The physiological role of the pro-myelocytic leukaemia (PML) gene product is poorly defined. Among other functions, PML is involved in haematopoietic differentiation and in control of cell growth and tumorigenesis. We investigated the regulation of human PML expression by interferons (IFNs) and IL-1 in various human haematopoietic lines (U937, THP1, HL60, NB4), in human diploid fibroblasts and in human peripheral blood leukocytes. Cytokine-induced modulation of PML expression was assessed by Northern blot analyses, flow cytometry studies and in situ immunolabelling. Our data show that IFNs and IL-1 upregulate PML transcript and protein expression in a time and dose-dependent manner. In situ immunolabelling revealed that upregulation of protein expression by IFN-α is a consequence of a marked increase in both the number and the intensity of the staining of so-called PML nuclear bodies. Our data suggest that stimulation of PML expression by interferons and IL-1 may account for upregulation of PML proteins observed in inflammatory tissues and in proliferative states.

Key words: PML, Interferons, IL-1

Introduction

The PML (pro-myelocytic leukaemia) gene was identified originally as a fusion partner of the RARα gene in the chromosomal translocation t(15;17) specific for acute promyelocytic leukaemia (APL).1–4 Several PML-cDNAs have been isolated, resulting from alternative RNA splicing in the 3′ coding region of a single gene.5 The encoded proteins have a predicted molecular weight ranging from 47 to 98 kDa. PML proteins belong to a heterogeneous family of DNA-binding proteins characterized by the C3HC4 zinc binding ring finger. PML protein expression features a typical speckled nuclear pattern which is the consequence of the localization of the protein in nuclear compartments named nuclear bodies.2 The function of the PML nuclear bodies is still unknown. Recently it has been shown that PML participates in regulation of haematopoietic differentiation and in control of cell growth and tumorigenesis.6 In addition, there is evidence that PML protein is aberrantly expressed in a variety of pathological conditions including malignancies.7,8 The level of PML expression is upregulated in inflammatory tissues and in benign or malignant proliferative states.7–9 Cytokines, especially interferons (IFNs), are likely to represent some of the mediators that may be involved in overexpression of PML under these circumstances. Interferons are polypeptides that mediate pleiotropic effects on sensitive cells (reviewed in Sen and Lengyel10). Major activities of IFNs include immunomodulating activities, antiviral effects and inhibition of cellular growth. Two families of IFNs can be distinguished: type I IFNs (IFN-α/β and IFN-ω) and type II IFN (γ). Type I IFNs are encoded by a family of over 20 genes, whereas type II IFN is structurally unrelated and encoded by a single gene.10 An important function of IFNs is regulation of cellular growth of normal and malignant cells. The molecular mechanisms involved in growth inhibition are poorly understood.

Here, we investigated modulation of PML expression by interferons, TNF-α and IL-1. We present evidence that PML gene expression is enhanced by type I and type II interferons and by IL-1. We analysed cytokine induced up-regulation of PML transcript and protein expression in human haematopoietic cell lines, in human diploid fibroblasts and in human peripheral blood leukocytes. Modulation of PML protein expression by IFN-α and IL-1 was assessed with immunofluorescence flow cytometry. Immunofluorescence in situ analysis of PML nuclear bodies revealed that IFN treatment resulted in up-regulation of both the intensity and the number of these nuclear structures.

Materials and methods

Cell culture and materials

HL60, NB4, U937 and THP1 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Peripheral blood mononuclear cells (PBMCs)
were isolated from heparinized venous blood from normal donors by Ficoll-Hypaque (Lymphoprep, Nyegaard, Norway) density separation as described.11 Human diploid foreskin fibroblasts were obtained from the Department of Dermatology, University of Mainz, Germany. All cells were grown in RPMI 1640 (Seromed, Munich, Germany) supplemented with 10% fetal calf serum (PAA, Cölbe, Germany), penicillin 50 IU/ml (Serva, Heidelberg, Germany), streptomycin 50 IU/ml (Serva), sodiumpyruvate 1 mmol/l, L-glutamine 2 mmol/l (Boehringer, Mannheim, Germany), 2-mercaptoethanol 0.05 mmol/l, HEPES 10 mmol/l, and nonessential amino acids (Seromed) at 37°C and 5% CO2 as described.11 rh-IFNα2b with a specific activity of 1.8 × 108 IU/mg was obtained from Essex Pharma (Munich, Germany). h-IFN-γ was purchased from Thomae (Biberach, Germany). rh-IL-1α was kindly provided by Hoffmann La Roche (Nutley, NJ), rh-TNF-α (6.6 × 106 IU/mg) by Knoll AG (Ludwigshafen, Germany) and rh-IFN-β (3 × 108 IU/mg) by Bioferon (Laupheim, Germany).

**Northern blot analysis**

Total RNA was isolated by the method of Chomczynski and Sacchi12 and Northern blot analysis was performed essentially as described.11 RNA (20 μg) was subjected to electrophoresis on a 1% formaldehyde agarose gel and transferred to nylon membranes (Hybond N, Amersham, Buckinghamshire, UK). All hybridizations were carried out at 65°C for 16 h in a solution containing 1% BSA, 7% SDS, 1 mM EDTA, and 0.5 M sodium phosphate buffer (pH 7.2). Filters were hybridized with PML cDNA insert radiolabelled by the random priming method. A partial cDNA probe for PML was synthesized by RT-PCR from total RNA of THP1 cells using primers S1: 5'-ACCAGTCGGTGCGTGAGTT-3' and AS1: 5'-TGGATCTCTGCGCTGATGTC-3', corresponding to nucleotides 525–544 and 715–734, respectively of the published sequence.13 This part of the PML sequence is 100% homologous for all PML genes. The synthesized cDNA was controlled by DNA-sequencing and was subcloned into pCRTM II using a commercial cloning kit (Invitrogen, Leek, Netherlands). For quantitative analysis of mRNA levels densitometric scanning of autoradiographs was applied by using the Quanti-scan® program. PML specific transcript levels were normalized to β-actin mRNA expression.

**Immunofluorescence analysis**

Quantitative immunofluorescence studies of PML expression were performed by flow cytometry (EPICS, Coulter, Germany) as described14 by using saturating concentrations of a PML specific mAb.14 Normal mouse IgG (2 μg/ml) (Coulter, Krefeld, Germany) was used as background control, and FITC-labelled goat-anti-mouse IgG (Coulter, Krefeld, Germany) as second reagent. A total of 50,000 cells was analysed for each determination and specific fluorescence intensity was calculated on the basis of background fluorescence with control antibodies by using the EPICS program. Mean specific fluorescence intensity is given as channel number on a log scale from 1 to 1024 or in percentage of the respective untreated control.

**Indirect immunofluorescence microscopy**

Cells were air dried overnight, fixed for 5 min with acetone followed by incubation with PG-M3 antibody14 as undiluted hybridoma supernatant or with normal mouse IgG (Coulter, Krefeld, Germany) at a concentration of 2 μg/ml for 5 min. Cells were then washed with PBS and incubated with a FITC labelled goat-anti-mouse antibody (Coulter, Krefeld, Germany) (5 μg/ml). Cells were analysed with a fluorescence microscope (Orthoplan, Leitz, Wetzlar, Germany).

**Results**

**PML mRNA levels are upregulated by type I and type II interferons**

To investigate whether IFN affects PML gene expression, we performed Northern blot analyses of total cellular RNA extracted from various haematopoietic cell lines cultured with and without IFNs. In all cell types tested including the myeloid cell lines HL60 and THP1, human diploid fibroblasts and human peripheral blood mononuclear cells a marked increase in the three major PML transcript levels was observed by treatment with both type I (α, β) and type II (γ) interferons (Figs 1–4). Dose response experiments

**FIG. 1. Induction of PML mRNA by interferon. Northern blot analysis of PML-specific mRNA levels from IFN-treated and untreated PBMNC. PBMNC were treated with the indicated dose of IFN-α, IFN-β, TNF-α or LPS for 6 h. Medium treated PBMNC and freshly isolated PBMNC without further incubation were included as controls. Size fractionated total cellular RNA (20 μg per lane) was blotted and hybridized to the indicated 32P-labelled cDNA probes. For quantitative analysis of mRNA levels densitometric scanning of autoradiographs was applied as described in Materials and Methods.
showed that upregulation of PML mRNA levels was already evident upon incubation with 30 IU/ml IFN-α for 6 h (Fig. 1). At IFN-α doses of 30 IU/ml and 3000 IU/ml, quantitative analysis employing densitometric scanning revealed a 3.2-fold and a 5.3-fold induction of PML mRNA levels, respectively (Fig. 1). As a control, PML transcript levels were monitored upon incubation for 6 h with TNF-α and LPS and were found to be unchanged (Fig. 1). In experiments (Fig. 2 and data not shown) investigating the kinetics of IFN-α induced PML mRNA upregulation in PBMNC, maximum enhancement of PML transcript expression was already observed upon incubation for 3 h (3000 IU/ml IFN-α). Thereafter PML transcript levels declined and were barely visible after 24 h despite continuous incubation with 3000 IU/ml IFN-α (Fig. 2). In contrast to IFN-α, IFN-β (3000 IU/ml) induced PML transcript induction increased gradually over time and maximum enhancement was reached at 24 h (Fig. 2). The time course of IFN-γ induced PML transcript enhancement during a 6-h-incubation period was similar to IFN-α (Fig. 3). Previous studies indicated that transcriptional regulation of the PML gene is involved in up-regulation of PML mRNA by IFNs. To confirm that IFN-induced modulation of PML mRNA is independent from de novo protein synthesis, we performed
Northern blot analyses with and without an inhibitor of protein synthesis (CHX). These experiments demonstrated that CHX had no effect on IFN-induced upregulation of PML mRNA (data not shown).

The influence of IL-1α on PML transcript expression was investigated in human foreskin fibroblasts (Fig. 4). Treatment with IL-1α at a dose of 100 IU/ml for 6h resulted in a 3.8-fold upregulation of PML-specific mRNA levels as analysed by densitometric scanning. A more pronounced enhancement of PML transcript levels (7.1-fold induction) was observed when cells were incubated with IFN-α. IL-1 in combination with IFN-α stimulated PML mRNA expression to more than twice the level obtained with IFN-α alone (15.3 fold induction relative to medium control), as estimated by densitometric scanning. Co-incubation with genistein, an inhibitor of tyrosine kinases, significantly inhibited the IFN-induced increase in the level of PML transcripts, whereas no effect of genistein on IL-1-induced PML mRNA expression was noted (Fig. 4). Similar results were obtained using the amnion fibroblast cell line WISH (data not shown).

Influence of IFN-α on PML protein expression

In an attempt to investigate modulation of PLM protein expression, we performed flow cytometry analyses. IFN-α treatment of the three myeloid cell lines HL60, NB4 and U937 resulted in upregulation of PML protein expression. For example, in HL60 cells, upregulation of PML protein expression was detectable upon incubation with IFN-α for 18h (Fig. 5). The time course of IFN-induced enhancement of PML protein expression was monitored in U937 cells (Fig. 6). Induction of PML was not detectable upon incubation with IFN-α (1000 IU/ml) for only 6h or 12h but started thereafter and reached a plateau at 20h (Fig. 6 and data not shown). Dose–response experiments in U937 cells revealed that incubation with 10 U/ml IFN-α for 20h already resulted in slight upregulation of PML protein expression to 113% of the untreated control (Fig. 7). Maximum upregulation of PML expression to 160% of that of untreated cells was achieved by incubation with doses greater than 500 IU/ml IFN-α (Fig. 7). These data indicate that half-

![Fig. 5. Induction of PML protein by IFN-α in HL60 cells. HL60 cells were treated with (B) and without (A) IFN-α (1000 IU/ml) for 18h. Expression of PML protein was analysed by flow cytometry using a specific monoclonal anti-PML antibody as described in Materials and Methods. Normal mouse IgG was used as isotype control.](image)

![Fig. 6. Kinetics of induction of PML protein by IFN-α in U937 cells. U937 cells were treated with and without IFN-α (1000 IU/ml) for the indicated time (A: medium control; B: IFN-α for 19.5h). Induction of PML protein was monitored by flow cytometry using a specific monoclonal antibody recognizing the aminoterminal domain of PML as described in Materials and Methods.](image)

![Fig. 7. Dose-response of induction of PML protein by IFN-α in U937 cells. U937 cells were incubated for 18h in the absence or presence of the indicated dose of IFN-α and PML protein expression was analysed by flow cytometry using a specific monoclonal antibody as described in Materials and Methods.](image)
maximum IFN-α response is reached at 50 U/ml IFN-α which corresponds to the IFN-α concentration needed to achieve a half-maximum response in other biological activities of IFN-α.10

When human foreskin fibroblasts were grown in the absence or in the presence of saturating concentrations of IL-1α, a nearly two-fold increase of PML protein expression was detected by flow cytometry analysis (Table 1). A similar result was obtained upon incubation with IFN-α (100 IU/ml). The combination of IL-1 and IFN-α stimulated PML protein expression to about three times the level observed in untreated controls (Table 1).

Analysis of PML nuclear bodies

Since it has been shown that PML proteins are located within subnuclear structures from active sites of transcription and splicing, the so-called PML nuclear bodies were monitored during IFN-α treatment using indirect immunofluorescence staining. PML-specific immunofluorescence varied in intensity between the cell lines tested. In all cell lines tested (NB4, HL60, U937) and in peripheral blood leukocytes the constitutive levels of endogenous proteins were detectable (Figs 8 and 9). Peripheral blood leukocytes revealed specific immunolabelling of PML nuclear bodies in neutrophils and monocytes (Fig. 8). When HL60 and U937 cells were incubated for 15 h with and without IFN-α (1000 U/ml) a marked enhancement of the immunolabelling was observed in treated cells. Both the intensity of the staining and the number of the labelled PML nuclear bodies was increased significantly (Fig. 9 and data not shown).

Discussion

Our data confirm and extend previous studies demonstrating that cytokines are involved in modulation of PML gene expression.15,16 We show that PML transcripts rapidly accumulate upon treatment with interferons in a variety of haematopoietic cell lines and in primary diploid cells. However, our experiments demonstrate a marked difference in the time course of PML-mRNA induction between IFN-α and IFN-β. Induction of PML in response to IFN was shown to be independent from de novo protein synthesis and actinomycin sensitive indicating that PML mRNA levels are transcriptionally regulated by IFNs.16 Recently, evidence has been provided that the PML gene is transcriptionally upregulated by IFNs through activation and binding of STATs to an ISRE and a GAS element in the untranslated first exon.18 IFNs activate STATs through tyrosine phosphorylation by members of the JAK tyrosine kinase family.19 Our results demonstrating inhibition of IFN-induced upregulation of PML transcripts by the tyrosine kinase inhibitor genistein are consistent with these findings. Flow cytometry analyses using a specific monoclonal anti-PML antibody recognizing the aminoterminal domain clearly demonstrate that IFNs induce PML

Table 1. Induction of PML protein by IFN-α, IL-1α or a combination of both in human diploid fibroblasts

| Treatment                          | Δ Mean X | x-fold induction |
|-----------------------------------|---------|-----------------|
| Medium                            | 1.29    | 1.0             |
| IFN-α (100 IU/ml)                 | 2.75    | 2.1             |
| IL-1α (100 IU/ml)                 | 2.31    | 1.8             |
| IL-1α (1000 IU/ml)                | 2.43    | 1.9             |
| IFN-α (100 IU/ml) + IL-1α (100 IU/ml) | 4.30  | 3.3             |
| IFN-α (100 IU/ml) + IL-1α (1000 IU/ml) | 3.80  | 3.0             |

Fibroblasts were incubated with and without the indicated cytokines at the indicated dose for 21.5 h. Induction of PML protein expression was analysed by flow cytometry using a specific anti-PML monoclonal mouse antibody and normal mouse IgG as control as described above. Given is the mean log specific fluorescence intensity as described in Materials and Methods.

FIG. 8. In situ immunofluorescence analysis of PML proteins in human peripheral blood leukocytes. Indirect immunofluorescence analysis of a peripheral blood smear was performed using the specific mouse monoclonal antibody PG-M3 (B) and normal mouse IgG (A) as control as described in Materials and Methods. Specific immunolabelling can be detected in neutrophils and monocytes which were identified by morphologic criteria.

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protein in a dose dependent manner. Recently, it has been shown that PML protein expression has a speckled nuclear pattern. This is due to localization of the protein in poorly defined nuclear organelles named nuclear bodies. The functions of the nuclear bodies are unknown except for the coiled bodies which act as storage compartments for splicing factors. To analyse expression of PML proteins within nuclear bodies we performed indirect in situ immunofluorescence studies. In situ immunolabelling of PML protein expression revealed an IFN-induced increase in number and intensity of so-called PML nuclear bodies. To the best of our knowledge induction of PML by IL-1 has not been described so far. Here we show that IL-1 rapidly induces PML mRNA expression in fibroblasts. Since IL-1 is a potent inducer of IFN-α and IFN-β it could be argued that the observed induction of PML mRNA expression is a consequence of IL-1 induced autocrine production of interferon-β. We addressed this issue by employing the tyrosine kinase inhibitor genistein. Genistein was shown to strongly inhibit IFN signalling. Our experiments clearly show that in fibroblasts genistein is not able to suppress the IL-1 induced upregulation of PML transcripts, whereas IFN-induced enhancement of PML-specific transcripts was strongly inhibited. Thus, indirect regulation of PML by IL-1 induced autocrine production of IFN-β appears unlikely. Flow cytometry analysis revealed that IL-1 and IFN-α act synergistically in induction of PML proteins. This regulation could be involved in the observed upregulation of PML proteins in inflammation. In contrast to normal tissues, in inflammatory tissues the level of PML proteins is upregulated. Local production of IL-1 has been demonstrated in inflammatory and autoimmune disease and this may contribute to tissue damage and repair. In fibroblasts, IL-1 mediates tissue repair after inflammation and injury by regulating key proteins necessary for formation of the extracellular matrix. The data presented here suggest that IL-1 induced PML expression may represent the molecular basis for the observed upregulation of PML proteins during inflammation. IFNs, as part of the cytokine network are indirectly induced by regulators such as tumour necrosis factor during immune responses and inflammation. This may contribute to upregulation of PML in inflammatory tissues by synergistic action of IFN-α and IL-1 in enhancing PML protein expression in fibroblasts.

FIG. 9. In situ immunofluorescence analysis of PML proteins in HL60 cells. HL60 cells were incubated with (C, D) and without (A, B) IFN-α (1000 IU/ml) for 15 h. Indirect immunofluorescence analysis was performed using the specific mouse monoclonal antibody PG-M3 (B, D) and normal mouse IgG (A, C) as control as described in Materials and Methods.
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