inhibit RA-induced transcription from some (but not all) promoters [19].

There is good evidence that RA-induced myeloid differentiation is indeed mediated by RARs. Several non-APL myeloid leukaemia cell lines can be induced to differentiate by retinoic acid. These include HL60 cells [23], which do not have a t(15;17) translocation and are classified as FAB M2 [77]. These cells express predominantly RAR-α, but also RAR-β [78]; since they only have one chromosome 17 they are haploid for RAR-α. A point mutation in this gene, which causes the expression of a receptor truncated at amino acid 411 (RAR-α411), has been found in an RA-resistant HL60 clone [79]. The expression of transfected RAR-α, -β, -γ or RXR-α were all able to reverse the resistant phenotype in a concentration-dependent manner [80]. The mutated RAR-α411 also displayed dominant negative activity on RAR-α-mediated transcription from a TRE-containing reporter in CV-1 cells. Another truncated RAR-α (RAR-α403) inhibits RA-induced differentiation after transfection into normal HL60 cells [81], and this receptor also inhibited RAR-α-mediated transcription from a TRE in NIH-3T3 cells, and transcription from a RARE by endogenous receptors in these cells. Strikingly, transfection of RAR-α403 into myeloid progenitor FDCP mix A4 cells changes their differentiation potential. These cells differentiate spontaneously at low frequency into monocytes and neutrophils, but expression of RAR-α403 induces differentiation into basophils and mast cells at high frequency [81]. This suggests that RAR-like activity may suppress this pathway of differentiation in multipotent progenitor cells.

Receptors do not necessarily require a functional DNA-binding domain to display dominant negative activity, and some TR mutants which only lack this region can inhibit RAR- and VDR-dependent transcription [60]. Similarly, the DNA-binding domain of RAR-α is not required for its ability to inhibit RXR-dependent transcription from the RXR response element in the CRBPII promoter [61].

Transcription of RA-responsive genes is also influenced by other promoter elements and transcription factors. Of particular interest are the COUP orphan receptors, which are also members of the steroid/thyroid hormone receptor superfamily. They are named after the chicken ovalbumin upstream promoter transcription factor (COUP-TF) which was the first to be identified. As yet a ligand for COUP receptors has not been identified, but in cell culture (containing fetal calf serum) they strongly inhibit transcription mediated by RARs, RXRs, TRs and the VDR [82, 83]. In fact, the COUP-TF binding site in the ovalbumin promoter functions as a RARE in the absence of COUP proteins [82]. Interestingly, COUP-TF appears unable to form dimers with RARs, TRs or VDR [83].

In summary, transcriptional activity is a complex function of the receptors which are present in a particular cell, the response element involved and its flanking sequences, the cellular environment, and the concentration and specificity of the activating ligand. The contribution made by the cellular environment in transient co-transfection assays is presumably due to the presence of interacting receptors and transcription factors, and metabolic effects upon the ligand. While the precise mechanisms which can cause dominant negative activity have not been elucidated, the E domain (which is required for receptor dimerisation and contains a ligand-dependent activation function) is usually required, and a DNA-binding region is sometimes required.

THE PML PROTEIN

PML has a proline-rich N-terminus, a cysteine/histidine-rich region, a coiled-coil motif and a serine-rich C-terminal domain (Fig. 1, [7–11]). The cysteine-rich region contains three cysteine clusters related to zinc fingers. The first is a newly recognised structure called a RING finger, having the general sequence $CX_2CX_{9-27}CXHX_2CX_2CX_{6-17}CX_2C$. The positions of the cysteine residues are highly conserved and proteins containing RING fingers make up a new class of DNA-binding proteins of diverse or unknown function [84, 85]. RING finger proteins contain a second zinc finger-like structure called a B box which has the general sequence $CX_2HX_7CX_7CX_2CX_5HX_2H$, and a more C-terminal coiled-coil putative dimerisation domain simi-

lar to that of PML. The second and third cysteine clusters in PML match the B box sequence and have been called B1 and B2, respectively (for review see [86]).

PML has highest homology with T18, a RING finger protein which is oncogenic when expressed as a climaeric T18/B-raf fusion protein, PML also has homology with MEL-18, which is preferentially expressed in transformed cells and Bmi-1 which is a related protein, with RET which has transforming potential when fused to a tyrosine kinase domain, the recombination-activating protein RAG-1, the 52K subunit of the RO-SSA (RO52K) ribonucleoprotein particle: a UV damage repair gene product from yeast called RAD18, RPT-1 which modulates expression of the IL-2 receptor, the herpes simplex virus gene regulatory protein ICPO, and the varicella zoster virus regulatory protein VZ61 ([11, and references therein]).

The coiled-coil region of PML has highest homology with the same motif in other RING finger proteins, but there is also significant homology with the leucine zipper of the FOS family of proteins [11]. The leucine zipper is required for dimerisation between FOS and JUN proteins and the stimulation of transcription from AP-1 sites [87].

The presence of proline-rich, cysteine-rich and coiled-coil motifs, which are all characteristic of transcription factors, suggests that PML may be able to act as a transcription factor. However, in the single reported direct analysis of this potential, a PML/Gal4 fusion protein was unable to stimulate transcription from a Gal4-responsive promoter [11].

THE PML/RAR-α FUSION PROTEIN

PML/RAR-α has been independently cloned by several groups [7–11]. The protein product consists of PML (N-terminal) fused to the B–F domains of RAR-α (C-terminal). It contains all the domains of RAR-α which are known to be required for activity, and all of PML except for the C-terminal serine-rich region. The differences in the cDNAs cloned by these groups result from the size heterogeneity of PML/RAR-α transcripts. In an extensive analysis of this phenomenon Pandolfi *et al.* [88] have characterised three breakpoint cluster (*bcr*) regions on chromosome 15. Names *bcr*-1, -2 and -3, they occur within the PML gene in intron 6, exon 6 and intron 3, respectively, and are rearranged in approximately 40, 20 and 40% of APL cases (Fig. 2). Further transcript size heterogeneity occurs within each patient due to alternative splicing of the PML portion of the fusion transcript, and alternative usage of two poly-A adenylation sites downstream of the RAR-α coding sequences.

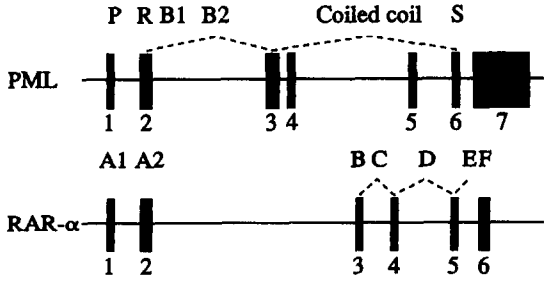

Fig. 2. Schematic diagram of the human RAR-α and PML genes. Exons (black boxes) are numbered according to Pandolfi *et al.* [88] and regions which encode protein domains (see Fig. 1) are indicated by broken lines. Domains are labelled as in Fig. 1; A1 and A2 are the alternative A domains of RAR-α. APL cells characteristically contain a t(15;17) chromosomal translocation in which exons 3 to 6 of the RAR-α gene on chromosome 17 are spliced to the PML gene. PML breakpoint cluster regions occur in intron 6 (*bcr*-1), exon 6 (*bcr*-2) and intron 3 (*bcr*-3). See text for details.

By contrast, the breakpoint in chromosome 17 consistently occurs between the exons encoding the A and B domains of RAR-α1. This region contains an alternatively spliced exon (encoding the A domain of RAR-α2) and although breakpoints have been mapped 5′ and 3′ of this exon it has not been detected in any of the PML/RAR-α RNA or cDNA sequences analysed [88].

Breakpoints in *bcr*-1 lead to a normal splicing event between exon 6 of PML and exon 3 of RAR-α, and translation of a 105 kD protein. Since the junction between these exons lies within a codon in the mature mRNA, *bcr*-1 rearrangement causes a change of one amino acid at the splice junction. Rearrangements in *bcr*-2 lead to the expression of a protein between 96 and 105 kD, depending on the exact position of the PML breakpoint. Some of these also generate single amino acid changes, and because normal splicing is interfered with, some produce mRNAs which contain sequences from intron 2 of the RAR-α gene. *Bcr*-3 rearrangements lead to splicing of PML exon 3 to RAR-α exon 3, causing a single amino acid change and translation of an 89 kD product. Each mRNA contains two potential AUG translation initiation sites separated by 23 codons [7–9, 11, 89]. It is, therefore, likely that several slightly different PML isoforms are translated.

Interestingly, a minor transcript encoding a truncated PML was detected in every APL sample analysed by Pandolfi *et al.* [88]. This truncated PML originates from the splicing of exon 4 in PML to exon in RAR-α (*bcr*-1 and -2), or exon 2 in PML to exon 3 in RAR-α (*bcr*-3). These splices generate in-frame termination codons a few amino acids downstream of the PML/RAR fusion boundary [88]. The authors suggests that PML breakpoints in *bcrs*-1, -2 and -3 may be selected in APL because they are the only ones which permit co-expression of PML/RAR-α and this truncated PML, implying that both proteins are necessary for leukaemogenesis. If correct, this also raises the intriguing possibility that APL may be caused by the expression of a truncated PML rather than the chimaeic PML/RARα.

THE ROLE OF PML/RAR-α IN LEUKAEMOGENESIS

Kastner *et al.* [11] transfected PML into COS-1 cells and determined its subcellular distribution by immunofluorescence. PML was mainly concentrated as discrete speckles in the nucleus although there was some cytoplasmic staining. Interestingly, the substitution of Glu59 Leu60 for Gln59 Cys60 within the RING finger caused a uniform nuclear distribution, which strongly suggests that this region has a specific DNA-binding function [85]. PML/RAR-α was detected in the cytoplasm and nucleus, and PML/RAR-α increased the cytoplasmic fraction of PML when both proteins were co-expressed; suggesting a direct interaction between the two proteins. Strikingly, RA restored the nuclear distribution of both PML and PML/RARα in such cells. In contrast to PML, RAR-α was entirely nuclear, uniformly distributed, and unaffected by RA.[^{3}H]RA-binding studies have confirmed the primarily nuclear localisation of PML/RAR-α and RAR-α [90].

Since RA can induce myeloid differentiation it is important to determine whether PML/RAR-α interferes with the induction of RA-responsive genes or RA-induced differentiation.

The first question has been addressed by the expression of exogenous RAR-α or PML/RAR-α in various cell types and determining their effects on transcription of co-transfected reporter genes in the presence or absence of RA. The detected

responses appears to be highly cell- and reporter construct-specific. While de The *et al.* [7] find PML/RAR-α inhibits RA stimulated transcription from reporter plasmids in HepG2 and HL60 cells, Kakizuka *et al.* [9] report that the presence of PML/RAR-α in either CV-1 or HL60 cells stimulates both basal and RA-stimulated expression of reporter genes under the control of a variety of RA- responsive promoters [9]. By contrast, Kastner *et al.* [11] show that in COS and HeLa cells the expression of PML/RAR-α reduces basal, but increases RA-stimulated expression from RA- responsive reporter plasmids [11]. Therefore, PML/RAR-α does not appear to have a consistent repressive or stimulatory effect on RA responsive genes.

The second question has been addressed by transfecting PML/RAR-α into HL60 cells, which can be induced to differentiate by a range of compounds including RA, dimethylsulphoxide (DMSO) and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). The expression of PML/RAR-α specifically inhibits RA-induced granulocytic differentiation, but does not affect granulocytic differentiation induced by DMSO, or macrophage-like differentiation induced by TPA (C. Chomienne, Hopital Saint Louis and F. Farzaneh).

It is not known how PML/RAR-α inhibits differentiation, and any model must account for the removal of this block by pharmacological concentrations of RA. If PML or RAR-α is involved in normal differentiation, then PML/RAR-α and/or the truncated PML [88] must inhibit this function—since normal PML and RAR-α proteins are expressed from the unrearranged alleles in APL cells [7, 9, 11]. Possible mechanisms include (a) inhibition of a normal RA signalling pathway, (b) inhibition of the normal function of PML, and (c) a new function specific to the chimera and requiring the contribution of both the PML and RAR-α components.

There are several ways in which PML/RAR-α could inhibit a normal RA signalling pathway. It might compete directly with RAR-α for RA or response elements, or bind and inactivate RAR-α or other proteins (such as RXRs) with which RAR-α normally interacts (either on or off RA-inducible promoters). However, it seems unlikely that competition for RA could be the full explanation because the affinities of both proteins for all-*trans* RA are similar [90], although PML/RAR-α protein does tend to be expressed at a higher level than RAR-α in APL cells [11].

Since PML/RAR-α retains the dimerisation domain of RAR-α it is likely to form complexes with the normal partners of RAR-α, and although this has not been demonstrated, it could be the basis of a dominant negative activity. PML/RAR-α does not consistently inhibit transcription from RA-responsive promoters [7, 9, 11], but it is possible that in promyelocytes the expression of specific genes which are required for differentiation may be inhibited. If this is so, how could high RA concentrations overcome this block? Possibilities include causing dissociation of PML/RAR-α from response elements or protein complexes (this possibility has not been addressed experimentally), or causing an up-regulation of RAR expression; both RAR-α2 and RAR-β are potentially inducible by RA [91]. It has also been suggested that PML/RAR-α could act as a normal RAR-α at high RA concentrations [7], but this implies that either PML/RAR-α has a lower affinity for RA than RAR-α, or more RA molecules are required for activation of transcription complexes which contain PML/RAR-α. The former now seems unlikely, since Nervi *et al.* [90] found little difference between RAR-α and PML/RAR-α in their binding specificities or affinities for a number of retinoids, including all-*trans* RA. Alternatively, since there appears to be some redundancy between RARs and RXRs with respect to their ability to mediate RA-induced differentiation, pharmacological concentrations of RA may alter a complex steady state distribution of receptors on gene promoters in favour of normal receptor signalling.

On the other hand, PML/RAR-α may inhibit the normal function of PML, possibly by binding to PML itself or to other proteins with which PML normally interacts. PML/RAR-α is known to occur predominantly in high molecular weight complexes when expressed in COS-1 cells [90], and the redistribution of PML which is caused by PML/RAR-α suggests there may be a direct interaction between these two proteins [11]. There are two attractive features of this model related to the induction of differentiation by RA. The first is the observation that pharmacological concentrations of RA restore the normal cellular distribution of PML protein, and the second is the theoretical possibility that PML/RAR-α has acquired a RA-inducible PML function. The ligand-binding domain of several steroid/thyroid hormone receptor family members can confer inducibility to a heterologous DNA-binding domain [34]. This model requires that normal PML function is necessary for myeloid differentiation past the promyelocyte stage, and although there is no direct evidence for this, the model is consistent with all the presently available facts.

It has been suggested [7] that the oncogenic activity of the PML/RAR-α fusion protein may depend on the co-expression of two functions in one molecule. Chimaeric proteins have been associated with other types of tumours [92, 93] and direct evidence for such a model is provided by the E26 retrovirus, in which the expression of a fusion myb/ets protein is much more oncogenic than the co-expression of myb and ets as separate components [94].

Finally, it is important to consider the possibility raised by Pandolfi *et al.* [88] that the truncated PML product may be the cause, or a contributory factor, in the generation of APL. In this case the effect of RA could be to inhibit the activity of this protein by influencing its interaction with PML/RARα.

OTHER CHROMOSOMAL ABNORMALITIES INVOLVING RAR-α?

Chromosome alterations other that the t(15;17) translocation have been reported in patients diagnosed as having APL by cell morphology and clinical characteristics [1]. Although chromosome 15 or 17 is often involved, neither is consistently rearranged. Furthermore, some cases of APL which do not have a detectable t(15;17) translocation are found to express chimaeric PML/RAR-α transcripts when analysed by PCR (E. Solomon, Imperial Cancer Research Fund).

A t(11;17) (q23–q21.1) translocation has been cloned from an APL patient who did not have a rearranged PML gene. The chromosome 17 splice-point is just downstream of the A2-encoding exon of the RAR-α locus, and a chimaeric protein is expressed consisting of the N-terminal sequences of a novel protein (PLZF) fused to the B-F domains of RAR-α [95]. The reciprocal gene products RAR-α1/PLZF and RAR-α2/PLZF are also expressed. PLZF does not appear to have any structural homology with PML, but is also has characteristics typical of transcription factors. It contains nine *Kruppel*-like (C_2–H_2) zinc fingers, and an acidic and a proline-rich region. The latter contains six putative proline-dependent phosphorylation sites. Highest detected homology is with the myeloid-specific zinc finger proteins MZF-1 and Evi-1 which may be involved in granulopoiesis [96–98]. PLZF is also related to the Krox gene

products which are involved in the regulation of homeobox (Hox) genes. Expression of PLZF transcripts (which are alternatively spliced) is restricted to myeloid cells and is very low in mature granulocytes. The expression of PLZF is down-regulated in HL60 and NB4 cells when they are induced to differentiate by treatment with RA [95]. PLZF/RAR-α retains all the putative DNA-binding motifs of PLZF. Since the original report [95], three additional APL patients have been identified who have similar t(11;17) chromosomal translocations. Unfortunately, none of these has responded either to conventional chemotherapy or to treatment with all-*trans* RA (A. Zelent, LRF Leukaemia Research Centre). This is consistent with the hypothesis that RA induces differentiation of APL cells (which express PML/RAR-α) by activating a PML function, although it does not completely rule out the alternative possibilities.

A number of other translocations also involving either chromosome 15 or 17 have been reported [8].

SUMMARY

While a great deal has been learned about APL over the last few years, many important questions remain unanswered. It has become clear that the PML/RAR-α fusion protein is expressed in most cases of APL, and this protein presumably contributes to leukaemia initiation and/or progression. PML/RAR-α appears to specifically block the further differentiation of myeloid progenitor cells, although the mechanism of its action is not known. It may inhibit the transcription of RAR- or PML-regulated genes, in which case expression must be restored in the presence of therapeutic RA concentrations. However, the possibility remains that PML/RAR-α may have a novel function.

In order to elucidate the molecular pathogenesis of APL, several important questions remain to be answered. These include whether PML is a transcription factor; the identification of its target genes and response elements, and the role of PML/RAR-α and RA in their regulation. Also whether the expression of PML/RAR-α in bone marrow cells (either by itself or in combination with other oncogenes) alters their tumourigenicity or differentiation potential. It is also important to determine the function(s) of PLZF and PLZF/RAR-α, and determine whether other APL patients with mutations involving PML or RAR-α (but not both) respond to therapy with all-*trans*-RA. Finally, it is important both for the understanding of the molecular biology of APL and its therapy, to determine the effects of other regulatory factors involved in the control of myeloid cell differentiation such as granulocyte colony stimulation factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF) on APL cells *in vitro* and *in vivo*, both at presentation and in the RA-resistant patients in relapse.

Note added in proof—*Perez et al.* (*EMBO J* 1993, **12**, 3171–3182) have recently demonstrated that PML/RAR-α can (a) heterodimerise with RXR, both on DNA response elements and in solution, (b) sequester RXR in the cytoplasm of transfected cells and (c) inhibit the binding of the VDR to a DRE.

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The Molecular Biology of Soft Tissue Sarcomas

Janet Shipley, Jayne Crew and Barry Gusterson

INTRODUCTION

SOFT TISSUE sarcomas are a heterogeneous group of malignant neoplasms which are generally considered to be derived from and which show features of mesenchymal tissue. The incidence of soft tissue sarcomas is in the region of 1–2 per 100 000 and they account for approximately 1% of all cancers and 2% of cancer deaths.

Although studies on soft tissue tumours have generally lagged behind those of more common malignancies such as breast and colon cancer, leukaemias and lymphomas, several key advances have been made in the last few years. Firstly, it has been well established that some inherited disorders can predispose to the development of certain soft tissue tumours, but only recently have some of the associated genes been cloned and characterised. Secondly, cytogenetic analyses have demonstrated the association of specific chromosomal changes with particular types of soft tissue tumours which may have important implications for diagnoses and, in some cases, prognoses. Lastly, gains and losses (or functional loss by mutations) of genetic material harbouring important genes are also implicated in tumorigenesis. These may be visible cytogenetically or detectable only at the molecular level.

This commentary illustrates how cytogenetic and molecular approaches can contribute towards a clearer understanding of

Correspondence to J. Shipley.
J. Shipley is at the section of Molecular Cytogenetics, F Block; J. Crew is at the section of Molecular Carcinogenesis; and B. Gusterson is at the section of Cell Biology and Experimental Pathology, Institute of Cancer Research, 15 Cotswold Road, Belmont, Sutton, Surrey SM2 5NG, U.K.
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