(Yamashita *et al.*, 1996). After culture for 2 weeks in the presence of G418, seven and four independent cell clones were isolated from cells transfected with pMT-NuMA or the empty vector, respectively. For the induction of NuMA expression, transfectants were cultured overnight in the presence of 0.1 mM $ZnSO_4$. Proteins were extracted from the transfectants and subjected to immunoblot analysis as described previously (Yamashita *et al.*, 1996); the latter was performed with rabbit polyclonal antibodies to NuMA (Gaglio *et al.*, 1997). The DNA profiles of cells were examined with a FACScan processor (BD Biosciences, San Jose, CA, USA) and cell cycle distribution was determined with the ModFIT program (BD Biosciences).

Subcellular localization of $p240^{NuMA}$

The *NuMA* cDNA spanning its total coding sequence was amplified by PCR with Pfu DNA polymerase (Stratagene, La Jolla, CA, USA), and inserted into the pEGFP-N1 vector (BD Biosciences), giving rise to pEGFP/NuMA that encodes a NuMA protein tagged C-terminally with EGFP. The human kidney 293 cells were transfected with pEGFP-N1 or pEGFP/NuMA by the calcium phosphate method, and cultured for 48 h. EGFP or NuMA-EGFP was then detected by the IX71 fluorescent microscope (Olympus, Tokyo, Japan) equipped with a digital CCD camera DP50 (Olympus).

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Bruton's Tyrosine Kinase Is Required For Lipopolysaccharide-induced Tumor Necrosis Factor α Production

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Abstract

Lipopolysaccharide (LPS), a product of Gram-negative bacteria, is potent mediator of tumor necrosis factor (TNF)α production by myeloid/macrophage cells. Inhibitors capable of blocking the signaling events that result in TNFα production could provide useful therapeutics for treating septic shock and other inflammatory diseases. Broad spectrum tyrosine inhibitors are known to inhibit TNFα production, however, no particular family of tyrosine kinases has been shown to be essential for this process. Here we show that the Bruton's tyrosine kinase (Btk)-deficient mononuclear cells from X-linked agammaglobulinemia patients have impaired LPS-induced TNFα production and that LPS rapidly induces Btk kinase activity in normal monocytes. In addition, adenoviral overexpression of Btk in normal human monocytes enhanced TNFα production. We examined the role of Btk in TNFα production using luciferase reporter adenoviral constructs and have established that overexpression of Btk results in the stabilization of TNFα mRNA via the 3′ untranslated region. Stimulation with LPS also induced the activation of related tyrosine kinase, Tec, suggesting that the Tec family kinases are important components for LPS-induced TNFα production. This study provides the first clear evidence that tyrosine kinases of the Tec family, in particular Btk, are key elements of LPS-induced TNFα production and consequently may provide valuable therapeutic targets for intervention in inflammatory conditions.

Key words: tyrosine kinase • adenovirus • TNFα • macrophage • X-linked agammaglobulinemia

Introduction

TNFα is a proinflammatory cytokine pivotal to the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease (1), and in inflammatory responses such as leukocyte migration, tissue resorption, the acute-phase response, and fever (2). The major producers of TNFα are cells of the mononuclear phagocyte lineage including macrophages, microglia, osteoclasts, and myeloid dendritic cells. Despite considerable efforts, relatively few signaling molecules, e.g., nuclear factor (NF)*-κB (3, 4) and p38 MAPK/MK2 (5, 6), have been shown to be essential for TNFα expression in macrophages. It is well documented that tyrosine kinase inhibitors are potent suppressors of LPS-induced TNFα production (7, 8) and that

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**Abbreviations used in this paper:* AdBtk, adenovirus-encoding human Bruton's tyrosine kinase; ARE, AU-rich element; Btk, Bruton's tyrosine kinase; IκBα, inhibitory protein κBα; MAPK, mitogen-activated protein kinase; m.o.i., multiplicity of infection; NF, nuclear factor; UTR, untranslated region; *xid*, X-linked immunodeficiency; XLA, X-linked agammaglobulinemia.

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the activation of tyrosine kinase activity is one of the earliest detectable events after LPS stimulation of monocytic cells (9–11). However, defining a causal relationship between a particular tyrosine kinase and TNFα production has been difficult. LPS can activate the Src family kinases $p58^{hck}$, $p53^{lyn}$, and $p59^{fgr}$, as well as $p72^{syk}$ (12–14), yet macrophages from mice deficient in these kinases do not show any impairment of LPS-induced TNFα production (15, 16). LPS has also been shown to activate the tyrosine kinase Pyk-2 (17), but its role, if any, in TNFα production has yet to be elucidated.

Another family of nonreceptor tyrosine kinases expressed by monocytic cells is the Tec family, and in particular, Bruton's tyrosine kinase (Btk). Recent studies have shown that the Tec family kinases are important components of both antigen receptor signaling and other cell surface receptors, resulting in the activation of numerous signal transduction pathways, regulation of the actin cytoskeleton, adhesion, migration, and transcriptional activation (18). Btk is found in all cells of the hematopoietic lineage except plasma and T cells (19), and is required for normal B cell development and signal transduction through cell surface molecules (20). Mutations in the *btk* gene result in aberrant B cell development, leading to the X-linked agammaglobulinemia (XLA) phenotype in humans and to the less severe X-linked immunodeficiency (*xid*) in mice. *xid* B cells show some hyporesponsiveness to LPS stimulation, although the precise cause has not been established (21, 22). Because the major phenotype of Btk deficiency is impaired B cell development and function, this cell type has been the major focus of interest to date. Earlier studies performed on *xid* mononuclear cells showed no obvious impairment of TNFα production in response to a series of inflammatory stimuli such as LPS and formalin-killed *Staphylococcus aureus* (23). However, bone marrow–derived cultured mast cells from *xid* mice have been shown to produce less TNFα in passive cutaneous anaphylactic reactions (24) and there is a recent report that *xid* peritoneal macrophages express reduced levels of TNFα and IL-1β in response to LPS (25). Additionally, *xid* mice are resistant to models of autoimmune diseases, e.g., collagen-induced arthritis (26, 27).

In humans, XLA is characterized by the absence of mature B cells in the periphery with a marked reduction in serum levels of all Ig isotypes resulting in a susceptibility to recurrent and severe bacterial infections (20, 28). Yet despite lacking Btk, XLA monocytes appear to develop normally and are present in expected numbers in the circulation. The importance of Btk in human monocyte/macrophages remains to be fully elucidated. As the XLA phenotype differs from that of the *xid* mice, we have examined the responses of XLA monocytes and macrophages to LPS stimulation. In addition, we have used an adenovirus expressing Btk in conjunction with TNFα luciferase reporter adenoviruses to analyze the contribution of Btk-dependent signaling events to the regulation of TNFα production.

Materials and Methods

Isolation and Culture of Cells

Isolation and Culture of PBMCs from XLA and Control Donors. Human blood samples were collected into lithium heparin vacutainers. Each blood sample was mixed with an equal volume of HBSS. PBMCs were prepared by ficoll-hypaque centrifugation on a lymphoprep gradient. PBMCs were cultured in RPMI containing 100 units/ml penicillin/streptomycin and 10% heat-inactivated FCS at 37°C in a humidified atmosphere containing 5% CO_2. Monocytes were isolated from the PBMCs by adherence to plastic for 1 h at 37°C in RPMI containing 100 units/ml penicillin/streptomycin and 10% FCS. Nonadherent cells were then washed off and the adherent monocytes were rested overnight and stimulated with LPS. B cells were depleted from PBMC using Dynabeads® coated with anti-CD19 antibodies. Cells were incubated with the beads for 1 h with constant agitation at 4°C. The beads were then magnetically removed and the remaining cells were subjected to FACS® analysis with FITC-conjugated anti-CD19 resulting in >90% B cell depletion.

Isolation of Monocytes by Elutriation. PBMCs were prepared from buffy coat fractions of a unit of blood from a single donor using ficoll-hypaque. The monocytes were then isolated by centrifugal elutriation as previously described (4). Monocyte fractions of >85% purity were routinely collected in this manner. Monocytes were cultured in RPMI containing 100 units/ml penicillin/streptomycin and 10% heat-inactivated FCS at 37°C in a humidified atmosphere containing 5% CO_2. For adenoviral infection, monocytes were treated with 100 ng/ml M-CSF (provided by G. Larsen, Genetics Institute, Boston, MA) for 72 h before viral infection.

Generation of Adenoviral Vectors and Cell Infection. Recombinant, replication-deficient adenoviral constructs encoding wild-type human Btk (AdBtk; cDNA provided by C. Kinnon, Institute of Child Health, London, United Kingdom) were prepared using the AdEasy system as previously described (29). In short, recombinant viral DNA was transfected into 293 cells in 6-well tissue culture plates using lipofectamine (GIBCO BRL) according to the manufacturer's instructions. Cells were overlaid with 2% agarose/DMEM 24 h after transfection and viral plaques were picked after 9–10 d. Viral clones were propagated in 293 cells and screened for transgene expression by Western blotting. Clonal viruses were then prepared from 20 175 cm^3 tissue culture flasks of 293 cells by ultracentrifugation through two caesium chloride gradients. Titres of viral stocks were determined by plaque assay and viral aliquots were stored at −70°C. The AdGFP adenovirus was prepared according to this protocol using AdTrackGFP with no insert. AdTrack0 (Ad0), pAdTrack-TNF 5′ promoter-Luc-3′ untranslated region (UTR; Ad5′3′luc), and pAdTrack-TNF 5′ promoter-Luc (Ad5′luc) were generated as previously described (30). The NF-κB luciferase adenovirus (AdNF-κB-luc) contains four tandem copies of the κ enhancer element located upstream of the firefly luciferase gene (31). This adenovirus was provided by P.B. McCray Jr. (University of Iowa, Iowa City, IA) and is a modification of the pNF-κB reporter vector (BD Clontech). Recombinant adenoviral vectors encoding *Escherichia coli* β-galactosidase (Adβgal) were provided by M. Woods and A. Bryne (Oxford University, Oxford, United Kingdom).

M-CSF–derived monocytes were plated in 96-well plates at 1.5×10^5 cells/well and allowed to settle for at least 4 h. The cells were washed in serum-free RPMI medium and then exposed to virus at different multiplicity of infection (m.o.i.) for 2 h

in serum-free RPMI at 37°C, after which cells were washed in RPMI and cultured in complete medium for 24 h before stimulation with LPS as previously described (4).

Immunoprecipitation and In Vitro Kinase Assay. Btk autokinase activity was measured in response to LPS in primary human monocytes. Cells were stimulated with LPS for various times as detailed. Cells were pelleted by centrifugation and lysed for 20 min on ice with 1% NP-40 lysis buffer containing 20 mM Tris, pH 8, 130 mM NaCl, 10 mM NaF, 1 mM DTT, 20 μM leupeptin, 100 μM sodium orthovanadate, 1 mM PMSF, 10 μM E64, and 2 mg/ml aprotinin. Lysates were microfuged for 10 min at 13,000 rpm at 4°C and supernatants were removed and precleared for 30 min at 4°C in 20 μl protein A–sepharose (previously washed in lysis buffer). After centrifuging the samples for 10 min at 1,300 rpm, the supernatants were removed and incubated with 2 μl rabbit polyclonal anti–Btk (provided C. Kinnon, Institute of Child Health, London, United Kingdom) for 1 h at 4°C. 30 μl protein A–sepharose was then added to each sample and incubated for an additional 1.5 h at 4°C. The beads were then washed three times in lysis buffer and once in kinase buffer (10 mM $MgCl_2$; 10 mM $MnCl_2$). Beads were mixed with kinase buffer containing γ^{32}P[ATP] and incubated for 15 min at room temperature. The reaction was stopped by the addition of 4× Laemmli buffer and the samples were boiled for 10 min. Samples were electrophoresed on an 8% SDS polyacrylamide gel. The gel was stained, fixed, dried, and autoradiographed. Tec autokinase activity was measured as previously described (32). Tec protein was immunoprecipitated, either with rabbit anti–Tec serum (32) or goat polyclonal anti–Tec (Santa Cruz Biotechnology, Inc.).

Western Blot Analysis. Cells were lysed in lysis buffer and debris pelleted as described above. Lysate protein concentration was assessed by Bradford assay and equivalent amounts of lysate protein were electrophoresed on 8% SDS polyacrylamide gels, followed by electrotransfer of proteins onto nitrocellulose membranes. Membranes were blocked in 10% Marvel/PBS/Tween (0.05%) and probed with either rabbit polyclonal anti–Btk (BD Biosciences) at 1 μg/ml in 5% Marvel/PBS/Tween (0.05%), anti–inhibitory protein κBα (IκBα; Santa Cruz Biotechnology, Inc.), anti-p54/JNK (Santa Cruz Biotechnology, Inc.), or rabbit anti–Tec for 2 h. After washing, membranes were incubated with anti–rabbit horseradish peroxidase at 1:5,000 in 5% Marvel/PBS/Tween (0.05%). After washing in PBS/Tween (0.05%), the membranes were developed using enhanced chemiluminescence according to the manufacturer's instructions (Amersham Biosciences).

ELISA. Supernatants were harvested 18 h after stimulation of human macrophages or PBMCs. The concentration of TNFα (BD Biosciences) was determined by ELISA according to the manufacturer's instruction. Absorbance was read and analyzed at 450 nM on a spectrophotometric ELISA plate reader (Labsystems Multiskan Biochromic) using the Ascent 2.4.2 software program. Results are expressed as the mean concentration of triplicate cultures ±SD.

Luciferase Assays. After LPS stimulation, cells were washed once in PBS and lysed with 100 μl CAT lysis buffer (0.65% [vol/vol] of NP-40, 10 mM Tris-HCl, pH 8, 0.1 mM EDTA, pH 8, 150 mM NaCl). 50 μl cell lysate were transferred into the well of a luminometer cuvette strip containing 120 μl luciferase assay buffer. Luciferase activity was measured with a Labsystem Luminometer by dispensing 30 μl luciferin (Bright-Glo luciferase assay system; Promega) per assay point. Cell lysates were assayed for protein concentration by Bradford assay and luciferase activity was adjusted accordingly.

Taqman RT-PCR. M-CSF–treated monocytes were plated at 5×10^5 cells/well in 24-well plates and infected as described above. Total RNA was extracted using RNeasy Kit (QIAGEN) according to the manufacturer's instructions. All semiquantitative RT-PCR was performed using an ABI PRISM 7700 Sequence Detection System, Taqman One Step RT-PCR reagent, and TNFα, IL-6, and GAPDH predeveloped assay reagents (Perkin-Elmer). ABI PRISM 7700 Sequence detector was programmed for the RT step of 30 min at 48°C followed by a 5-min deactivation step at 95°C. Subsequent PCR amplification consisted of 40 cycles of denaturation at 94°C for 15 s and annealing/extension at 60°C for 60 s. The cycle number at which the amplification plot crosses a fixed threshold above baseline is defined as threshold cycle (C_t). To control variation in mRNA concentration, all results were normalized to the housekeeping gene, GAPDH. Relative quantitation was performed using the comparative $\Delta\Delta C_t$ method according to the manufacturer's instructions.

Results

Btk Deficiency Results in Impaired TNFα Production. PBMCs from XLA blood were used to see if Btk deficiency had any effect on LPS-induced TNFα production. When compared with PBMC from normal age and sex-matched controls, the LPS response of XLA cells was impaired. At the lowest dose of LPS (0.1 ng/ml), XLA and normal PBMCs produced similar levels of TNFα, but increasing concentrations of LPS failed to generate the increased response seen in the normal cells. At 10 ng/ml, LPS-induced TNFα production by XLA PBMCs was only 15% of the control cells, whereas at 100 ng/ml LPS the response was only 10% of control (Fig. 1 A). Although B cells are not known to produce TNFα in response to LPS, the involvement of B cells from the normal PBMCs in LPS-induced TNFα production was investigated to negate any secondary effects due to the absence of B cells in XLA PBMCs. Depleting B cells from normal PBMC showed no effect on LPS-induced TNFα production (Fig. 1 B). Furthermore, we found that the T cells, the major component of PBMCs, did not produce TNFα in response to LPS (unpublished data) and these cells do not express Btk (33). Although the amount of blood available made purifying the monocytes from XLA blood difficult, we managed to separate the monocytic cells using adherence. LPS-induced TNFα production was again significantly reduced, although at 50% of normal controls, the impairment was not as great as seen in PBMCs (Fig. 1 C).

Stimulation with LPS Induces Btk Kinase Activity In Vitro. To further establish a role for Btk in LPS-induced TNFα production, we attempted to determine whether LPS was able to regulate Btk activity. Autokinase assays of enzyme immunoprecipitated from human primary monocytes demonstrated that LPS could rapidly activate Btk (Fig. 2 A). Btk autokinase activity was also observed in the murine macrophage cell line RAW 264.7 after LPS stimulation (unpublished data). Additionally, the effect of overexpression of Btk was examined by infecting 48-h M-CSF–treated monocytes (4) with increasing amounts of adenovirus-encoding human Btk (AdBtk). Infection with AdBtk

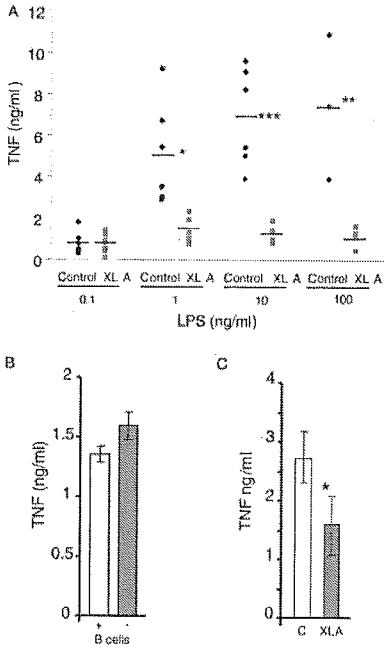

Figure 1. LPS-induced TNFα expression in PBMCs/monocytes from XLA and normal donors. (A) PBMCs from XLA and normal male donors (age range 17–38 yr). (B) Total PBMCs and PBMCs depleted of B cells from normal donors. (C) Monocytes prepared by adherence from normal and XLA PBMCs. Cells were stimulated with 10 ng/ml LPS (unless otherwise stated) for 18 h and supernatants were assayed for TNFα production by ELISA. In A, each point represents a donor, B is representative of three separate experiments, and C shows the combined results of five donors of each type (error bars ± SD). Results from Student's *t* test P values: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. TNFα production from unactivated cells was undetectable.

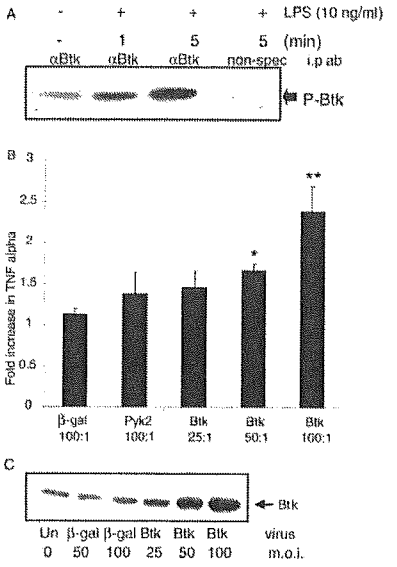

Figure 2. Btk is activated by LPS stimulation and modulates TNFα production. (A) Primary monocytes were stimulated with LPS for the indicated intervals. Cell lysates were immunoprecipitated and immune complex kinase assays were performed. Autophosphorylated Btk proteins were detected by autoradiography. This result is representative of three separate experiments. (B) Monocytes were infected with adenoviruses encoding either wild-type Btk (Btk WT), *β-galactosidase* (β-Gal), or an unrelated tyrosine kinase, Pyk2. Cells were stimulated with LPS for 18 h and the supernatants were assayed for TNFα expression. Data is presented as fold activation compared with uninfected LPS stimulated control cells (±SD). *, $P < 0.05$; **, $P < 0.01$ compared with LPS-activated uninfected controls. (C) Cell lysates from the infected cells were examined for Btk expression by immuno-Western blotting. Data is representative of at least four different donors.

alone did not induce TNFα production (unpublished data). However, infection with increasing amounts of virus synergized with LPS to increase TNFα expression, resulting in a two- to threefold increase in TNFα at the highest m.o.i. of 100 (Fig. 2 B). This effect correlated with the increased amounts of Btk protein seen in the infected cells (Fig. 2 C). No effect on TNFα production was seen with control viruses encoding either β-galactosidase or Pyk2, another LPS-activated tyrosine kinase (17), at the highest m.o.i. of 100 (Fig. 2 B).

LPS-induced IκBα Degradation Is Intact in XLA PBMCs. Overall, the data strongly suggested that there was a role for Btk in LPS-induced TNFα production. However, there was the possibility that this was the result of some development defect, rather than a direct effect of an absent kinase. As others have reported (34), FACS® analysis showed no impairment of CD14 expression, a component of the LPS binding on XLA monocytes (unpublished data). We also used the LPS-induced degradation of IκBα as a measure of signaling function, as this event does not appear to require tyrosine kinase activity (35, 36) and therefore should be intact. As shown in Fig. 3 A, there was a similar degradation of IκBα in XLA and normal monocytes in response to LPS, indicating no obvious impairment of the NF-κB activation pathway. We further investigated the involvement of the NF-κB signaling pathway by coinfecting M-CSF–treated monocytes with AdBtk and an NF-κB reporter virus that encodes luciferase under the control of a series of NF-κB binding sites (31). M-CSF monocytes were simultaneously infected with these viruses and controls, and the luciferase activity and TNFα production were measured after 18 h of LPS stimulation. Although we still observed an increase in TNFα production in these cultures (Fig. 3 C), there was no corresponding increase in luciferase activity (Fig. 3 B), indicating that Btk was able to promote TNFα production independently of the NF-κB activation.

Overexpression of Btk Acts via the 3′ UTR of TNF mRNA. To establish the mechanism of Btk action on TNFα production, the activity of the human TNF promoter in stimulated human macrophages was studied using 5′ promoter-luciferase and 5′ promoter-luciferase-3′ UTR adenoviral TNF gene reporter constructs (30). Primary human macrophages were simultaneously infected with reporter viruses at an m.o.i. of 40, whereas the Ad0 or AdBtk were used at an m.o.i. of 100. 24 h after adenoviral infection, macrophages were treated with LPS or left unstimulated for 18 h before assay of luciferase activity and ELISA for TNFα pro-

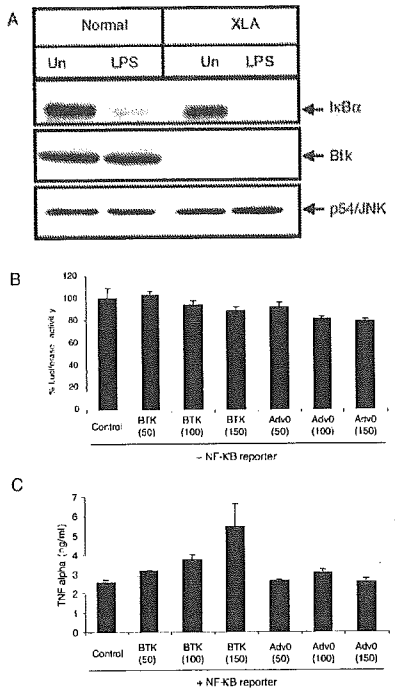

Figure 3. LPS-induced IκBα degradation in XLA and normal PBMCs. (A) PBMCs stimulated with 10 ng/ml LPS for 20 min were lysed and sequentially immunoblotted for the indicated proteins. This result is representative of three different experiments. (B and C) M-CSF monocytes were simultaneously infected with NF-κB luciferase reporter construct (m.o.i. of 100) alone, or coinfected with either AdBtk or Ad0 at various m.o.i. 24 h after adenoviral infection, cells were treated with LPS for 18 h before assay of (B) luciferase activity and (C) TNFα production. Data are means of triplicate cultures ± SD and are expressed as a percentage of the control level. This graph is representative of five experiments performed using different donors.

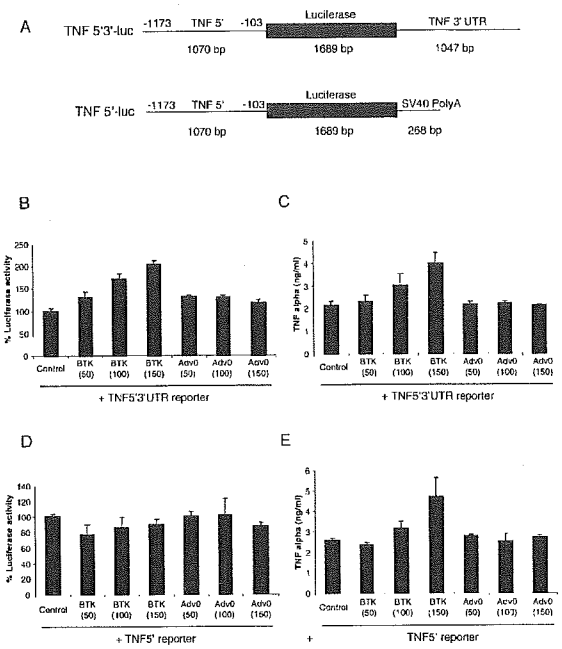

Figure 4. Btk overexpression enhances TNFα via the 3′ UTR. (A) Schematic representation of the human TNF 5′ promoter-luciferase-3′ UTR and TNF 5′ promoter-luciferase adenoviral constructs. Activity of the human TNF 5′ 3′ UTR (B and C) or TNF 5′ promoter (D and E) in stimulated human macrophages was tested by simultaneously infecting primary human macrophages with reporter virus at an m.o.i. of 40 and either Ad0 or AdBtk at various m.o.i. 24 h after adenoviral infection, macrophages were treated with LPS for 18 h before assay of luciferase activity (B and D) and TNFα production (C and E). Data are means of triplicate cultures ± SD and are expressed as a percentage of the control level. Each graph is representative of five experiments performed using different donors.

duction. There was a threefold increase in luciferase activity in the cells containing the TNF5′ 3′ UTR reporter construct compared with the Ad0 control cells (Fig. 4 A). However, there was no enhancement of luciferase activity from the TNF 5′ promoter construct (Fig. 4 C). Overexpression of Btk enhanced LPS-stimulated TNFα production by threefold in the presence of either TNF reporter construct (Fig. 4, B and D). There was no significant difference in TNF production and luciferase activity when comparing Ad0-infected macrophages with controls infected with the reporter constructs alone. The lack of enhanced luciferase activity from the 5′ promoter construct after LPS stimulation indicated that the 3′ UTR of TNFα mRNA was required for the action of Btk.

Overexpression of Btk Leads to Stabilization of TNFα mRNA. The importance of the 3′ UTR in the stability of TNFα mRNA has been studied extensively (37, 38). Because Btk appeared to be acting via the 3′ UTR, it seemed likely that it was also affecting the stability of TNFα mRNA. To further analyze the mechanism by which Btk determines the levels of TNFα, we blocked transcription in LPS-stimulated M-CSF monocytes by using actinomycin D and analyzed half-life of TNFα mRNA by Taqman RT-PCR analysis. As a control, GAPDH mRNA was detected and used to normalize the TNFα mRNA levels. For each of the controls, uninfected Ad0 and PykM, the half-life of TNFα mRNA was <45 min after actinomycin D treatment. However, in the presence of Btk, TNFα mRNA levels were maintained at 70–100% of the time 0 control for the duration of the 120-min time course (Fig. 5 A). These data demonstrate the ability of Btk to stabilize TNFα mRNA and consequently this may result in the elevated protein expression observed in these cultures.

As the stability of TNF mRNA is known to be associated with p38 mitogen-activated protein kinase (MAPK) activation, we examined the ability of PBMCs from XLA patients to phosphorylate p38 MAPK in response to LPS treatment. We were able to observe consistently low levels of p38 MAPK phosphorylation in XLA PBMCs (Fig. 5 B), however there was considerable variability in the degree of p38 MAPK phosphorylation from person to person in the normal controls. There was no difference in p38 MAPK phosphorylation after LPS treatment of normal PBMCs in either the presence or absence of B cells (unpublished data). Due to the limitation of patient samples we were unable to

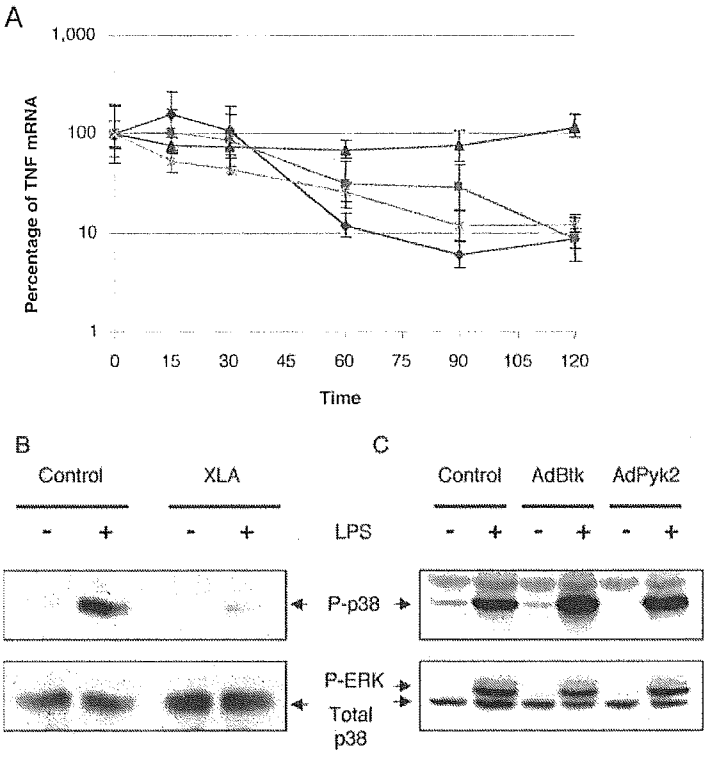

Figure 5. Btk overexpression stabilizes TNFα mRNA. (A) Cells were either uninfected or infected with Ad0, Ad PykM, or AdBtk (m.o.i. of 100) and activated with LPS for 4 h. 5 μg/ml actinomycin D was added to stop any further mRNA synthesis and the cells were incubated for an additional 0, 15, 30, 60, 90, or 120 min, after which time they were harvested in RNA lysis buffer and the supernatants were reserved for TNFα ELISA. Total mRNA was prepared and Taqman RT-PCR was used to access the quantity of TNFα. The results were normalized to 100% at the 0-min time point. The lines are representative of Control (◆), Ad0 (■), Btk (▲), and PykM (x). Data are means of triplicate reactions ± SD expressed as a percentage of the control and are representative of three experiments performed using different donors. (B) PBMCs from XLA and normal male donors or (C) M-CSF monocytes infected with AdBtk or AdPyk2, were stimulated with LPS for 20 min, lysed, and sequentially immunoblotted for the indicated proteins. These results are representative of three different experiments.

examine the kinetics of p38 MAPK activation in XLA patients compared with normal controls. To further establish the link between p38 MAPK and Btk, we used adenoviral overexpression of Btk to determine if there was any increase in p38 MAPK activity in normal M-CSF–treated monocytes. As shown in Fig. 5 C, we were able to observe a 1.5–2-fold increase in phosphorylated p38 MAPK.

Tec Is Phosphorylated in Response to LPS. Although we have demonstrated that Btk is able to modulate TNFα expression, XLA patients do not show a serious impairment in their innate immune function. Treatment of XLA monocytes for 2 d with M-CSF greatly enhanced their production of TNFα in response to LPS to levels similar to those obtained with normal monocytes (Fig. 6 A). Consequently, we examined the mechanism of the increased responsiveness of monocytes after M-CSF treatment. Because members of the Tec family of kinases can functionally complement each other (39), we tested whether M-CSF can regulate the expression of an alternative Tec family kinase to Btk. An obvious candidate was Tec kinase, as this enzyme is expressed in myeloid cells and does, to some degree, complement Btk activity in *xid* B cells (40). Western immunoblot analysis showed that Tec kinase was expressed at low levels in untreated monocytes from both normal donors and XLA patients (Fig. 6 B), however, after treatment with M-CSF for 48 h the expression of Tec protein was increased. Counter blots confirmed that the expression of Btk was restricted to the normal cells (Fig. 6 B). Next, the response of Tec kinase to LPS in M-CSF–treated monocytes was investigated using autokinase assays of immunoprecipitated enzyme. Like Btk, Tec kinase activity was increased in response to LPS treatment of human M-CSF–treated monocytes (Fig. 6 C).

Discussion

Our data has shown that stimulation of human monocytes with LPS triggers the activation of the nonreceptor tyrosine kinase, Btk. Moreover, in XLA monocytes that lack functional Btk, there is a failure to respond to LPS treatment as determined by their reduced TNFα production after stimulation. Our initial findings led us to further examine the contribution of Btk in controlling TNFα production. Adenoviral gene transfer has been used extensively to investigate the role of different transgenes in primary cells due to the ability of adenoviruses to infect both quiescent and dividing cells at high efficiency (4). Overexpression of Btk resulted in a consistent two- to threefold increase in TNFα production from M-CSF monocytes. In B cells, activation of the NF-κB transcription complex by signals derived from the B cell antigen receptor act via Btk to control B cell development, survival, and antigenic responses. However, the actions of Btk in macrophages appear to be independent of NF-κB activation and there is no defect in IκBα degradation in response to LPS in XLA monocytes.

TNF biosynthesis is under the control of multiple and complex regulatory mechanisms. Because the Tec family is

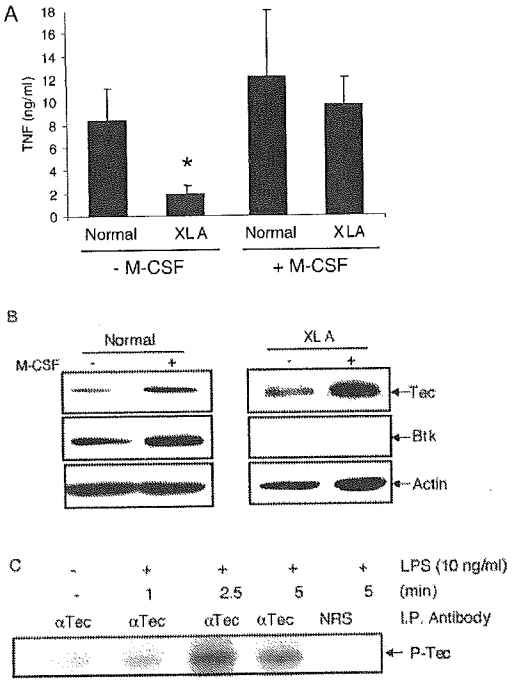

Figure 6. The effect of M-CSF treatment on LPS-stimulated TNFα production and Tec expression. (A) Adherent monocytes from XLA and normal donors were either stimulated with LPS for 2 h, or incubated with M-CSF for 48 h before LPS stimulation for 2 h. Supernatants were assayed for TNFα production. (B) Adherent monocytes from normal (lanes 1 and 2) and XLA donors (lanes 3 and 4) were either lysed immediately, or after M-CSF treatment for 48 h. Western blot analysis was performed using either α-TecSH3, α-Btk, or α-actin antibody. (C) Elutriated monocytes from normal donors were stimulated with LPS for the indicated time periods. Tec was immunoprecipitated and in vitro autokinase assay was performed. Each study is representative of at least three separate experiments.

known to interact with a wide range of signal transduction molecules (18), we took the alternate approach of examining the effect of Btk on the UTRs of TNFα. Using the 5′ promoter-luciferase and 5′ promoter-luciferase-3′ UTR adenoviral reporter constructs, we were able to show that the ability of Btk to enhance TNFα production required the 3′ UTR. There is considerable evidence indicating that some of these may occur at the translational level and be mediated by AU-rich elements (AREs) in the 3′ UTR of the mRNA. Certain inflammatory gene mRNAs, including cyclooxygenase-2, IL-6, IL-8, and TNFα mRNAs, are stabilized by activation of the p38 MAPK pathway by stimuli such as IL-1 and LPS (37, 41–43). Studies with mRNA reporter constructs have shown that the p38 MAPK-mediated stabilization directly involves AREs (38). Thus, AREs confer instability on mRNAs. However, after activation of the p38 MAPK pathway, they allow mRNA stabilization and hence increased protein expression. There is also a strong association between Btk and p38 MAPK in B cells showing that the activation of p38 MAPK was completely inhibited in cells deficient in Lyn and Btk, and the introduction of wild-type Btk, but not kinase-inactive Btk, restored the p38 MAPK activation in response to 280 nm UV irradiation in chicken DT40 B cells (44). The precise regions of the TNF 3′ UTR involved in this interaction with Btk and the potential involvement of p38 MAPK and other intermediary molecules is a field of ongoing research in our laboratory.

The proposition that Btk is essential for LPS-induced TNFα production is not easily reconciled with the XLA phenotype that shows no obvious gross defect of the innate immune system. However, there have been limited reports that XLA patients have reduced inflammatory responses (45). This is in contrast to patients with common variable immunodeficiency who are prone to multiorgan granulomatous disease, although they too have a type of antibody deficiency similar to the XLA patients (28, 46). In humans, Btk is absolutely required for the progression of developing B cells through the pro-B to pre-B stage. However, in *xid* mice the absence of Btk alone does not result in the same phenotype and it is only when Tec is also ablated that the same B cell deficiency is observed (40). It would be of interest to investigate LPS responsiveness in monocyte/macrophages from these mice. This suggests that Tec might be able to substitute for the absence of Btk in mouse pro-B cells, but at this stage of development in man, Tec is either unavailable or is incapable of substituting for Btk (47). It is of interest to note that monocyte differentiation toward macrophages ablates the differences observed between the XLA patients and normal donors (Fig. 6 A) and that this is accompanied by an increase in Tec levels (Fig. 6 B). The observation that Tec kinase is expressed in resting monocytes from XLA and normal individuals, although at a lower level, may explain why there is some response to LPS in the Btk-deficient cells. The data obtained here would suggest that without an increase in Tec kinase expression induced by M-CSF, there is insufficient kinase activity in the resting XLA monocytes to support a full LPS response. This hypothesis is supported by the observation that at the lowest LPS concentration used in this study (0.1 ng/ml), TNFα production by XLA and normal cells was similar (Fig. 1 A). The majority of the studies in mice (23, 24), with the exception of the study by Mukhopadhyay et al. (25), have been unable to find any differences in LPS responsiveness between *xid* and normal macrophages. We also examined the responsiveness of peritoneal macrophages from *xid* mice versus normal mice and were unable to observe any differences in TNFα production and NF-κB DNA binding activity in response to LPS (unpublished data). Taken together, these data suggest that rather than a difference between murine and human cells, that it is in fact a difference between monocytes and macrophages. It is therefore possible that the up-regulation of Tec, or other Tec kinase family members, in human macrophages may explain why there is no major impairment of innate immunity in XLA patients although further work is required to confirm this hypothesis.

In summary, this study has demonstrated that Btk is not only another tyrosine kinase activated by LPS, but also that deficiency in its expression is associated with impairment of

TNFα expression. Furthermore, overexpression of Btk in macrophages synergizes with LPS to induce TNFα production by stabilizing TNFα mRNA. Although these data imply that Btk is the first described tyrosine kinase having a key role in LPS-induced production of TNFα by monocytes, this role is probably not restricted to Btk alone. The intriguing question remains as to what role Tec kinases may have in mediating TNFα production by myeloid cells to other stimuli, particularly those involved in inflammatory conditions like rheumatoid arthritis and Crohn's disease, where TNFα is a validated therapeutic target.

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DNA microarray analysis of stage progression mechanism in myelodysplastic syndrome

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Summary. Myelodysplastic syndrome (MDS) is a clonal disorder of haematopoietic stem cells. Despite the high incidence of MDS in the elderly, effective treatment of individuals in its advanced stages is problematic. DNA microarray analysis is a potentially informative approach to the development of new treatments for MDS. However, a simple comparison of 'transcriptomes' of bone marrow mononuclear cells among individuals at distinct stages of MDS would result in the identification of genes whose expression differences only reflect differences in the proportion of MDS blasts within bone marrow. Such a 'population shift' effect has now been avoided by purification of haematopoietic stem-like cells that are positive for the cell surface marker AC133 from the bone marrow of healthy volunteers and 30 patients at various stages of MDS. Microarray analysis with the $AC133^+$ cells from these individuals resulted in the identification of sets of genes with expression that was specific to either indolent or advanced stages of MDS. The former group of genes included that for PIASy, which catalyses protein modification with the ubiquitin-like molecule SUMO. Induction of PIASy expression in a mouse myeloid cell line induced apoptosis. A loss of PIASy expression may therefore contribute directly to the growth of MDS blasts and stage progression.

Keywords: DNA microarray, myelodysplastic syndrome, haematopoietic stem cell, acute myeloid leukaemia, apoptosis.

Myelodysplastic syndrome (MDS) is a clonal haematological disorder that mainly affects elderly people (Lowenthal & Marsden, 1997) and is characterized by dysplasia in multiple lineages of blood cells, including myeloid, erythroid and megakaryocytic-platelet lineages. MDS is therefore thought to result from the malignant transformation of pluripotent haematopoietic stem cells (HSCs). Another important characteristic of MDS is the co-existence of increased cellularity in bone marrow (BM) and cytopenia in peripheral blood, a condition referred to as 'ineffective haematopoiesis'. Immature BM cells of individuals with MDS may thus be defective with regard to differentiation or undergo apoptosis before giving rise to a sufficient number of progeny.

The clinical course of MDS can be divided into several distinct phases (Harris *et al*, 1999). In the early, indolent stage, affected individuals manifest only cytopenia and may not require any specific treatment; this phase is referred to as 'refractory anaemia' (RA) or 'RA with ringed sideroblasts' (RARS), depending on the absence or presence of ringed sideroblasts in BM. After experiencing the indolent phase for several years (or even decades), a proportion of patients undergoes transformation to a leukaemic state. As the percentage of leukaemic blasts in BM increases, patients are diagnosed first with 'RA with excess blasts' (RAEB) (5–20% blasts in BM) and, finally, with MDS-associated 'acute myeloid leukaemia' (> 20% blasts in BM). The malignant cells of individuals with such MDS-associated leukaemia are refractory to chemotherapy, and the median survival time of these patients is < 1 year.

The development of an effective treatment for MDS will require the characterization of the molecular mechanism that underlies stage progression. Although point mutations in *RAS* proto-oncogenes have been detected in the transformed cells of individuals with MDS, the prognostic value of these mutations is unclear (Horiike *et al*, 1994; Neubauer *et al*, 1994). Allelic loss of the *P53* tumour-suppressor gene and point mutations in the remaining allele have been detected in the advanced stages of MDS, but only in a small

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percentage of patients (Fenaux *et al*, 1996). Transcriptional repression through promoter silencing has also been demonstrated in MDS patients for the tumour-suppressor gene that encodes $p15^{INK4b}$ (Quesnel *et al*, 1998). However, none of these gene alterations has been shown to be relevant to stage progression in MDS.

DNA microarray analysis enables the 'transcriptome' of a given cell type or tissue to be monitored. The expression levels of thousands of genes can thus be quantified simultaneously with this technology (Duggan *et al*, 1999). Such large-scale screening offers the possibility of identifying genes whose expression changes in a stage-dependent manner during the clinical course of MDS. The identification of such stage-specific genes would facilitate the molecular diagnosis of MDS and the prediction of stage progression, as well as providing insight into the molecular events that underlie the expansion of leukaemic blasts in individuals with this condition. However, a simple comparison by microarray analysis among BM mononuclear cells (MNCs) derived from patients at different stages of MDS is likely to generate a large number of pseudopositive results, given that the proportion of leukaemic blasts varies widely among such individuals. If, for instance, the transcriptome of BM MNCs is compared between patients with RA and those with MDS-associated leukaemia, the analysis would mistakenly indicate that genes with expression that is specific to immature blood cells are induced in MDS-associated leukaemia. These results would reflect the expansion of the immature leukaemic cell population in the BM of individuals with MDS-associated leukaemia rather than an increase in the mRNA copy number of the corresponding genes per cell.

Such a 'population shift' effect could be prevented by purification of background-matched cell populations from clinical specimens before microarray analysis. Given that malignant transformation occurs in HSCs of individuals with MDS, we hypothesized that HSCs would be a good target for such 'background-matched population' (BAMP) screening (Miyazato *et al*, 2001) of MDS. Analysis of HSCs should thus enable the direct comparison of the transcriptomes of MDS blasts, irrespective of the blast population size within BM or the differentiation capacity of the blasts. To achieve this goal, we took advantage of the HSC-specific surface marker AC133 (Hin *et al*, 1997) and began to purify and store, in a depository known as the 'Blast Bank', AC133-positive HSC-like fractions from individuals with leukaemia or leukaemia-related disorders, including MDS (Miyazato *et al*, 2001). Using Blast Bank cells, we have identified previously genes whose expression helps in the differential diagnosis between MDS-associated leukaemia and *de novo* acute myeloid leukaemia (Miyazato *et al*, 2001). We have also identified genes whose expression is stage dependent in chronic myeloid leukaemia (Ohmine *et al*, 2001).

The expression levels of 2304 genes have now been compared among Blast Bank samples derived from 30 MDS patients (11 with RA, five with RAEB and 14 with MDS-associated leukaemia) and healthy volunteers. We identified a set of genes whose expression was high in cells from control individuals and RA patients but reduced in those from patients at advanced stages of disease. These genes include the one for PIASy, an E3 ligase for the small ubiquitin-like protein known as SUMO and a potential inhibitor of signal transducer and activator of transcription (STAT) 1 (Liu *et al*, 2001). Further investigation revealed that expression of PIASy induced apoptosis in the mouse myeloid cell line 32D (Greenberger *et al*, 1983). A reduction in the level of PIASy expression may therefore contribute to the growth of leukaemic blasts and stage progression in MDS.

PATIENTS AND METHODS

RNA preparation and DNA microarray analysis. $AC133^+$ cells were purified from BM MNCs of MDS patients using MicroBeads conjugated with antibodies to AC133 (Miltenyi Biotec, Auburn, CA, USA), as described previously (Miyazato *et al*, 2001). $AC133^+$ cells were also purified from BM MNCs of two healthy volunteers and then mixed for use as a 'healthy control' sample. Total RNA was extracted from $AC133^+$ cells and subjected to two rounds of amplification with T7 RNA polymerase (Van Gelder *et al*, 1990); the fidelity of the RNA amplification procedure was confirmed as described previously (Ohmine *et al*, 2001). The amplified cRNA (1 μg) was converted to double-stranded cDNA, which was then used to synthesize biotin-labelled cRNA with the ExpressChip labelling system (Mergen, San Leandro, CA, USA). The labelled cRNA was allowed to hybridize both with a DNA microarray (HO-3, Mergen) that contains oligonucleotides based on genes that encode mostly transcription factors as well as with our custom-made array (Mergen) that contains oligonucleotides corresponding to genes for membrane proteins or proteins that mediate cell signalling or redox regulation. The names and database accession numbers for the 2304 genes represented on the arrays are available (Supplementary material, Table SI). Detection of hybridization signals and analysis of the digitized data were performed with a 418 array scanner (Affymetrix, Santa Clara, CA, USA) and GENESPRING 4.1.0 software (Silicon Genetics, Redwood, CA, USA) respectively. In a hierarchical clustering analysis, similarity was measured by the standard correlation with a separation ratio of 0·5.

Real-time polymerase chain reaction (PCR) analysis. Portions of unamplified cDNA were subjected to PCR with SYBR green PCR core reagents (PE Applied Biosystems, Foster City, CA, USA). Incorporation of the SYBR green dye into PCR products was monitored in real time with an ABI Prism 7700 sequence detection system (PE Applied Biosystems), thus allowing determination of the threshold cycle (C_T) at which the exponential amplification of PCR products begins. The C_T values for cDNAs corresponding to the β-actin gene and PIASy were used to calculate the abundance of the PIASy transcript relative to that of β-actin mRNA. The oligonucleotide primers for PCR were as follows: 5′-CCATCATGAAGTGTGACGTGG-3′ and 5′-GTCCGCCTAGAAGCATTTGCG-3′ *for* β-actin cDNA; and 5′-AACTACGGCAAGAGCTACTCGGTG-3′ and 5′-GTTCATCTGCAGGTAGAAGACGGC-3′ for PIASy cDNA.

Conditional expression of PIASy. The mouse myeloid cell line 32D was maintained in Roswell Park Memorial Institute (RPMI)-1640 medium (Life Technologies, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) and interleukin 3 (IL-3; 25 U/ml). For the induction of granulocyte differentiation, cells were cultured in RPMI 1640 supplemented with 10% FBS and 1 ng/ml granulocyte colony-stimulating factor (G-CSF). The cDNA encoding human PIASy (Helix Institute, Chiba, Japan) with a C-terminal FLAG epitope tag (PIASy-F) was inserted into the pMX-tetOFF retroviral vector (Ohmine *et al*, 2001), yielding pMX-tetOFF/PIASy-F. Either pMX-tetOFF or pMX-tetOFF/PIASy-F was then transiently introduced into the packaging cell line BOSC23 (Pear *et al*, 1993) in order to produce the ecotropic retroviruses MX-tetOFF and MX-tetOFF/PIASy-F respectively. 32D cells were then infected with the virus-containing supernatant of BOSC23 cells for 24 h in the presence of retronectin (Takara Shuzo, Shiga, Japan). The cells were subsequently harvested and cultured in RPMI 1640–FBS–IL-3 medium supplemented with blasticidin-S (5 μg/ml; Funakoshi, Tokyo, Japan) and tetracycline (1 μg/ml; Boehringer Mannheim, Mannheim, Germany). The expression of PIASy-F was induced by changing the culture medium to RPMI 1640 supplemented with 10% FBS, 2 μmol/l 17β-oestradiol (Sigma, St Louis, MO, USA) and appropriate cytokines.

Protein analysis was performed as described previously (Ohmine *et al*, 2001). Total-cell lysates (10 μg per lane) were fractionated by 7·5% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to immunoblot analysis with antibodies to FLAG (Eastman Kodak, New Haven, CT, USA).

Apoptosis of 32D cells. 32D cells infected with MX-tetOFF or MX-tetOFF/PIASy-F were cultured for 8 d in the presence of G-CSF and 17β-oestradiol, after which the cells were stained with Wright–Giemsa solution or with propidium iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated annexin V (annexin V–FITC apoptosis detection kit; BD Biosciences, San Jose, CA, USA) in order to evaluate apoptotic changes with a FACScan processor (BD Biosciences). Differentiation of 32D cells was evaluated by FACScan processor analysis with antibodies to the granulocyte-specific marker Gr-1 (BD Biosciences).

RESULTS

Transcriptome analysis of MDS samples

For this analysis, we prepared a custom-made microarray that contains oligonucleotides corresponding to a total of 1152 human genes encoding for membrane proteins and proteins involved in redox regulation or cell signalling. Membrane proteins were chosen because diagnosis by flow cytometry with antibodies to MDS-specific cell surface markers, if found, would be of great clinical value. We also reasoned that, from the point of view of resistance mechanisms to chemotherapeutic reagents, cell surface proteins as well as those involved in redox regulation should be focused on.

Many cases of disease-specific chromosome translocation found in leukaemias lead to deregulation in the expression of transcriptional factors or to the generation of fusion products containing the transcriptional factors as the components (Alcalay *et al*, 2001). These data suggest an important role for the altered functions of transcriptional factors in leukaemogenesis. We therefore used a commercially available microarray (HO3) containing oligonucleotides based on 1152 genes encoding mainly transcriptional factors.

The expression profile for a total of 2304 genes was thus obtained for Blast Bank samples purified from 11 patients with RA, five patients with RAEB and 14 patients with MDS-associated leukaemia. The pooled $AC133^+$ cells from two healthy volunteers were used as a healthy control sample. Expression intensity data for the various genes were normalized relative to the median expression value of all genes in each hybridization. The resulting data were then used to generate a dendrogram, or 'gene tree', in which genes with similar expression profiles were clustered near each other (Fig 1A). About 50% of the genes analysed were transcriptionally silent throughout the clinical course of MDS. Several clusters of genes, however, were expressed in a manner dependent on MDS stage. For example, genes in the cluster designated #1 were highly expressed only in RA patients 3 and 7, whereas those in cluster #2 were expressed at a higher level in patients with RAEB or MDS-associated leukaemia than in healthy controls or RA patients. These genes were thus potential molecular markers for the corresponding MDS stage.

To facilitate the identification of genes that are expressed in a stage-dependent manner, we calculated the mean expression value of each gene for RA, RAEB and MDS-associated leukaemia groups and then used these values to generate another dendrogram, an 'average tree' (Fig 1B). From the resulting, readily identifiable, gene clusters with stage-specific expression profiles, we then attempted to select those genes with stage-dependent expression that was statistically significant by Welch analysis of variance (ANOVA). However, most genes whose stage-dependent expression was significant ($P < 0{\cdot}001$) were found not to be appropriate stage-specific markers. Such analysis thus tended to select genes whose expression level exhibited a small deviation within each MDS stage; the expression intensities for these genes, however, did not exhibit large differences among disease stages.

Genes induced during stage progression

An important goal of this study was to clarify the molecular events that underlie the progression from indolent RA to therapy-refractory RAEB and MDS-associated leukaemia. We thus focused our attention on identifying differences in the transcriptome between disease stages with good prognosis (healthy control and RA) and those with poor prognosis (RAEB and MDS-associated leukaemia). The mean expression intensity of each gene was thus calculated for the good prognosis group and the poor prognosis group, and the differences in the resulting values are represented in Fig 1C.

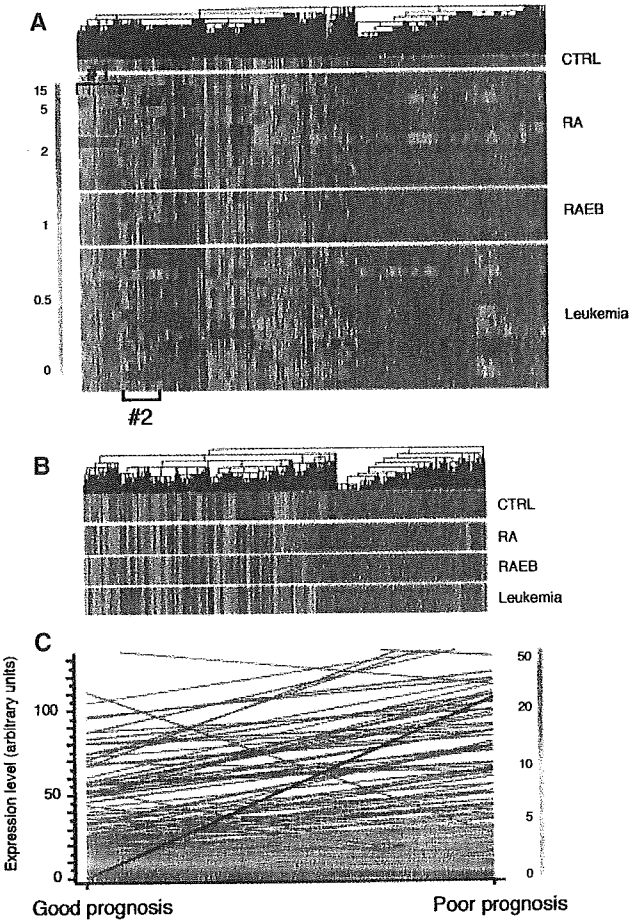

Fig 1. Expression profiles of 2304 genes in the MDS blasts. (A) Hierarchical clustering of 2304 genes on the basis of their expression profiles in Blast Bank samples derived from healthy controls (CTRL) and 30 individuals with MDS [11 with RA, five with RAEB and 14 with MDS-associated leukaemia (Leukaemia)]. Each column represents a single gene on the microarray, and each row a separate patient (or control) sample. The fluorescence intensity of each gene was normalized relative to the median fluorescence value for all spots in each hybridization, and the normalized value is colour-coded as indicated on the left. The positions of clusters of RA-specific genes (cluster #1) and of RAEB- or MDS-associated leukaemia-specific genes (cluster #2) are indicated. (B) The mean expression levels of each gene were calculated for RA, RAEB and MDS-associated leukaemia groups and then used to generate another dendrogram, or 'average tree.' Clusters of genes whose expression was specific to different groups are evident. (C) Comparison of gene expression levels between good prognosis (healthy control and RA) and poor prognosis (RAEB and MDS-associated leukaemia) groups. The mean expression values of each gene were calculated for the good prognosis and bad prognosis groups and then compared between the two groups. Each line corresponds to a single gene and is coloured according to the mean expression level of the gene in the good prognosis group. The hypothetical 'poor prognosis-specific gene' is shown in blue.

We first attempted to identify genes with expression that was induced in the $AC133^+$ cells of the poor prognosis group, compared with that in the corresponding cells of the good prognosis group. The GENESPRING software was used to search for genes whose expression profiles were statistically similar, with a minimum correlation of 0·99, to that of a hypothetical 'poor prognosis-specific gene' (blue line in Fig 1C) that exhibits a mean expression level of 0·0 arbitrary units (U) in the good prognosis group and 100·0 U in the poor prognosis group. From a total of 96 such genes, we then selected those whose expression level was < 20·0 U in all samples from the good prognosis group and > 50·0 U in at least one sample from the poor prognosis group.

Eleven such genes were finally identified (Fig 2A), including those for NADH-ubiquinone oxidoreductase flavoprotein 1 (NDUFV1), LIM-Hox2 (LH2) and paraneoplastic antigen MA2 (PNMA2). Expression of *NDUFV1*, for example, was highly specific to the poor prognosis group; its expression level was 4·60, 1·50 ± 0·92, 26·29 ± 11·30 and 10·36 ± 9·04 U (means ± standard deviation, SD) for the control, RA, RAEB and MDS-associated leukaemia samples respectively. The difference in *NDUFV1* expression level between the good and poor prognosis groups was statistically significant ($P = 0·0061$, Mann–Whitney *U*-test). NDUFV1 is a component of NADH:ubiquinone oxidoreductase, which participates in mitochondrial electron transport (Ali *et al*, 1993). Increased expression of *NDUFV1* may therefore reflect an increased rate of mitochondrial respiration in the transformed blast cells.

LH2 is a homeobox-containing transcription factor. Although neither its target genes nor its *in vivo* functions have been identified, LH2 has been suggested to participate in mitogenic signalling on the basis of the observations that the corresponding gene is aberrantly expressed in chronic myeloid leukaemia (Wu *et al*, 1996) and that the protein is expressed in immature, but not in mature, B lymphocytes (Xu *et al*, 1993). PNMA2 was originally identified as a serological marker produced by testicular cancer cells (Voltz *et al*, 1999) and may function as an antigen that causes paraneoplastic syndromes. Expression of these three genes only in the advanced stages of MDS may provide a basis for the differential diagnosis of MDS at the molecular level.

Genes silenced during stage progression

Given the importance of functional deficiency of various tumour-suppressor genes in malignant transformation, a

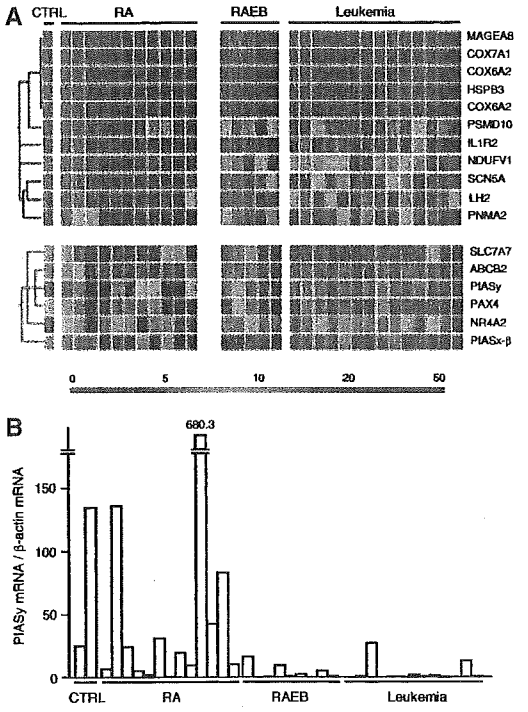

Fig 2. Identification of stage-dependent genes. (A) The expression profiles of 11 poor prognosis-specific genes (top) and six good prognosis-specific genes (bottom) are shown colour-coded as indicated by the scale below. Each row corresponds to a single gene, with the columns indicating the corresponding expression level in $AC133^+$ cells obtained from healthy controls (CTRL) or individual RA, RAEB and MDS-associated leukaemia (Leukaemia) patients. The similarity of expression profiles among the poor prognosis-specific or the good prognosis-specific genes is indicated by the purple or blue dendrogram, respectively, shown on the left. The names, accession numbers and expression intensity data for the genes are available (Supplementary material, Tables SII and SIII). (B) Quantification of *PIASy* transcripts in $AC133^+$ blasts. Complementary DNA was prepared from the blasts of 37 individuals, comprising two healthy volunteers (CTRL) as well as 13 RA, nine RAEB and 13 MDS-associated leukaemia (Leukaemia) patients, and was subjected to real-time PCR analysis with the primers specific for *PIASy* or the β-actin gene. The ratio of the abundance of *PIASy* transcripts to that of β-actin mRNA was calculated as 2^n, where n is the C_T value for β-actin cDNA minus the C_T value of *PIASy* cDNA.

decrease in the expression of certain genes may contribute directly to stage progression in MDS. We therefore searched for genes whose expression profiles were statistically similar to that of a hypothetical 'good prognosis-specific gene' that exhibits a mean expression level of 100·0 U in the good prognosis group and 0·0 U in the poor prognosis group. A total of 182 such genes were identified. We then selected those genes with an expression level of > 40·0 U in at least one sample from the good prognosis group and of < 20·0 U in all samples from the poor prognosis group; six such genes were finally identified (Fig 2A).

Among these six genes were those for PIASy and PIASx-β, members of the PIAS family of signalling proteins (Shuai, 2000). PIASy was identified as a binding protein and inhibitor of STAT1 (Liu *et al*, 2001). More recent studies have indicated that PIAS proteins perform a wide spectrum of functions, including modification of the activity of the androgen receptor (Gross *et al*, 2001) or p53 (Nelson *et al*, 2001) and sumoylation of the transcription factor LEF1 (Sachdev *et al*, 2001). Moreover, forced expression of PIASy in human kidney 293T cells was shown to be accompanied by induction of apoptosis (Liu & Shuai, 2001), suggesting that PIASy also possesses proapoptotic activity. Our data indicated that *PIASy* is active in normal HSCs or pluripotent stem cells in the indolent stage of MDS, but that expression of the gene is suppressed in these cells on transition to the advanced stages of MDS. If the proapoptotic activity of PIASy is required *in vivo* to prevent deregulation of the growth of HSCs in normal individuals or RA patients, then the loss of *PIASy* expression may allow the accelerated growth of blastic cells within BM, the hallmark of RAEB and MDS-associated leukaemia. The expression level of *PIASy* may thus not only prove useful as a molecular marker for stage diagnosis or prediction of prognosis in individuals with MDS but could also contribute directly to the mechanism of transformation to the advanced stages of this disease.

We therefore attempted to verify the stage-dependent expression of *PIASy* by quantitative real-time PCR analysis. The abundance of *PIASy* transcripts was determined relative to that of β-actin mRNA in the $AC133^+$ blasts from 37 individuals, including two healthy volunteers as well as 13 RA, nine RAEB and 13 MDS-associated leukaemia patients. Consistent with the results obtained by microarray analysis, PCR showed that the expression of *PIASy* was markedly greater in the blasts from control subjects or RA patients compared with that in those from RAEB or MDS-associated leukaemia patients ($P = 0{\cdot}043$, Mann–Whitney U-test) (Fig 2B). *PIASy* is thus a potential candidate for a stage-dependent molecular marker in MDS.

Inhibition of 32D cell growth by overexpression of PIASy

Given the potential proapoptotic activity of PIASy, sustained expression of the corresponding gene might influence the growth or differentiation of immature blood cells. To examine directly the effect of *PIASy* expression in such cells, we took advantage of the pMX-tetOFF retrovirus vector (Ohmine *et al*, 2001), which allows the dual regulation of exogenous gene expression by tetracycline and β-oestradiol (Iida *et al*, 1996). The mouse myeloid cell line 32D grows without differentiation in the presence of IL-3, but exhibits a reduction in growth rate and undergoes terminal differentiation to granulocytes in response to stimulation with G-CSF. We therefore infected 32D cells with the viruses MX-tetOFF or MX-tetOFF/PIASy-F, the latter of which encodes FLAG epitope-tagged PIASy, and maintained the cells under non-inducing (presence of tetracycline, absence of β-oestradiol) or inducing (minus tetracycline, plus β-oestradiol) conditions with regard to PIASy-F expression. Immunoblot analysis with antibodies to the FLAG epitope revealed the expression of PIASy-F at a high level only in the cells that were infected with

MX-tetOFF/PIASy-F and maintained under the inducing condition (Fig 3A).

The effect of PIASy expression on cell growth was examined in virus-infected 32D cells cultured in the presence of IL-3. No difference in cell growth, viability, morphology or surface marker expression was apparent between cells infected with MX-tetOFF/PIASy-F that were maintained under inducing conditions and those that were cultured under non-inducing conditions (data not shown).

We next examined the effect of PIASy expression on virus-infected cells cultured in the presence of G-CSF. Under the non-inducing condition, both mock-infected and MX-tetOFF/PIASy-F-infected cells grew in a similar manner (Fig 3B). The induction of PIASy-F expression, however, resulted in growth inhibition. Although β-oestradiol exhibited a slight inhibitory effect on the growth of the mock-infected cells, the effect of this agent on the MX-tetOFF/PIASy-F-infected cells was markedly greater. Concomitant with its inhibitory effect on cell growth, PIASy-F expression induced a substantial decrease in cell viability (Fig 3B).

PIASy-induced apoptosis in 32D cells

To clarify the mechanism by which PIASy inhibits the growth and reduces the viability of 32D cells in the presence of G-CSF, we examined the morphology of cells maintained for 8 d under inducing conditions by staining with Wright–Giemsa solution (Fig 4A). Mock-infected cells cultured in the presence of IL-3 were of medium to large size and exhibited a high nucleus-to-cytoplasm ratio. When cultured in the presence of G-CSF, > 50% of the mock-infected cells displayed the differentiated phenotype; some cells thus had a lower nucleus-to-cytoplasm ratio and neutrophilic cytoplasm and, in others, the nucleus was segmented. In the presence of IL-3, cells expressing PIASy-F showed a phenotype similar to that of the mock-infected cells. Culture of the cells expressing PIASy-F in the presence of G-CSF, however,

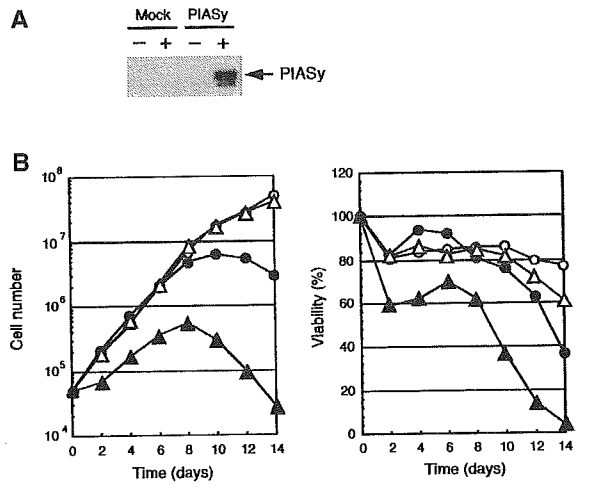

Fig 3. Effects of PIASy expression on cell growth and viability. (A) 32D cells were infected with MX-tetOFF (mock) or MX-tetOFF/PIASy-F (PIASy) retroviruses and cultured in the presence of IL-3, blasticidin-S and tetracycline. The blasticidin-S-resistant mass culture was then maintained overnight under the same condition (–) or in medium containing β-oestradiol instead of tetracycline (+), after which the cells were harvested and subjected to immunoblot analysis with antibodies to the FLAG tag. (B) 32D cells infected with MX-tetOFF (circles) or MX-tetOFF/PIASy-F (triangles) were cultured in the presence of G-CSF under either non-inducing (open symbols) or inducing (closed symbols) conditions. Total cell number (left) and cell viability assessed on the basis of trypan blue exclusion (right) were determined at the indicated times. Data are means of triplicates from a representative experiment.

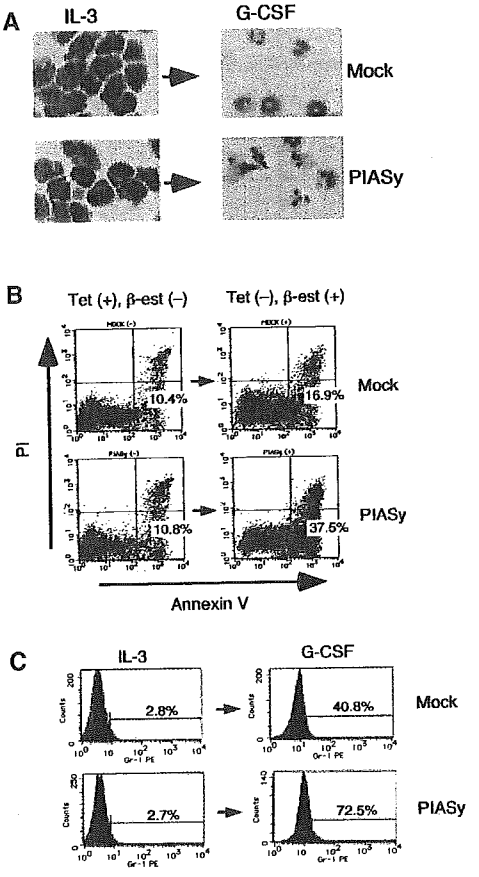

Fig 4. Induction of apoptosis by PIASy. (A) 32D cells infected with MX-tetOFF (mock) or MX-tetOFF/PIASy-F (PIASy) were incubated for 8 d under the inducing condition in the presence of either IL-3 or G-CSF, as indicated. The cells were then stained with Wright–Giemsa solution. Original magnification, 200 ×. (B) 32D cells infected with MX-tetOFF or MX-tetOFF/PIASy-F were incubated for 8 d with G-CSF under the non-inducing [Tet (+), β-est (–)] or inducing [Tet (–), β-est (+)] condition. They were then stained with annexin V–FITC and PI and subjected to flow cytometry. The percentages of apoptotic cells (annexin V-positive, PI-negative) are indicated. (C) 32D cells infected with MX-tetOFF or MX-tetOFF/PIASy-F were cultured under the inducing condition in the presence of either IL-3 or G-CSF. The proportion of differentiated cells was then evaluated by flow cytometry with phycoerythrin–conjugated antibodies to the granulocyte-specific marker Gr-1. The percentages of Gr-1$^+$ cells are indicated.

resulted in a marked increase in the number of cells with apoptotic characteristics, including condensation and fragmentation of nuclei and cell shrinkage.

To determine the extent of apoptosis induction by PIASy in 32D cells cultured in the presence of G-CSF, we subjected cells that had been incubated for 8 d with this cytokine to staining with annexin-V and PI. The translocation of phosphatidylserine from the inner leaflet to the outer leaflet of the lipid bilayer of the cell membrane, a characteristic of apoptosis, was then evaluated by flow cytometry. When mock-infected 32D cells were cultured with G-CSF under non-inducing or inducing conditions, 10·4% or 16·9%, respectively, of the cells were characterized as apoptotic (phosphatidylserine-positive, PI-negative). Expression of PIASy-F in cells cultured with G-CSF increased the proportion of apoptotic cells from 10·8% to 37·5% (Fig 4B). The proportion of all dead cells (phosphatidylserine-positive, PI-negative or -positive) was 15·5%, 23·8%, 18·4% or 49·8% for the non-induced mock-infected cells, induced mock-infected cells, non-induced MX-tetOFF/PIASy-F-infected cells and induced MX-tetOFF/PIASy-F-infected cells respectively. These data thus support the notion that PIASy inhibits the growth of differentiating 32D cells through the induction of apoptosis.

To determine whether PIASy expression also promotes the differentiation of 32D cells, we measured the proportion of differentiated cells by flow cytometry with antibodies to the granulocyte-specific surface protein Gr-1. When cultured in the presence of IL-3 under inducing conditions, the proportion of mock-infected or MX-tetOFF/PIASy-F-infected cells that were positive for Gr-1 was $< 3\%$ (Fig 4C). Incubation with G-CSF resulted in expansion of the Gr-1^+ population in both mock-infected (40·8%) and MX-tetOFF/PIASy-F-infected (72·5%) cells, with the effect in the latter cells being more marked. The peak fluorescence intensities were similar, however, for the mock-infected and MX-tetOFF/PIASy-F-infected cells, suggesting that a relative decrease in the size of the Gr-1^- fraction of PIASy-F-positive cells (probably as a result of apoptosis) may have contributed to the overall increase in the percentage of Gr-1^+ cells. Examination of cell morphology by Wright–Giemsa staining also revealed that the time courses for the detection of terminal granulocytes with segmented nuclei were similar for the two groups of infected cells (data not shown). The principal mechanism by which PIASy inhibits 32D cell growth thus appeared to be the induction of apoptosis rather than the acceleration of cell differentiation.

DISCUSSION

In the present study, we purified the AC133$^+$, HSC-like fraction from a large number of MDS patients (including those with MDS-associated leukaemia) and used these cell preparations to compare the expression profiles of genes for cell surface proteins, signalling molecules and transcription factors. We have shown previously that such BAMP screening with purified blasts yields fewer pseudopositive results than do comparisons of MNC preparations (Miyazato *et al*, 2001; Ohmine *et al*, 2001). The BAMP screening applied here to MDS resulted in the identification of sets of stage-specific genes, one of which is the gene for PIASy. The association of the loss of *PIASy* expression with advanced stages of MDS suggests that PIASy functions as a tumour suppressor and prevents stage progression in MDS.

To provide further support for this hypothesis, we examined the effects of PIASy overexpression in the mouse myeloid cell line 32D. The induction of PIASy expression in these cells incubated in the presence of G-CSF resulted in apoptotic cell death. The PIAS family of proteins has been shown previously to induce apoptosis through the activation of c-Jun N-terminal kinase (JNK) 1 (Liu & Shuai, 2001). However, we did not detect any effect of PIASy overexpression on the activity of JNK1 in 32D cells (data not shown). Furthermore, PIASy-induced apoptosis in 32D cells was not prevented by the forced expression of a dominant-negative form of JNK1 (data not shown). It is therefore unlikely that JNK1 is a principal mediator of PIASy-induced apoptosis in these cells.

It was also possible that PIASy affects cell viability through the inhibition of STAT proteins (Liu *et al*, 2001). However, an electrophoretic mobility shift assay did not reveal any inhibition of STAT1 activity by PIASy in 32D cells (data not shown). Moreover, our observation that the G-CSF-induced differentiation of 32D cells was not inhibited by PIASy suggests that the intracellular signalling pathway involving the G-CSF receptor and STAT1 was, at least partially, intact.

Both PIASy and PIAS1 have been shown to function as E3 ligases for SUMO (Kahyo *et al*, 2001; Sachdev *et al*, 2001), as has a yeast PIAS-related protein (Johnson & Gupta, 2001; Takahashi *et al*, 2001). SUMO is a ubiquitin-like molecule that is conjugated to a variety of proteins including RanGAP1, PML, IκB-α and p53 (Hay, 2001). Although, like ubiquitin conjugation, modification with SUMO appears to control the proteolysis of target proteins, sumoylation may also play additional regulatory roles. Given the diversity of sumoylated proteins, the various actions of PIASy described to date may be the result of PIASy-mediated sumoylation. The phenotype associated with PIASy expression may thus depend on the cellular context and the expression of PIASy substrates. The identification of binding partners of PIASy in 32D cells might provide important insight into the molecular mechanism of PIASy-induced apoptosis in these cells.

In conclusion, our microarray analysis with purified Blast Bank samples is an efficient system for the identification of molecular markers for the various stages of MDS. Furthermore, this analysis has yielded important information on the molecular mechanism of transformation in this disease.

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SUPPLEMENTARY MATERIAL

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/bjh/bjh4601/bjh4601sm.htm

Table SI. The names and GenBank accession numbers for the genes observed on our custom-made array.

Table SII. Expression intensity of the genes suppressed in the advanced stages of MDS.

Table SIII. Expression intensity of the genes activated in the advanced stages of MDS.

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