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The \(t(15;17)\) translocation alters a nuclear body in a retinoic acid-reversible fashion

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Nuclear bodies (NBs) are ultrastructurally defined granules predominantly found in dividing cells. Here we show that PML, a protein involved in the \(t(15;17)\) translocation of acute promyelocytic leukaemia (APL), is specifically bound to a NB. PML and several NB-associated proteins, found as auto-antigens in primary biliary cirrhosis (PBC), are co-localized and co-regulated. The APL-derived PML–RAR\(\alpha\) fusion protein is shown to be predominantly localized in the cytoplasm, whereas a fraction is nuclear and delocalizes the NB antigens to multiple smaller nuclear clusters devoid of ultrastructural organization. RA administration (which in APL patients induces blast differentiation and consequently complete remissions) causes the re-aggregation of PML and PBC auto-antigens onto the NB, while PML–RAR\(\alpha\) remains mainly cytoplasmic. Thus, PML–RAR\(\alpha\) expression leads to a RA-reversible alteration of a nuclear domain. These results shed a new light on the pathogenesis of APL and provide a molecular link between NBs and oncogenesis.

Key words: auto-immune disease/leukaemia/nuclear matrix/protein traffic/transdominant mutant

Introduction

Eukaryotic cells are divided into many physical and functional compartments. In the cytoplasm, the role of the different organelles and the traffic between them is extensively studied and basically known. In the nucleus, a growing body of evidence strongly suggests that DNA replication, RNA transcription and hnRNA splicing also take place in defined nuclear domains whose nature and interconnections, however, are far from being understood (Puvion and Moyné, 1981; Nakayasu and Berezney, 1989; Leonhardt et al., 1992; Jackson et al., 1993; Jimenez-García and Spector, 1993; Rosbash and Singer, 1993; Wansink et al., 1993). Little is known concerning the structural bases for this nuclear compartmentalization. Ultrastructural analysis of the nucleus, however, detected within the interchromatin space a few structures of unidentified function, known as nuclear bodies (NBs), (originally described by de Thé et al., 1960; reviewed in Brash and Ochs, 1992). While NBs are usually defined as electron dense regions in the nucleus, the use of other criteria has led to some inconsistencies in literature. Some NBs have been extensively studied, such as the nucleolus-derived coiled bodies shown to contain certain splicing components as well as a specific protein, p80-coilin (Andrade et al., 1991). Other less well characterized bodies were recently described using a variety of human auto-antibodies (Szosteki et al., 1987; Asconi and Maul, 1991; Fusconi et al., 1991; Saunders et al., 1991) and monoclonal antibodies (Asconi and Maul, 1991; Stuurman et al., 1992) which recognize distinct antigens of unknown function (such as Sp100, Szosteki et al., 1990). Although some of these bodies are induced in lectin-activated, hormone-stimulated or interferon-treated cells, their function remains elusive (Guldner et al., 1992; reviewed in Brash and Ochs, 1992).

Acute promyelocytic leukaemia (APL) is characterized by a specific \(t(15;17)\) translocation which fuses the PML gene to that of the retinoic acid receptor \(\alpha\) (RAR\(\alpha\)) (Borrow et al., 1990; de Thé et al., 1990; Goddard et al., 1991; Kakizuka et al., 1991; Pandolfi et al., 1991; de Thé et al., 1991; Kastner et al., 1992; reviewed in Warrell et al., 1993). The PML–RAR\(\alpha\) fusion protein might interfere with either or both of the PML and RAR pathways. As retinoic acid (RA) induces the differentiation of several myeloid cell lines, a defect in the retinoid signal transduction pathway could impair normal myeloid differentiation. Recently, this proposal has received some experimental support (Grignani et al., 1993; Rousset et al., 1994). Strikingly, administration of pharmacological doses of RA induces the differentiation of the leukaemic clone and leads to complete remissions in patients (Warrell et al., 1993). This effect clearly relates to the presence of the PML–RAR\(\alpha\) fusion protein, but its molecular mechanism remains undetermined. RA therapy of APL represents one of the first examples of differentiation therapy and, although APL is a rare disease, constitutes a key model system in cancer biology.

We have previously demonstrated that PML had a speckled nuclear distribution, which, in APL, is disrupted in a RA-reversible fashion (Daniel et al., 1993). In this report, we show that PML is associated with a specific NB. PML co-localizes and is co-regulated with auto-antigens of primary biliary cirrhosis (PBC) including a cloned protein: Sp100 (Szosteki et al., 1990). Overexpression of one of these proteins induces both the swelling of the structure and an increased labelling of the other antigens. PML–RAR\(\alpha\) induces the scattering of NB antigens within the nucleus and a marked reduction in both the number and labelling of normal NBs. In APL, the NB-associated antigens return to
their normal location after RA treatment. These observations of a RA-dependent alteration in PML distribution shed a new light on the pathogenesis of APL. Despite our ignorance of the molecular anatomy of the structure described here, the identification of some of its components and of PML–RARα as a trans-dominant mutant may contribute to the elucidation of NB function.

**Results**

**PML co-localizes with other antigens on a NB**

Immunoelectron microscopy was performed to determine precisely the localization of PML on stable CHO clones overexpressing PML. This revealed an exclusive association with a specific NB (Figure 1A–C) which is neither a coiled

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Fig. 1. Immunoelectron microscopic analysis of the NB detected by anti-PML antibodies in CHO cells stably expressing PML. Two aspects of NBs showing specific labelling of the periphery of the capsule. (A) The inner core displays a finely fibrillar organization. Bar, 0.2 μm. (B) Nuclear body with a neighbouring unlabelled cluster of interchromatin granules (IG). Bar, 0.2 μm. (C) A low power view of the cell showing the specificity of NB labelling. Bar, 0.5 μm; C, cytoplasm; N, nucleus; Nu, nucleolus.
Fig. 2. Double-labelling of transfected Cos7 cells incubated with antibodies to NB-associated proteins. (A and B) Transfection with a PML expression vector (A is stained with 5E10 and B with PBC2). Note the increase in dot size and in fluorescence when compared with an untransfected Cos cell (arrow in A or E). The cytoplasmic labelling in (B) is due to the anti-mitochondrial auto-antibodies. (C–F) Transfection with a PML–RARαS expression vector. (C and D) double-labelling with monoclonal antibody 5E10 (which does not recognize PML–RARαS) (C) and an anti-RARα antisera (D). Note that in the four transfected cells (top and right) the speckled pattern of endogenous PML proteins (detected with 5E10) is lost and that PML–RARα and PML do not apparently co-localize. The transfected PML–RARα fusion protein is principally perinuclear. This observation demonstrates that PML–RARα disorganizes the NB. (E) PML–RARαS transfected cells with a micro-speckled pattern of PML–RARα; Sp100 staining. Note that the typical micro-punctate pattern contrasts with that of the endogenous PML/Sp100 profile (arrow). (F) RA treatment: while in most cells RA administration led to perinuclear aggregation of the fusion protein (not shown), in rare cases a typical PML pattern was found as shown in (F). This demonstrates that RA can retarget PML–RARα to PML sites. In the above experiments, identical results were obtained using either 5E10, anti-PML, anti-Sp100 or PBC sera.
nor a beaded body. Similar images were obtained on normal haematopoietic cells (Figure 7A and B). This nuclear spherical mass of 0.3–1 μm diameter consists of an outer electron dense fibrillar capsule (selectively labelled by the anti-PML antibody) which surrounds a fibrillar or finely tubular core. The presence of a capsule defines a complex as opposed to a simple NB. In some cases, a very dense intermediate layer could be seen between the PML-labelled capsule and the core (not shown). In CHO cells overexpressing PML, the uranyl staining of the capsule was more pronounced than in normal cells, which may suggest that a high PML expression modifies the composition of the capsule (Figure 1A and B compare with Figure 7A and B). Labelling of the NB was very specific (Figure 1C). Thus, the speckled immunofluorescence pattern of PML is the result of binding to specific ultrastructurally defined nuclear domains.

Several antigens known to be NB-associated were shown to co-localize with PML on this structure: 5E10, a mouse monoclonal antibody raised against nuclear matrix from rat liver (Stuurman et al., 1992), two sera from patients with PBC and a rabbit polyclonal against a cloned auto-antigen in PBC (Sp100, Szoestecki et al., 1990) (Figures 2 and 3, and data not shown). These reagents were previously shown to co-localize with yet another set of auto-antibodies (Ascoli and Maul, 1991; Stuurman et al., 1992). To our surprise, Western blot analysis of PML and PML–RARα transfected cells consisted 5E10 to be an anti-PML monoclonal antibody. Importantly, the 5E10 antigen was shown to co-fractionate with the nuclear matrix, implying that PML belongs to this structure. We could map the 5E10 epitope between amino acids 448 and 466 (data not shown). Accordingly, this antibody is unable to detect PML–RARα type S (de Thé et al., 1991) or the bcr3 PML–RARα (Kakizuka et al., 1991; Kastner et al., 1992) as the epitope is absent due to the position of the breakpoint (bcr3) or to the absence of an alternatively spliced exon (PML–RARαS). The two sera from PBC patients recognize neither overexpressed PML nor Sp100 under denaturing or non-denaturing conditions (Western blot or slot blot, data not shown), suggesting that at least three different antigens from this NB can be monitored.

The structure described here is distinct from sites of DNA replication, as no co-localization was found between PML and de novo incorporated BrdU (not shown). A number of auto-immune sera with anti-SS-A/Ro, anti-Sm, anti-RNP, anti-La and anti-PIKA (Saunders et al., 1991) specificities were tested and again no co-localization was observed (not shown). These observations (as well as previous work with auto-antibodies and the 5E10 monoclonal (Ascoli and Maul, 1991; Stuurman et al., 1992)] imply that this NB is distinct from previously identified domains.

**Overexpression of PML or Sp100 increases NB size**

The availability of two cloned genes, whose products are associated with NbS, allowed a genetic analysis of this structure. Overexpression of PML led to a considerable swelling of the NB accompanied by a dramatic increase in staining of the structure with all antibodies (Figure 2A and B). This observation suggests that NB antigens are recruited (or perhaps induced or stabilized) by PML overexpression. Reciprocally, interferon α treatment (which transcriptionally up-regulates Sp100 (Guldner et al., 1992)) increased PML staining 5- to 10-fold (not shown). The sharp increase in fluorescence at the periphery of the dots suggests that these proteins are all located on the capsule of the NB, consistent with the immunoelectron microscopy data. As the level of antigen expression paralleled the size of the structure, PML and Sp100 may contribute to the structural scaffolding of the bodies. The co-regulation of the different antigens is consistent with the idea that the proteins form a complex. However, no binding of in vitro translated Sp100 to *Escherichia coli* produced GST–PML or MBP–PML fusion proteins could be detected under conditions where both PML and PML–RARα bound. Performing the reaction in the presence of total cell extracts also did not induce Sp100 binding (data not shown). Moreover, immunoprecipitation of co-translated in vitro PML and Sp100 by either anti-PML or anti-Sp100 antibodies failed to demonstrate any direct interactions between these two proteins (T.Sternsdorf and H.Will, unpublished data). These experiments confirm previous work on the dimmerization of PML and PML–RARα (Kastner et al., 1992) but strongly suggest that PML and Sp100 are not in direct contact.

**PML–RARα expression disrupts NB structure**

A natural alteration of the PML gene is found in APL where PML–RARα fusion proteins are formed as the consequence of the t(15;17) translocation. Previous work in transiently transfected Cos7 cells suggested that PML–RARα was targeted to nuclear sites distinct from those of PML, and that in some transfected cells, PML–RARα was predominately cytoplasmic (Kastner et al., 1992; Daniel et al., 1993). In stable expression systems, the PML–RARα pattern was less variable and consisted of hundreds of very small nuclear dots (hereafter referred to as micro-punctate or micro-speckled) (Daniel et al., 1993). Monoclonal antibody 5E10, which recognizes neither PML–RARαS nor bcr3 fusion proteins, allows the monitoring of endogenous PML proteins in cells transfected with either construct. Expression of these forms of PML–RARα in Cos7 or HeLa cells disrupted the endogenous PML pattern. As shown in Figure 2C and D monoclonal 5E10 revealed a diffuse PML fluorescence in PML–RARαS transfected Cos cells (which were identified with anti-RARα antibodies). In such transfected cells PML fluorescence was occasionally found in large, unstructured nuclear aggregates (not shown). Speckles were lost in all cells where PML–RARαS was cytoplasmic or perinuclear (these cells are those which express very high levels of the fusion protein). In some instances (when PML–RARαS was not so highly expressed, or in transfected HeLa cells), a distinct nuclear micro-speckled pattern was found (as in Figure 2E, note the difference with both the normal Cos pattern (arrow) and the previously described diffuse pattern (Figure 2C)). In those cases, endogenous PML (but also Sp100 or PBC) strictly co-localized with PML–RARαS (Figure 2E). In our hands, colocalization between PML–RARα and endogenous PML was only observed when the fusion protein was nuclear, contrasting with previous studies using co-transfected PML–RARα and epitope-tagged PML (Kastner et al., 1992). Our results establish that in all cases PML–RARα dominantly delocalizes PML, Sp100 and the other NB antigens from their usual nuclear sites.

The latter findings were extended to the APL cell line NB4 and to primary APL blasts. Through serial double labelling experiments (either by standard immunofluorescence or confocal microscopy), we could show that the RARα, PML,
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Fig. 3. Effect of RA on NB-associated antigens in APL. (A and B) Double-labelling of untreated NB4 cells with 5E10 (A) and anti-Sp100 (B). (C) Primary APL blasts labelled with anti-PML. (D and E) Double labelling of RA-treated NB4 cells: (D) 5E10, (E) anti-RARα. Note that the PML/Sp100 profile changes as the result of RA treatment to form the typical PML speckles found in non-APL cells. The cell in the centre of the figure has the typical micro-speckled profile of untreated NB4 cells. Note that this cell is still labelled with anti-RARα, while the others have lost most (right side) or almost all anti-RARα labelling (top and left side). (F) RA-treated NB4 cell labelled with PBC1, reaggregation of the antigen is evident (compare with B). Again, identical results were obtained using either 5E10, anti-PML, anti-Sp100 or PBC sera. (G, H and I) Phase-contrast microscopy of undifferentiated (G), differentiating (H) NB4 cells or primary APL blasts (I) at the same magnification. Note the respective sizes of the nucleus and cytoplasm as well as the RA-induced nuclear shrinking.

5E10, PBC and Sp100 nuclear patterns were identical and micro-punctate (Figures 3A, B and C, and 4A, and data not shown). In primary APL blasts, a distinct cytoplasmic PML labelling was apparent in half of the cells (Figure 3C). To facilitate identification of the cellular compartments, phase contrast images (Figure 3G, H and I) as well as confocal images (Figure 4C) are shown. These results extend our previous work on PML expression in APL (Daniel et al., 1993). Cell fractionation (by a variety of techniques) followed by Western blotting showed that PML-RARα was ~10 times more abundant in the cytoplasm than in the nucleus of APL blasts or NB4 cells (Figure 5). No significant differences in the distribution of the fusion protein were noted after 36 or 96 h of RA treatment (Figure 5 and data not shown). This strongly suggests that the weak cytoplasmic fluorescence seen in APL blasts is specific and is consistent with our previous immuno-histochemical data showing a strong PML labelling in the cytoplasm of APL cells (Daniel et al., 1993). Thus, immunofluorescence likely over-emphasizes the importance of the aggregated nuclear form.
of PML and underestimates the cytoplasmic form (either as the consequence of bleaching or insufficient sensitivity); on the other hand, Western blotting or immunochemistry points to the presence of a cytoplasmic form of PML—RARα. In conclusion, in APL cells, the fusion protein is predominantly cytoplasmic; but a fraction is nuclear and located in micro-speckles together with delocalized endogenous PML, Sp100 and the other putative PBC auto-antigens.

The nature of the micro-speckles was investigated by immunoelectron microscopy. In NB4 cells, normal NBs with labelled peripheral capsules were very rarely found (1 in 50 sections) and faintly labelled, whereas distinct clusters of gold grains were scattered throughout the nucleoplasm (Figure 6A). The labelled regions were devoid of a detectable ultrastructure. An APL patient’s bone marrow sample was analysed by the same technique. Prominently labelled normal bodies were found in sparse normal cells [an erythroblast (Figure 7A), a lymphocyte (Figure 7B)], while the rest of the nucleoplasm was devoid of specific labelling. In leukaemic blasts, rare faintly labelled normal bodies could be detected together with numerous clusters of gold grains in unstructured areas of the nucleoplasm (Figure 7C, compare with 6A), consistent with the immunofluorescence data (Figure 3C). This suggests that in APL blasts, a few intact bodies persist with a diminished PML content, while most of the NB antigens are scattered in the nucleus.

**RA treatment of APL cells**

Retinoic acid induces the terminal differentiation of APL cells by a yet poorly understood mechanism. In PML—RARαS transfected Cos cells, RA induces a perinuclear shift of the fusion protein (Kastner, 1992; and data not shown). In some rare cases, typical PML dots could be labelled with anti-RARα (as well as anti-PML), implying that RA can re-target PML—RARα to PML sites (Figure 2F) consistent with our previous data on stable CHO clones (Daniel et al., 1993). In PML—RARαS (or bcr3) transfected HeLa cells, RA administration led to the disappearance of anti-RARα stained cells and to a normalization of the endogenous PML pattern (data not shown). A similar situation was found in NB4 cells. The micro-punctate nuclear pattern was lost and substituted by a normal PML pattern as detected with PML, Sp100 and PBC antibodies as early as 24 h after RA exposure (Figures 3D—F and 4B) extending our previous data (Daniel et al., 1993). Surprisingly, nuclear anti-RARα labelling was (nearly) lost in 90% of cells. In the remaining 10% of cells

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**Fig. 5.** Western blot analysis of RA-treated or untreated NB4 or APL cells. Cytoplasmic (C) or nuclear (N) extracts (15 μg) of RA-treated or untreated NB4 cells were loaded on a 15% acrylamide gel. Cos/PML—RARα is a whole extract of transfected Cos cells. The fusion protein is revealed with anti-PML antibodies. The size of the PML—RARα fusion product is indicated. Note that PML—RARα is predominantly located in the cytoplasm; on longer exposures, the nuclear form can be detected (not shown). RA treatment does not significantly modify the abundance of PML—RARα. Identical results were found in APL cells (in that case, the patient was treated *in vivo* for 1 week). Reprobing of the blot with anti-RARα gave similar results (not shown).

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**Fig. 4.** Confocal microscopy analysis of NB4 cells during RA-induced differentiation. (A) Untreated cells. Co-localization of PML and RARα labelling in micro-speckles. (B) Three days RA treatment. PML is relocated in a small number of macro-speckles, whereas the RARα labelling is greatly diminished in intensity, but still co-localizes with PML in the most brightly PML-stained speckles. (C) Untreated or RA-treated cells stained with anti-PML and the cytoplasmic dye DiO6. Left panel, the majority of the micro-speckles are located in the nuclei. Note the mitotic cell bodies. Right panel, note the enlargement of the cytoplasm and the nuclear speckles.
prominently labelled staining RARα made on seen those 6. Fig. localized structures with 24 h. Note the co-existence of a large complex NB (arrow) with prominent labelling of the peripheral capsule and of a simple structure similar to those seen in untreated cells (arrowhead). Nu, nucleolus; C, cytoplasm (bar, 1 μm).

Fig. 6. Immunoelectron microscopy analysis of RA induced NB4 differentiation with anti-PML antibodies. (A) Untreated NB4 cells. Distinct small labelled structures (arrows) are present in the interchromatin space. Nu, nucleolus; C, cytoplasm (bar, 0.5 μm). (B) NB4 cells exposed to RA for 24 h. Note the co-existence of a large complex NB (arrow) with prominent labelling of the peripheral capsule and of a simple structure similar to those seen in untreated cells (arrowhead). Nu, nucleolus; C, cytoplasm (bar, 1 μm).

Discussion

The results presented in this report shed a new light on APL by implying the existence of RA-reversible alterations in the nuclear matrix. They also provide an unexpected link between the nuclear scaffold, NBs and oncogenesis.

The PML protein is identified here as a NB-associated protein. PML belongs to a gene family defined by a C3HC4 motif found in a large group of proteins, some of which (T18, RFP, bmi-1, LYAR and PML) are involved in transformation (Takahashi, et al., 1988; Haupt et al., 1991; Miki et al., 1991; Van Lohuizen et al., 1991; Su et al., 1993; reviewed in Lovering et al., 1993). Among this C3HC4 family, intracellular localization was studied for ICPO, RO-52K, PML and the Drosophila polycomb group, and shown to be speckled nuclear (Gelman and Silverstein, 1987; Ben-Chetrit et al., 1988; Paro et al., 1990). A sub-family, comprising seven proteins (PML, RO-52K, T18, RFP, RPT-1, XNF7 and PwA33) is defined by both a complex cysteine-rich region (including the C3HC4 motif) and a coiled-coil dimerization domain (Reddy et al., 1992; Bellini et al., 1993). It is of major interest that RFP was also shown to co-fractionate with the nuclear matrix (Isomura et al.,...
Fig 7. Ultrastructural analysis of an APL patient’s bone marrow sample. (A) An erythroblast shows a normal body (arrow). (B) Two NBs in a lymphocyte (arrows). (C) An APL blast cell with a faintly labelled normal body (arrow) and several clusters of gold particles (arrowheads). Note the similarity with Figure 6A. Bar, 0.5 μm. Identification of the cells was performed on a low magnification view by morphological criteria (M.T. Daniel, unpublished).
Moreover, T18 and RFP are also found as fusion proteins associated with transformation (Takahashi et al., 1988; Miki et al., 1991). Whether the members in this family are targeted to nuclear sub-structures (like PML and RO-52K) and whether the deletion/fusion process alters the intracellular localization of T18 and RFP would be interesting to determine. Thus, this sub-family is not only connected through structural similarities but also through similar localization, alteration and possibly function.

At present, the approach to NBs has been essentially descriptive and primarily based on ultrastructural observations. Previous work had identified some markers of NBs, principally auto-antigens from PBC patients (Szosteki et al., 1990; Asconi and Maul, 1991; Fusconi et al., 1991; Stuurman et al., 1992). However, the absence of an established function for Sp100 and the poor characterization of the other PBC auto-antibodies had hampered further progress in this field. While the various antigens used in this study are all located in the capsule of the NB, the nature of the inner core is unknown. Treatment of the nuclei with RNase, DNase or micrococcal nuclease left the PML bodies intact suggesting that nucleic acids are not a major component of this structure (Ascoli and Maul, 1991; Stuurman et al., 1992; data not shown). The cross-regulation and co-localization of these antigens (even when they are no longer NB-associated) strongly suggest that they form a complex. We note that the N-terminal part of Sp100 harbours a potential coiled-coil which could be used to form multimeric complexes (M.Koken, unpublished data). Despite our inability to demonstrate direct interactions between PML and Sp100, it cannot be excluded that they might bind each other possibly through the putative PBC auto-antigens which could function as adapters.

PML – RARα expression leads to the delocalization of the NB antigens studied here. In transfected cells, as well as in APL blasts, PML – RARα relocates endogenous Sp100, PML and PBC antigens to nuclear micro-speckles. This is accompanied by a great reduction in both number and labelling of the normal NBs. Such disruption is likely achieved through PML/PML – RARα heterodimer formation and delocalization of a large, multimeric protein complex off the NB core (Figure 8). Several molecular mechanisms could account for the corrective effect of RA. The most straightforward model would assume that RA disrupts the nuclear PML/PML – RARα heterodimers. This could be the consequence of the previously noted RA-induced conformational change in PML – RARα (Kastner et al., 1992; Perez et al., 1993). However, the in vitro binding of PML – RARα to GST – PML was RA independent (data not shown) and RA did not relocate endogenous PML in PML – RARα transfected Cos cells. These two observations are not in favour of this hypothesis. An alternative model would be that in the presence of RA, the fusion protein is targeted to normal PML sites. Indeed, in some RA-treated NB4 cells or PML – RARα transfected cells, a normal PML pattern is observed with anti-RARα antibodies (Figures 2F, 3E and 4B). However, the drastic decrease in RARα fluorescence found in RA-treated cells (Figures 3E and 4B) suggests that only a fraction of PML – RARα does so or that recognition of the fusion protein by the antisera is greatly diminished in the presence of RA. On the basis of the available data, neither of the hypotheses is fully satisfactory.

The actual mechanism may be a combination of these two models.

The data presented in this report may contribute to elucidate the pathogenesis of APL. Most models infer that PML – RARα should interfere with either PML or nuclear receptors in a RA-dependent fashion (Mangelsdorf and Evans, 1992; Kastner et al., 1992; de Thé and Dejean, 1992; Warrel et al., 1993). Expression of PML – RARα impairs nuclear receptor function and several models were proposed to account for the molecular mechanism (Kakizuka et al., 1991; de Thé et al., 1991; Kastner et al., 1992; Grignani et al., 1993; Perez et al., 1993; Rousselot et al., 1993). Our data indicating that the fusion protein is predominantly cytoplasmic, could strengthen the proposal that PML – RARα sequesters RXRs in the cytoplasm. We cannot completely exclude a leakage of an exclusively nuclear protein from the nucleus to the cytoplasm during the fractionation procedure as occurs for other proteins (Gorski et al., 1986; Barberis et al., 1989). However, taken together, the cytoplasmic localization of transfected PML – RARα, the immunocytochemical analysis on APL blasts and the various techniques of cell fractionation used make such an artefact relatively unlikely. By forming heterodimers with RXRs, cytoplasmic PML – RARα could impair the function of
nuclear receptors that require RXRs for DNA binding (Kastner et al., 1992). However, RA-induced rerouting of RXRs to the nucleus is likely not the basis for the restoration of nuclear receptor function as transfection studies have shown that RA does not affect the stability of (PML—RARα) — RXR complexes (Perez et al., 1993). On the other hand, RA-reversible alterations in NB structure are likely accompanied by the loss of the unknown function of this nuclear domain. In that sense, this fulfills the proposal that fusion of PML to RARα may render PML RA-dependent (Kakizuka et al., 1991). Little is known about the function of NBs, although previous studies on mitogenic, hormonal or interferon activation (Ascoli and Maul, 1991; Fusconi et al., 1991; Brach and Ochs, 1992; Guldner et al., 1992; as well as our own, M.Koken, manuscript in preparation) have shown a drastic increase in NBs, suggesting a link with cellular activity. Should NBs be implicated in growth control, their disruption could relate to transformation.

Previous studies on APL had principally focused on alterations of RA response and differentiation induction. By demonstrating the existence of RA-reversible alterations of the nuclear ultrastructure in APL, our observations could shed a new light on the pathogenesis of the disease. The experiments reported here provide the first evidence for genetically induced alterations in NB structure and provide a molecular link between NBs and oncogenesis. Apart from its role as a model for differentiation therapy, APL might also become a key system to study the genesis of NBs and may contribute to the elucidation of their functions.

Material and methods

Antibodies

Generation of affinity-purified rabbit, and mouse anti-PML antibodies, monoclonal antibody 5E10 and the Sp100 antiserum was described elsewhere (Szostecki et al., 1990; Sturman et al., 1992; Daniel et al., 1993). Two sera from PBC patients showing either an exclusively speckled nuclear pattern (PBC1) or an additional anti-mitochondrial labelling (PBC2) were used. Anti-RARα antiserum was kind gift of R. Evans (affinity-purified rabbit polyclonal), P. Chambon (mouse monoclonal antibody Ab9ααF) and J. Grippi (mouse monoclonal antibody α against the F region of RARα). Identical results were found with those three reagents. For immunofluorescence all antibodies were used at dilutions of 1/100 to 1/400, with the exception of the affinity-purified anti-PML serum, used at a 1/5 dilution. Cells were fixed in 4% paraformaldehyde for 15 min at 4°C, followed by 5 min 100% methanol at 4°C. Immunofluorescence was performed as described (Daniel et al., 1993). The dye DiO6 (Sigma) was used to stain cytoplasmic membranes (Terasaki et al., 1984).

Electron microscopy

Electron microscopic analysis was performed on stable CHO clones expressing a high level of PML protein (Daniel et al., 1993). Briefly, cells fixed with 1.6% glutaraldehyde in PBS for 20 min at 4°C were embedded in Lowicryl K4M at 30°C. Thin sections were incubated with affinity-purified anti-PML antibodies (1/2 in PBS) or 5E10 (1/10 in PBS) and subsequently in gold-labelled (10 nm particles) goat anti-rabbit (or mouse) IgG, followed by uranyl acetate staining. An identical procedure was used for NB4 cells and APL bone marrow. Both antibodies gave the same results.

Confocal microscopy

Observations were made using a MRC-600 (Bio-Rad, UK) confocal scanning laser microscope using the Comos software package. Discrete photon counting allowed a sharp visualization of weak label even with the highest magnification. A multiple Argon-ion laser beam was operated at full power and attenuated with one, two or three neutral density filters.

Cell culture and protein analysis

Transfections were performed using Lipofectone (Gibco/BRL) according to the manufacturer’s instructions. All-trans retinoic acid was used at a concentration of 10−8 M for 24 h unless otherwise indicated. Fractionation of NB4 cells was performed as follows: pellets of cells were resuspended in an equal volume of hypotonic lysis buffer (10 mM Tris, pH 8; 10 mM KCl; 20 mM NaMoO4; 1.5 mM EDTA; protease inhibitors) in the presence or absence of 18% Ficoll (Lue and Kornberg, 1987) in a 1 ml dosage followed by 2G2 — PML with 2G2 with peptide A. The supernatant yielded the cytoplasmic fraction. Following extensive washes of the pellet in lysis buffer (or centrifugation over a 30% sucrose gradient in lysis buffer), the nuclei were lysed in an appropriate volume of Laemmli sample buffer. Western blotting was performed by standard procedures (Sambroek et al., 1989) and revealed using ECL (Amersham). Production of glutathione-S-transferase—PML (GST—PML) and maltose binding protein—PML (MBP—PML) fusions in E.coli was performed as described (Aubusel et al., 1991). Binding of E.coli expressed PML fusions and in vitro translated PML, PML—RARα and Sp100 (Aubusel et al., 1991) were performed in NTEN buffer containing 150 or 250 mM NaCl. In some experiments Triton (0.1% or 1%) or a total cell extract of Cos7 cells was also included. RA was used at concentrations of 10−7 M and 10−6 M.

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**Note added in proof**